Handbook of Fermented Functional Foods

Second Edition

EDITED BY

Edward R. Farnworth

FUNCTIONAL FOODS AND NUTRACEUTICALS SERIES

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Handbook *of* Fermented Functional Foods

Second Edition

FUNCTIONAL FOODS AND NUTRACEUTICALS SERIES

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Contents

Series Editor's Preface

The Functional Foods and Nutraceuticals Book Series, launched in 1998, was developed to provide a timely and comprehensive treatment of the emerging science and technology of functional foods and nutraceuticals, which are shown to play a role in preventing or delaying the onset of diseases, especially chronic diseases. The first eleven volumes in the Series have received broad acceptance by food, nutrition, and health professionals. They are: *Functional Foods: Biochemical and Processing Aspects. (Volumes 1 and 2)*, *Herbs Botanicals and Teas*, *Methods of Analysis for Functional Foods and Nutraceuticals (1st and 2nd Editions), Handbook of Fermented Functional Foods (1st Edition*), *Handbook of Functional Dairy Products*, *Handbook of Functional Lipids, Dictionary of Functional Foods and Nutraceuticals, Processing Technologies for Functional Foods and Nutraceuticals, and Functional Food Carbohydrates*.

The latest volume, *Handbook of Fermented Functional Foods, 2nd Edition,* edited by Dr. Edward R. Farnworth (Agriculture and Agri-Food Canada, Saint Hyacinthe, Quebec, Canada) is organized into 20 chapters, contributed by 39 leading scientists from 12 counties.

The second edition of this exceptional book on fermented functional foods provides updated, in-depth treatments of the scientific and technological information on all the topics covered in the first edition (history of fermented foods; challenges facing development of probiotic-containing functional foods; properties of *Enterococcus faecium* and fermented milk; kefir; yogurt and immunity; health properties of milk fermented with *Lactobacillus casei*; biologically active peptides released in fermented milk; cheese and its potential as a probiotic food; *natto*, a soybean food made by fermenting cooked soybeans with *Bacillus subtilis* (*natto*); fermented meat; miso production, properties and health benefits; Korean fermented foods, *kimchi* and *doenjang*; the role of *Lactobacillus Plantarum* in foods and human health; sauerkraut; and the future for fermented foods), as well as five new chapters addressing traditional Chinese fermented foods; *tempeh* or *tempe kedelee*, a mold-modified food made by the fermentation of dehulled, cooked soybeans with *Rhizopus oligosporus*; table olive processing for quality control and functional proprieties; Thai fermented foods that include fermented fish, meat, and plant products; and production of probiotic cultures and their addition in fermented foods.

Dr. Farnworth has assembled a group of outstanding international contributors in the forefront of fermented food science and technology, and together they have produced an outstanding reference book that is expected to be a valuable resource for researchers, teachers, students, food, nutrition and health practitioners, and all those working in the functional food and nutraceutical industry.

To Dr. Farnworth and all the contributors I extend my sincere thanks, and I hope that the reader will find this book informative and stimulating.

G. Mazza, Ph.D., FCIFST, FIAFoST
Series Editor

Preface to Second Edition

The role of food in health and well-being is becoming more and more evident. At the same time, our understanding of how the complex microbiota in the gastrointestinal tract functions is expanding. Digestion, metabolism, diseases resistance—all appear to be influenced by the bacteria that live in our gastrointestinal tracts (autochthonous bacteria) and by live bacteria that we eat in our food (allochthonous bacteria). The properties of each individual bacterial species often are unique; results from experiments using even closely related bacteria often are different. As a result, a consensus of opinion about the beneficial effects of probiotics is hard to achieve.

We are seeing new fermented foods in the marketplace as the concept of probiotics is gaining credibility, and as food manufacturers find innovative ways to protect bacteria in an ever wider variety of foods and beverages. As has been described in the chapters of this book, people have been eating fermented foods around the world for centuries. This continues today, and may be even more important in the future.

This second edition updates chapters that appeared in the first edition with new findings and interpretations that point even more clearly to the important role fermented foods play in our diets. Additional chapters have been added that include descriptions of additional fermented foods that are common in the diets of consumers around the world.

About the Author

Dr. Edward (Ted) Farnworth is a senior research scientist at the Agriculture and Agri-food Canada Food Research Development Centre in St. Hyacinth, Quebec. He received his Ph.D. in nutrition at the University of Guelph and since joining AAFC has carried out research on the nutritional quality of canola oil, sow and piglet nutrition, and most recently on functional foods and probiotics. Dr. Farnworth is a past-president of the Canadian Society for Nutritional Sciences and currently program leader for the Functional Foods and Nutraceuticals program at his center. His publication list includes articles in chemistry, microbiology, food science, flavor chemistry, and animal and human nutrition and metabolism. His team carries out research on the composition (chemical, microbiological) of various fermented foods, and using in vitro, animal models, and clinical trials, they are showing the efficacy and mode of action of potential probiotics and prebiotics.

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1 The History of Fermented Foods

Jashbhai B. Prajapati and Baboo M. Nair

CONTENTS

1.1 INTRODUCTION

The history of fermented foods is lost in antiquity. It may have been a mere accident when people first experienced the taste of fermented food. The first fermentation must have started with the storage of surplus milk, which resulted in a fermented product the next day. After drying, fermentation is the oldest food preservation method. Fermentation became popular with the dawn of civilization because it not only preserved food but also gave it a variety of tastes, forms, and other sensory sensations. Slowly, people have realized the nutritional and therapeutic value of fermented foods and drinks, and this has made fermented foods even more popular.

It seems that the art of fermentation originated in the Indian subcontinent, in the settlements that predate the great Indus Valley civilization. During the Harappan spread or pre-Vedic times, there are indications of a highly developed system of agriculture and animal husbandry. Artefacts from Egypt and the Middle East also suggest that fermentation was known from ancient times in that region of the world. It is believed that the knowledge written in the four *Vedas* (sacred Hindu writings) came from the experiences, wisdom, and foresightedness of sages, which had been preserved by verbal tradition. As there is no written proof, controversies exist among historians in predicting the probable date of the *Vedas*. Based on astronomy, Lokmanya Tilak estimated it as the period between 6000 to 4000 V.P. (V.P. stands for the Hindu calendar of Vikram); using other methods of calculations, it is approximately 2500 V.P.[1]

The cow was referred to 700 times alone in the *Rig Veda* (the oldest and most important of the sacred books of the Hindus). It is a symbol of endless bounty in numerous contexts, and the importance of milk, curds, buttermilk, and country butter was emphasized.

For the most part, the cultures of the South Asian countries have left relatively few artefacts, and this has led to an over-emphasis on the cultural advances of other regions, such as the Middle East, Central America, and even sub-Saharan Africa in comparison to South Asia.[2] The skills of food preservation existed among the native people of many areas, and the knowledge was propagated orally. During the Middle Ages, the varieties of fermented foods and drinks developed depended upon the availability of raw materials, environmental conditions, and the taste preferences of the local people. Knowledge of some of the products that originated in ancient times has developed, and these products are now being manufactured on a large commercial scale. However, many products are still poorly understood, and their technology needs to be refined and commercialized in order for their health benefits to be realized by society. The important milestones in the history of fermented foods are presented in Table 1.1.

As a process, fermentation consists of the transformation of simple raw materials into a range of value-added products by utilizing the phenomena of the growth of microorganisms and their activities on various substrates.[3] This means that knowledge of microorganisms is essential to understand the process of fermentation. Such knowledge has only existed since 1680, when Antony Van Leeuwenhoek first demonstrated the use of a microscope and described the existence of microorganisms. Louis Pasteur, in the middle of 19th century, contributed significantly to the understanding

TABLE 1.1
Milestones in the History of Fermented Foods

Milestone	Development/Location
ca.10,000 B.C. to Middle Ages	Evolution of fermentation from salvaging the surplus, probably by pre-Aryans.
ca. 7000 B.C.	Cheese and bread making practiced
ca. 6000 B.C.	Wine making in the Near East
ca. 5000 B.C.	Nutritional and health value of fermented milk and beverages described
ca. 3500 B.C.	Bread making in Egypt
ca. 1500 B.C.	Preparation of meat sausages by ancient Babylonians
2000 B.C.–1200 A.D.	Different types of fermented milks from different regions
ca. 300 B.C.	Preservation of vegetables by fermentation by the Chinese
500–1000 A.D.	Development of cereal-legume based fermented foods
1881	Published literature on *koji* and *sake* brewing
1907	Publication of book *Prolongation of Life* by Eli Metchnikoff describing therapeutic benefits of fermented milks
1900–1930	Application of microbiology to fermentation, use of defined cultures
1970–present	Development of products containing probiotic cultures or friendly intestinal bacteria

Source: Data compiled from Joshi, V.K. and Pandey, A., *Biotechnology: Food Fermentations*, Vol. 1, Educational Publishers and Distributors, New Delhi, 1999, 1–24; Pederson, C.S., *Microbiology of Food Fermentations*, AVI, Westport, CT, 1971, 1–274; IDF, Fermented Milks, *IDF Bull*., No. 179, 16–32, 1984; Metchnikoff, E., *The Prolongation of Life*, G.P. Putnam's Sons, New York, 1908; Steinkraus, K.H., *Handbook of Indigenous Fermented Foods*, Marcel Dekker, Inc., New York, 1983; Padmaja, G. and George, M., in *Biotechnology: Food Fermentations*, Vol. II, Joshi V.K. and Pandey, A., Eds., Educational Publishers and Distributors, New Delhi, 1999, 523–582.

of the phenomenon of fermentation; he established the role of microbes in fermentation and also proved that there are many different kinds of fermentations. Since the time of Pasteur, there have been manifold increases in the knowledge of the microbiology, biochemistry, technology, and food engineering aspects of food fermentations. At present, we have a number of fermented foods and drinks including fermented milks, fermented cereals, fruits, vegetables, fish, meat, and many other mixed products, which emerged in very early times.

1.2 FERMENTED MILKS

Rock drawings discovered in the Libyan Desert, believed to have been made about 9000 B.C., depict cow worship and cows being milked.[4] Some of the oldest records suggest development of dairying in ancient India, Mesopotamia, and Egypt. It is apparent from writings, drawings, and friezes dating back to 6000 B.C. from the Sumerians of Mesopotamia, that dairying was highly developed. A sculptured relief dating back to 2900–2460 B.C., found at Teil Ubaid in the Middle East in the territory of ancient Babylonia, shows development of a system for processing milk. It could be deduced from all these pieces of evidence that the souring of milk was used to produce butter, and probably milk was also consumed in a soured form.[5] A great

many of today's fermented milk products were originally developed by nomadic Asian cattle breeders.

Nearly every civilization has developed fermented milk products of some type. The terms *dahi, butter milk, yogurt, leben*, and *acidophilus milk* are familiar to many people, but those who first produced these foods did not know that they were fermented by bacteria. Fermented milk products originating from different countries are listed in Table 1.2.

TABLE 1.2
Origin of Some Important Fermented Milk Products

Product	Country of origin	Period	Characteristics and use
Dahi	India	6000–4000 B.C.	Coagulated sour milk eaten as a food item; an intermediate product for making country butter and ghee (clarified butter)
Chhash (Butter milk)	India	6000–4000 B.C.	Diluted dahi or the butter milk left after churning of dahi into butter; used as beverage after or with meal
Laban zeer/Khad	Egypt	5000–3000 B.C.	Sour milk, traditionally coagulated in earthenware vessels
Leben	Iraq	ca. 3000 B.C.	Traditional fermented milk containing yogurt bacteria; whey partially drained by hanging the curd
Zabady	Egypt and Sudan	2000 B.C.	Natural type yogurt; firm consistency and cooked flavor
Cultured cream	Mesopotamia	1300 B.C.	Naturally soured cream
Shrikhand	India	400 B.C.	Concentrated sour milk, sweetened and spiced; semisolid mass eaten with meals as sweet dish
Kishk	Egypt and Arab world	—	Dry fermented product made from Laban zeer and par boiled wheat; small round irregular pieces, yellowish brown in color with hard texture; highly nutritious with high amino acids and vitamin content
Kumys, Kumiss	Central Asia (Mongol, Russia)	400 B.C. (probably known around 2000 B.C.)	Traditionally mares' milk fermented by lactobacilli and yeast; sparkling beverage containing lactic acid, alcohol, and carbon dioxide
Mast	Iran	—	Natural type yogurt; firm consistency and cooked flavor
Villi	Finland	—	High viscosity fermented milk with lactic acid bacteria and mold
Taette	Norway	—	Viscous fermented milk also known as cellarmilk
Langfil, Tattemjolk	Sweden	—	Milk fermented with slime-producing culture of lactococci

Product	Country of origin	Period	Characteristics and use
Ymer	Denmark	—	Protein fortified milk fermented by Leuconostocs and lactococci; whey is separated
Skyr	Iceland	870 A.D.	Made from ewes' milk by addition of rennet and starter; today concentrated by membrane technology
Prostokvasha	Soviet Union	—	Fermented milk made from ancient times by fermenting raw milk with mesophilic lactic bacteria
Kefir	Caucasusian China	—	Milk fermented with kefir grains; foamy effervescent product with acid and alcoholic taste
Yogurt (Kisle mliako)	Bulgaria	—	Cow's or ewe's milk fermented by *Str. thermophilus* and *Lb. bulgaricus*
Yogurt	Turkey	800 A.D.	Custard like sour fermented milk
Bulgarian milk	Bulgaria	500 A.D.	Very sour milk fermented by *Lb. bulgaricus* alone or with *Str. thermophilus*
Trahana	Greece	—	Traditional Balkan fermented milk; fermented ewe's milk mixed with wheat flour and then dried
Churpi	Nepal	—	Fermented milk is churned and the buttermilk remaining is heated to form a solid curd; may be further dried
Airan	Central Asia, Bulgaria	1253–1255 A.D.	Cow's milk soured by *Lb. bulgaricus*, used as refreshing beverage
Yakult	Japan	1935 A.D.	Highly heat treated milk fermented by *Lb. casei* strain Shirota; beverage and health supplement

Source: Data compiled from Pederson, C.S., *Microbiology of Food Fermentations*, AVI, Westport, CT, 1971, 1–274; IDF, Fermented Milks, *IDF Bull*., No. 179, 16–32; Yegna Narayan Aiyar A.K., *Indian Dairyman*, 5, 77–83, 1953; Koroleva, N.S., *IDF Bu*ll., No. 227, 96–100, 1988; Rasic, J.L.J. and Kurmann, J.A., *Yogurt—Scientific Grounds, Technology, Manufacture, and Preparations,* Technical Dairy Publishing, Copenhagen, 1978, pp. 11–15.

1.2.1 Dahi

Dahi (Sanskrit: *dadhi*) is a popular Indian fermented milk product that is quite analogous to plain yogurt in appearance and consistency. It is popular with consumers due to its distinctive flavor and because it is believed to have good nutritional and therapeutic value. It is utilized in various forms in many Indian culinary preparations. The use of dahi has been prevalent since Vedic times, and it is mentioned in ancient scriptures like the *Vedas, Upanishads*, and various hymns.[6] During Lord Krishna's time (ca. 3000 B.C.), dahi, butter milk, and country butter were highly regarded. Dahi is also traditionally used as an article in rituals and an ingredient of *panchamrut* (five nectors). *Ayurveda*, the traditional scientific system of Indian

medicine, in its treatises, *Charaka Samhita* and *Sushruta Samhita*, discusses various properties of cow and buffalo milk dahi and emphasizes its therapeutic characteristics.[7,8] Ayurveda also describes the properties of various types of *chhash* (stirred diluted dahi) and their role in the control of intestinal disorders.[9] Dahi, which came into use as a means of preserving milk nutrients, was probably used by Aryans in their daily diet, as it reduced putrefactive changes and provided an acidic, refreshing taste. Dahi is consumed with rice in South India, and with wheat preparations in the north; it is also used as a beverage or dessert. Dahi is also prepared from the milk of the yak and the zomo in the Himalayas.[10] Dahi is still made by local *halwais,* shops, and restaurants and in homes by traditional methods. Some dairies have started its commercial manufacture in India.

Chakka is a concentrated product obtained after draining the whey from dahi. When it is blended with sugar and other condiments, it becomes *shrikhand*, referred to as *shikhrini* in old Sanskrit literature. This has been a very popular dessert in Western India for several hundred years.

1.2.2 Kefir

Kefir is a refreshing drink that originated on the northern slopes of the Caucasus Mountains. The product is made using kefir grains, which according to the legend, were given to orthodox people by Mohammad. Mohammad strictly forbad the secret of kefir preparation to be given outside the faith; otherwise, it was said, the grains would lose their magic strength. This may be the reason why the method of kefir preparation was kept a secret for such a long time.[11] Traditional kefir was made in skin bags. The milk was poured in daily and a natural fermentation took place. It was customary to hang the bag near the door, and everyone who came in or out had to push or kick the bag in order to mix the liquid. The finished product has high acidity and varying amounts of alcohol and carbon dioxide. Kefir can be produced by a type of continuous process where the kefir is taken out and fresh milk is added.[11,12] Commercial production of kefir now occurs in many countries, particularly in Eastern Europe. (See Chapter 4 for more details on the production and health properties of kefir.)

1.2.3 Kumys

Kumys (*kumiss*) prepared from mares' milk is an ancient drink widely consumed throughout Eastern Europe and the Asiatic regions. The name *kumiss* was derived from a tribe called Kumanes, who lived along the river Kumane in the Asiatic steppes. Scythian tribes that roamed in southeast Russia and Middle Asia used to drink mares' milk in the form of kumys some 25 centuries ago.[11] It was consumed as food as well as an alcoholic drink. Marco Polo mentioned kumys as being a pleasant milk drink.

1.2.4 Yogurt (Yoghurt)

As is the case with many other fermented milk products, no precise records are available regarding the origin of yogurt. It is believed that the ancient Turkish people in Asia, where they lived as nomads, first made yogurt. The first Turkish name for

this product appeared in the eighth century as "yoghurut" and was subsequently changed in the eleventh century to its present spelling. One legend tells that an angel brought down a pot that contained the first yogurt, while another source claims that the ancient Turks, who were Buddhists, used to offer yogurt to the angels and stars who protected them.[13] According to Chomakow[14] and others, yogurt originates from the Balkans. The inhabitants of Thrace used to make soured milks called *prokish* from sheep's milk, which later became yogurt. In the Bible, it is recorded that when the Patriarch Abraham entertained three angels, he put before them soured and sweet milk (Genesis VIII, 8). The ancient Greeks and Romans were also acquainted with preparations of soured milks. The bibliography of Roman Emperor Elagabalum (204 to 222 A.D.) mentions two recipes for soured milk.

Ancient physicians of the Near and Middle East prescribed yogurt or related soured milks for curing disorders of the stomach, intestines, and liver and for stimulation of the appetite.[13] Records also exist of the use of soured milks, particularly yogurt, for preservation of meat against spoilage during the summer.[15] Earlier writers of the Middle East mention the use of soured milks as cosmetics for Persian women. However, systematic studies on the therapeutic properties of fermented milks started after the publication of the book *Prolongation of Life* by Metchnikoff.[16] In this book, Metchnikoff attributed the long life of Bulgarian people to the consumption of large quantities of Bulgarian milk containing *Lactobacillus bulgaricus*. Later, it was found that *Lb. bulgaricus* cannot be implanted in the intestines. In the search for another milk-souring organism, Moro in 1900 described *Lb. acidophilus*, which was isolated from the faeces of infants and is a normal inhabitant of human intestines. This organism could be implanted in the intestinal tract and hence was selected as a more suitable candidate for making fermented milk with a higher therapeutic value.

One of the first industrial productions of yogurt in Europe was undertaken by Danone in 1922 in Madrid.[13] After World War II, and particularly since 1950, the technology of yogurt and understanding its properties have advanced rapidly. The yogurt made in the United States for many years was a soft-curd product quite different from the custard-like yogurt prepared in the Middle East.[4] The organisms involved in this first commercial yogurt were *Lb. bulgaricus* and *Streptococcus thermophilus*. Fermentation was carried out at a lower temperature than those prevalent in the Middle East. This product resembled the soft-curd product commonly used in northern areas of Europe. The method of preparation varied considerably, but the basic process, using high acid-producing lactic acid bacteria, was the same.

New criteria have been introduced for culture selection in yogurt production. Supplementing yogurt flora with *Lb. acidophilus* and *Bifidobacterium bifidus* for the purpose of increasing the product's health-promoting value resulted in new cultured milks variously called ACO-yogurt, acidophilus-bifidus yogurt, Bioghurt, and Biogarde.

(See Chapters 5 and 7 for more details on the production and health properties of yogurt.)

1.2.5 Cheese

According to an ancient legend, cheese was accidentally made by an Arabian merchant when he put his supply of milk into a pouch made of a sheep's stomach when

he set out on a long day's journey across the desert. The rennet in the lining of the pouch combined with the heat of the sun caused the milk to separate into curd and whey. This story seems to have occurred approximately 7000 yr B.C. in the Fertile Crescent situated between the rivers Euphrates and Tigris in Iraq. The earlier records in Vedic hymns in India (6000 to 4000 B.C.), Egyptian records (4000 B.C.), and Babylonian records (2000 B.C.) clearly show references to milk, butter, and cheese. However, it is believed that with the advance of civilization, the art of cheese making spread via the Mediterranean basin to the rest of the world.[17] There is reference to cheese in biblical times (Job 10:10 [ca. 1520 B.C.] and Samuel 1:17:18 and 2.17:29 [ca. 1017 B.C.]), but written history is scarce until the periods of the Greek and Roman Empires, when various authors left written evidence.[18] Greek records go back to about 1550 B.C. and Roman records to 750 B.C., indicating milk and cheese were important components of the diets of these people. By the beginning of the Christian era, milk and cheese were used for food throughout Europe.[4]

Milking operations and curdling of milk are depicted in an early Sumerian frieze from El-Ubaid. A food material found in the Tomb of Hories Aha (ca. 3000 B.C.) has been proven to be cheese.[18] A scene on the walls of a Ramesid tomb (100 B.C.) depicts goats being led to pasture and also skin bags suspended from poles. Such bags were traditionally used to ferment milk by nomadic tribes. During fermentation, drainage of whey through cloth or perforated bowls allowed the collection of curds which, when salted, became cheese. Bowls with perforated bases, presumably used for draining whey, have been found in several locations in Europe and Asia. Baskets from reeds and other stems have also been found. Such baskets are used today in India for making both Surati paneer and Dacca curds.

Impressions of baskets found at Windmill Hill in Dorset, England (ca. 1800 B.C.) indicate that cheese was made in England well before the arrival of the Romans. Cheese was included in the offering of ancient Greeks to the gods at Mount Olympus, and cheese making was clearly a well-established craft at the time of Homer's writing. Homer, ca. 1184 B.C., referred to cheese made from the milk of sheep and goats in caves by the cyclops Polyphemus.[19] Such cheese may have been the ancestor of feta cheese made widely in Greece today.[8] Later, Herodotus, 484 to 408 B.C., refers to the Scythian cheese made from mares' milk, whereas Aristotle (384 to 322 B.C.) noted that Phrygian cheese was made from the milk of mares and asses. The trade of cheese between countries became important during the rule of the Roman emperor Diocletian (284 to 205 A.D.).

By the 14th century, cheese making was a considerable industry in Switzerland, but export was forbidden. At this time, a cheese market was operating in Gouda, Holland. It is reported that the first cooperative cheese factory was started at Voralberg in the Balkans about 1380.[18] By 1500, it is recorded that the expansion of cheese making in England, France, Germany, and Holland resulted in Italy's losing its dominant position as a cheese maker.[17]

Cheddar cheese originated in the village Cheddar in Somerset, England, and was popular during the reign Queen Elizabeth I (1558 to 1603), although it has been said to have been known from three centuries earlier. The period of 1860 to 1880 saw the introduction of a factory system throughout the cheese-making world. In 1851, the first cheese factory was established in Oneida County, New York, and it proved

so successful that within a few years several other factories had been established.[20] In 1870, the first British cheese factory was opened in Derbyshire.[21]

Some cheeses were developed only later to become extinct. Changes in agricultural practice, environment, food habits, sociological conditions, etc., have been given as reasons for the disappearance of some cheese varieties, but consumer reaction is probably the major reason. The craft was traditionally handed down, usually from mother to daughter, by word of mouth, or by practical teaching, but the art of cheese making was kept alive by monasteries in Europe. Occasionally, some new varieties of cheeses were produced, but these were not as successful as those recorded by monks. The monasteries, through the interchange of monks, spread the knowledge of the methods of cheese making. Six countries—Italy, France, England, Holland, Switzerland, and Germany—are considered as the "big six" in the history of cheese making. Most of the cheese varieties consumed today come from these countries.

Sir Joseph Lister first isolated the milk bacterium now known as *Lactococcus lactis* in 1878. After his discovery, attempts were made to use selected cultures of lactic acid bacteria for making cheese, butter, and fermented milks. The first instance of the use of a selected culture to make fermented milk is reported in 1890 by the Danish scientist Storch,[22] who used selected strains for souring cream for butter making. Prior to the use of cultures, cheese was made by:

1. Natural souring with adjustment of temperature
2. Addition of milk of sour whey or buttermilk
3. Adding homemade starter.

About 1870, Hansen in Denmark put a commercial rennet preparation on the market, and in the beginning of 1900, he put commercial cultures for cheese making on the market. This gave a boost to the manufacture of cheese on a wider scale.[17]

There are about 2000 names assigned to cheeses based on the area of the cheese's origin, country, source of milk, method of production, moisture content, cultures used, inventor, method of ripening, etc. Of these, about 800 varieties have been well established. They can be classified into 18 distinct types. Description of more than 400 varieties has been given by the U.S. Department of Agriculture (USDA).[23] Table 1.3 shows some of the cheese varieties, their countries of origin, and estimates of when they were first made.

(See Chapter 8 for more details on the production and health properties of cheese.)

1.3 CEREAL- AND LEGUME-BASED FERMENTED FOODS

Cereals and legumes are important contributors of carbohydrates and proteins to the diet, especially for the vegetarian population of the world. Ancient peoples in Asia used the techniques of hydrolyzing starch and proteins in these products to improve the digestibility and organoleptic properties of their food. Asians have been pioneers in the development of fermented plant proteins to produce meatlike flavors; Indonesians developed fermentation methods to introduce a meat-like texture to vegetable products; Egyptians developed wheat bread leavened with yeasts; and Indians discovered methods for souring and leavening cereal–legume batters.[24]

TABLE 1.3
History of Some Important Cheese Varieties

Cheese	Period of origin	Country of origin	Characteristics
Karish cheese	ca. 3200 B.C.	Egypt	Soft cheese made from sour milk; consumed fresh or after pickling
Mish Cheese	ca. 3200 B.C.	Egypt	Made by pickling Karish cheese in pickling medium in earthenware for more than one year; yellowish brown color, sharp flavor and high salt content
Feta (old Cyclops)	ca. 1184 B.C.	Greece	White, pickled soft cheese
Domiati	332 B.C.	Egypt	Soft, white, pickled cheese
Emmenthal	ca. 58 B.C.	Switzerland	Hard, pressed-curd cheese with eyes
Gorgonzolla	879 A.D.	Italy	Blue-green veined
Roquefort	1070 A.D.	France	Blue-veined, semi-soft to hard
Marolles	1174 A.D.	France	Soft, cows' milk cheese cured for 3–5 months
Grana (Permesan)	1200 A.D.	Italy	Granular body and texture, sharp flavor, small eyes
Cheddar	1500 A.D.	England	Hard cheese ripened for 3–6 months
Gouda	1697 A.D.	Holland	Sweet curd, semisoft to hard
Stilton	1785 A.D.	England	Hard, mild, blue-veined
Camembert	1791 A.D.	France	Soft, surface mold ripened
Limberger	1800 A.D.	Belgium	Semi-soft surface bacterial ripened with strong aroma and flavor

Source: Data compiled from Davis, J.G., *Cheese Vol. 1. Basic Technology,* Churchill Livingstone, Edinburgh, 1964, 1–16; Scott, R., *Cheese Making Practice,* 2nd ed., Elsevier, London, 1986, 1–11; Galloway, J.H., and Crawford, R.J.M., in *Microbiology of Fermented Foods* Vol. 1, Wood, B.J.B., Ed., Elsevier, London, 1985, 11–166; USDA, Cheese Varieties and Descriptions, USDA, Washington, D.C., 1978, 1–140.

The traditional methods for fermenting cereals and legumes are simple and inexpensive. However, these methods are changing rapidly through modern microbial technology. Soybeans, black grams, mung beans, and Bengal gram are the principal legumes, and rice is the main cereal used in the preparation of a variety of fermented foods in different parts of the world. Wheat was mainly used for making breads. Some of these fermented cereal–legume based foods, which originated from different regions of the world, are shown in Table 1.4.

1.3.1 Bread

Bread is one of the most widespread and ancient cereal products fermented by yeasts. The art of modern bread making came from the Egyptians about 3500 yr ago.[25] The Egyptians were probably the first to observe fermentation and leavening when bread dough was allowed to stand for hours. Bread was the principal food of the Egyptians and it was also given out in lieu of wages. The Romans were probably

TABLE 1.4
Some Cereal- and Legume-Based Fermented Foods

Product	Country/region	Substrate	Microorganisms(s) involved	Nature of product	Product use
Ang-kak	China	Red rice	*Monascus purpureus*	Powder	Dry red powder used as colorant
Bagni	Caucasus	Millet	—	Liquid	Drink
Banku	Ghana	Maize & cassava	Lactic acid bacteria, yeasts	Solid	Used as a staple food
Bhallae	India	Black gram	Lactic acid bacteria, yeasts	Deep fried patties	Snack after soaking in curd or water
Bhatura	India	White wheat flour	Lactic acid bacteria, yeasts	Deep fried bread	Breakfast
Bongkrek	Central Java	Coconut press cake	*Rhizopus oligosporus*	Solid	Roasted or fried in oil, meat substitute
Burukutu	Savannah region of Nigeria	Sorghum & cassava	Lactic acid bacteria *Candida* sp., *S. cerevisiae*	Liquid	Liquid creamy drink
Chee-fan	China	Soybean whey curd	*Mucor* spp., *A. glaucus*	Solid	Eaten fresh, like cheese
Chickwa-ngue	Congo	Cassava roots	Bacteria	Paste	Staple food
Darassum	Mongolia	Millet	—	Liquid	Drink
Dawadawa	West Africa, Nigeria	African locust bean	Spore-forming bacteria, lactic acid bacteria, yeast	Solid	Eaten fresh or in stews
Dhokla	India	Bengal gram and wheat	Lactic acid bacteria, yeasts	Solid/spongy	Spongy condiment
Dosai/dosa	India	Black gram and rice	*Leuconostocs, Lb. fermentum, Saccharomyces*	Solid	Spongy fried breakfast food
Fermented rice	India	Rice	Lactic acid bacteria	Semi-solid	Breakfast
Fufu	Africa	Cassava roots	*Lactobacillus* sp., *Leuconostoc* sp., *S. cerevisiae*	Paste	Eaten with soup, sauce, or stews
Gari	West Africa	Cassava roots	*Corynebacterium, Geotrichum candidum, Lb. plantarum, Leuconostocs, Alcaligenes* sp., *Candida* sp.	Granular powder	Granular wet paste eaten as a staple with stews

(continued)

TABLE 1.4
Some Cereal- and Legume-Based Fermented Foods (continued)

Product	Country/region	Substrate	Microorganisms(s) involved	Nature of product	Product use
Hama natto	Japan	Soybeans, wheat flour	*Aspsergillus oryzae, Streptococcus* sp., *Pediococcus* sp.	Soft	Raisin-like flavoring agent for meat or fish or eaten as a snack
Hopper (Appa)	Sri Lanka	Rice or wheat flour and coconut water	Baker's yeast, acid-producing bacteria	Semi-solid	Breakfast
Idli	India	Black gram and rice	*Leuconostocs, Saccharomyces* sp.	Solid	Spongy, steam-cooked breakfast food
Injera	Ethiopia	Wheat, barley, teff, maize	*Candida guiliermondii*	Solid/spongy	Bread substitute
Jalebies	India, Nepal, Pakistan	Wheat flour	Yeasts, lactobacilli	Solid	Syrup-filled confectionery
Kanji	India	Rice and carrots	*Hansenula anomala*	Liquid	Sour liquid added to vegetables
Kecap	Indonesia and nearby regions	Soybeans, wheat	*A. oryzae, Lactobacillus* sp., *Hansenula* sp., *Saccharomyces* sp.	Liquid	Condiment and seasoning agent
Kenima	Nepal, North East India	Soybeans	—	Solid	Snack food
Kenkey	Ghana	Maize	*Corynebacteria, Saccharomyces, Molds*	Solid	Steamed, eaten with vegetables
Ketjap	Indonesia	Black soybeans	*A. oryzae*	Syrup	Seasoning agent
Khaman	India	Bengal gram	*Leuconostocs, Lactobacilli*, yeasts	Solid	Cake-like breakfast food
Kisra	Sudan	Sorghum flour	Yeasts, lactobacilli, Acetobacter	Spongy bread	Staple food
Kulcha	North India and Pakistan	White wheat flour	Lactic acid bacteria, yeasts	Flat bread	Staple food
Lafun	West Africa, and Nigeria	Cassava roots	*Leuconostocs, Corynebacteria, Candida*	Paste	Staple food
Lao-chao	China, Indonesia	Rice	*R. oryzae, R. chinensis Chlamydomucor oryzae, Saccharomyces* sp.	Solid	Soft, glutinous, eaten with vegetables

Mahewu	South Africa	Maize	Lactic acid bacteria	Liquid	Drink
Meitauza	China, Taiwan	Soybean cake	*Actinomucor elegans*	Solid	Fried in oil or cooked with vegetables
Meju	Korea	Soybeans	*A. oryzae, Rhizopus* sp.	Paste	Seasoning agent
Merissa	Sudan	Sorghum	*Saccharomyces* sp.	Liquid	Drink
Minchin	China	Wheat gluten	*Paecilomyces* sp., *Aspsesrgillus* sp., *Claadosporium* sp., *Fusarium* sp.	Solid	Condiment
Miso	Japan, China	Rice and soybeans	*Aspsergillus* sp., *Torulopsis etchellsii, Lactobacillus* sp., *Saccharomyces rouxii*	Paste	Paste, soup base
Nan	India, Pakistan, Afghanistan, Iran	Unbleached wheat flour	Yeasts	Solid	Snack food
Natto	Japan	Soybeans	*Bacillus natto*	Solid	Cake used as a meat substitute
Ogi	Nigeria West Africa	Maize	Lactic acid bacteria *Cephalosporium* sp., *Fusarium* sp., *Aspsergillus* sp., *Penicillium* sp., *S. cerevisiae*	Paste	Staple breakfast food
Ontjom	Indonesia	Peanut cake	*Neurospora intermedia, R. oligosporous*	Solid	Roasted or fried food used as meat substitute
Peujeum	Java	Cassava roots	Yeasts, molds	Solid	Acidic product with alcoholic flavor, eaten as such or after baking
Poi	Hawaii	Taro corms	*Lactobacillus* sp., *Candida vini, Geotrichum candidum*	Semi-solid	Taken with fish or meat
Pozol	Mexico	White maize	Molds, yeasts	Solid	Beverage or porridge
Puda/ Pudla	India	Bengal gram, mung, wheat	Lactic acid bacteria, yeasts	Solid	Pancake snack food
Puto	Philippines	Rice	*Leuconostocs, Streptococcus faecalis, S. cerevisiae*	Solid	Snack food

(continued)

TABLE 1.4
Some Cereal- and Legume-Based Fermented Foods (continued)

Product	Country/region	Substrate	Microorganisms(s) involved	Nature of product	Product use
Sufu	China, Taiwan	Soybean whey curd	*A. elegans, Mucor hiemalis, M. silvaticus, M. subtilissimus*	Solid	Soybean cake, condiment
Shamsy bread	Egypt	Wheat flour	Yeasts	Spongy bread	Staple food
Soy sauce	Japan, China, Philippines and Oriental countries	Soybeans and wheat	*A. oryzae, A. sojae, Lactobacillus* sp., *Saccharomyces rouxii*	Liquid	Seasoning agent for meat, fish and cereals
Tao-si	Philippines	Wheat flour, soybeans	—	Semi-solid	Seasoning agent
Taotjo	East Indies	Roasted wheat meal or glutinous rice, soybeans	—	Semi-solid	Condiment
Tape	Indonesia and nearby regions	Cassava roots or rice	*S. cerevisiae, H. anomala, R. oryzae, Mucor* sp., *Endomycopsis fibuliger*	Solid/paste	Soft, solid eaten as a staple
Tempeh	Indonesia and nearby regions	Soybeans	*Rhizopus* sp.	Solid	Fried in oil, roasted, as a meat substitute
Uji	Kenya, Uganda and Tanzania	Maize, sorghum or millet flour	Lactobacilli, Pediococci, *Leuconostocs*	Semi-solid	Breakfast and lunch
Vadai	India	Black gram	*Leuconostocs, H. anomala, Saccharomyces*	Deep fried patties	Snack
Waries	India	Black gram flour	*Candida* sp., *Saccharomyces* sp.	Solid	Spongy, spicy condiment

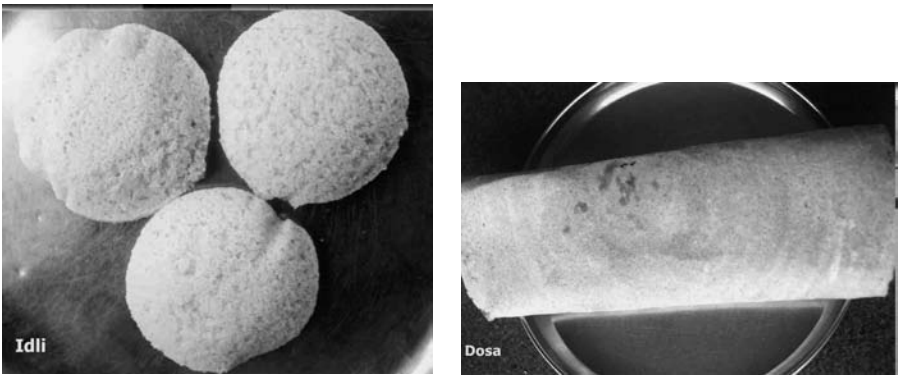
Source: Modified from Soni, S.K. and Sandhu, D.K., in *Biotechnology: Food Fermentations*, Vol. II, Joshi, V.K. and Pandey, A., Eds., Educational Publishers and Distributors, New Delhi, 1999, pp. 895–950; Padmaja, G. and George, M., in *Biotechnology: Food Fermentations*, Vol. II, Joshi, V.K. and Pandey, A., Eds., Educational Publishers and Distributors, New Delhi, 1999, pp. 523–582.

the first to commercialize bread making by using yeasts separated from wine. It is estimated that 250 bakeries existed in Rome around 100 BC.[4] The ancient Greeks prepared leavened bread that contained barley flour. With the development of leavened bread, the use of barley declined because it does not produce the light airy bread typical of wheat bread.

The early Europeans made a flat sour rye bread using sour rye starter cultures as early as 800 B.C.[26] Sour rye bread has survived the centuries and is still very popular in many parts of Europe as well as in North America. One of the most unusual starter cultures is known as "mother sponge," which is used to make San Francisco sourdough French bread. This culture contains yeasts and bacteria in ratio of 1:100. The origin of this natural culture is not known, but it has been used continuously for over 140 yr.[25]

1.3.2 Idli

Whereas the ancient Egyptians developed wheat breads, the people of India discovered methods of leavening cereal and legume batters with bacterial and yeast fermentations. The people of the Middle East discovered that sour milks combined with wheat resulted in dried soup ingredients with superior nutritional value and excellent keeping quality. The cereal- and legume-based mixed fermented products have complimentary nutritional value. *Idli* (Figure 1.1a) and dosa (Figure 1.1b), staple foods of South India prepared from rice and legumes, have each had a long history, though not every detail can be clearly traced. Idli is frequently mentioned; in 1025 A.D., the poet Chavundaraya described it unequivocally as *urad dal* (black gram) soaked in buttermilk, ground to a fine paste, mixed with the clear water of curds, cumin, coriander, pepper, and asafoetida and then shaped.[27] The *Manasollasa* of about 1130 A.D., written in Sanskrit, describes idli as made from fine urad flour, fashioned into small balls, fried in ghee, and then spiced with pepper powder, cumin powder, and asafoetida.[28]



(a) (b)

FIGURE 1.1 Some fermented foods of Asia.

(c)

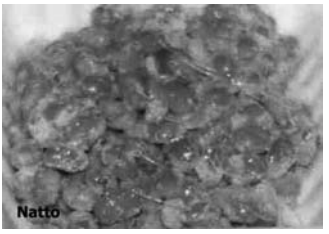
(d)

(e)

(f)

(g)

(h)



(i)

FIGURE 1.1 Some fermented foods of Asia. (continued)

1.3.3 Dosa

Dosa is a pancake made from the batter of rice and black gram, and is first noted in the Tamil (India) Sangam literature about the sixth century A.D.[29] The use of pulses to make the well-known *dhokla* (steam-cooked fermented spicy cake) of today was first mentioned in 1066 A.D. (Figure 1.1c). In northern India, dried spicy hollow balls called *warries* (Figure 1.1d) have been made for more than 100 yr. They are prepared from black gram paste fermented for 3 to 10 d. *Nan, bhatura,* and *kulcha* are a type of staple foods made from fermented wheat dough and shaped as flattened breads in India, Pakistan, Afghanistan, and Iran (Figures 1.1e to 1g).

1.3.4 Soy Foods

1.3.4.1 Soy Sauce

Soybeans are one of the most important protein sources for millions of people in the Orient; one of the most popular products for centuries has been soy sauce. Soy sauce is a liquid food condiment prepared from fermented rice or wheat and soybean with the help of molds, bacteria, and yeasts. Soy sauce is known as *ch'au yau* or *pak yau* in China, *shoyu* in Japan, *ketjap* in Malaysia, *kecap* in Indonesia, *kenjang* in Korea, *toyo* in the Philippines, and *see-ieu* in Thailand.[30] It is said that soy sauce became popular in Japan as a result of the introduction of Buddhism from China. The Chinese have been using soy sauce for over 3000 yr.[31]

1.3.4.2 Miso

Miso (see Figure 1.1h) is a fermented soybean paste that is believed to have originated in China in 600 A.D. or earlier. It is known as *chiang* in China, *miso* in Japan, *jang* or *deoenjang* in Korea, *tauco* in Indonesia, *taochieo* in Thailand, and *tao-si* in the Philippines. Most of these products contain rice or barley fermented by *Aspergillus oryzae*, which makes *koji*. This is mixed with soybeans and fermented for several months. Miso has been popular in Japan for over 1000 yr and is used as a base for soups, a sauce served with meat, or poultry, seafood, and vegetable dishes. (See Chapter 11 for more details on the production and health properties of miso.)

1.3.4.3 Tempeh

One of the most important products of Indonesia is *tempeh*, which is made from soybean fermented with the mold *Rhizopus*. It is particularly important in Java and Bali. It is also produced in Malaysian villages, Singapore, Canada, Holland, the West Indies and the United States.[31] Tempeh, the mold-fermented cake, has been consumed as a meat analogue for several centuries. Prinsen Geerlings[32] was the first to identify the tempeh mold. Later, Boorsma[33] analyzed tempeh and soybeans to determine the changes that were occurring to the substrates during fermentation. (See Chapter 17 for more details on the production and health properties of tempeh.)

1.3.4.4 Natto

Natto in Japan and *thua-nao* in Thailand are ancient whole soybean fermented products. This fermented food is also known as *tu-si* in China, *tao-si* in the Philippines, and *tao-tjo* in East India (see Figure 1.1i). Foods of this type are consumed with boiled rice or used as a seasoning agent with cooked meat, seafood, and vegetables. Natto products are dark in color and have a pungent but pleasant aroma. They are inexpensive and highly nutritious foods that serve as a substitute for fermented fish and meat. *Hama natto* is reported to have come to Japan by way of Korea approximately 350 yr ago at the time of the Japanese invasion.[34] The word *natto* means "contributed beans." It is believed that the ancestors of the owners of the Yamaya Brewery and the Saito Mido plant of Hamanatsn inherited the process of natto making from Buddhist monks.[31] (See Chapter 9 for more details on the production and health properties of natto.)

1.3.4.5 Sufu

Manufacture of soybean curd, known as *sufu*, began during the era of the Han Dynasty in China. In the *Pen Ts'as* or Chinese *Meteria Medica* of 1596, it was implied that soybean curd was invented by Lin An (179 to 122 B.C.), king of Wainan. Literally, sufu means "molded milk" and *tosufu* means "molded bean milk." In the West, sufu has been referred to as "Chinese cheese." Because of the numerous dialects used in China and the difficulties of phonetic rendering from Chinese to English, the synonyms *tosufu, fu-su, fu-ru, foo-yue*, etc. are found. It is called as *chao* in Vietnam, *tahuri* in the Philippines, *takaoan* in Indonesia, *tao-hu-yi* in Thailand and Taiwan. The major molds used in sufu fermentation are *Actinomucor, Rhizopus*, and *Mucor.*[35]

1.4 FERMENTED PLANT ROOT PRODUCTS

Several tuber crops in Africa and other countries are traditionally fermented to produce nutritious and safe foods. Cassava (*Manihot esculenta* ssp. *esculenta*) is the most abundant and important staple in tropical regions of Africa, Latin America, and Asia, where it is a common food for more than half a billion people.[36] Cassava is a perishable crop and contains toxigenic cyanogenic glucosides, linamarin, and lotaustralin. Many traditional fermented foods are made from this crop, as fermentation has the dual advantage of preserving it for a longer time and reducing the cyanogen content. Other tuber crops, such as, potato, sweet potato, yams, and taros have a longer shelf life and generally fermented food items are not prepared from them.

1.4.1 Gari

Gari is one of the most popular fermented cassava products known traditionally in many West African countries. The indigenous technology of gari making was introduced into West Africa over a century ago by the immigrant freed slaves from Brazil, who were used to *farinha de Mandioca*, an analogue of gari in South America.[37] The traditional preparation of gari is mainly done by village women by fermenting

peeled and grated cassava pulp. The prepared pulp is put into cloth bags, tied, and heavy stones are placed on the sack to press out cassava juice, and the remaining solids are allowed to ferment for 3 to 5 days. The fermented mash is sifted and then roasted. The final product is a dry, farinaceous, cream-colored powder. *Candi* and *kpokpogari* are similar traditional products made from cassava.[38]

1.4.2 Fufu

Fufu, prepared in West Africa, is also a solid cake, ball shaped or granular product of fermented cassava. *Lafun* is a similar powdery product made in Nigeria, whereas *chickwangu* is popular in Zaire and *penjeum* is a traditional product of Java.[38] In South India and Sri Lanka, the mother liquor prepared from toddy (coconut wine) and curd are used to ferment cassava and make sour cassava flour.[39]

1.5 FERMENTED FRUITS AND VEGETABLES

It seems that the development of fermented fruit and vegetable products took place from the time ancient people started collecting and storing food. Fresh fruits and vegetables are difficult to store. Fruits are naturally rich in juices and sugars and are slightly acidic. The components induce growth of yeasts and are naturally used for making alcoholic beverages. In the case of vegetables, people first added salt or seawater that resulted in extended shelf life. Before history was recorded, it was known that salt preserved foods and enhanced their organoleptic qualities.

Vegetable fermentation may have started in China, which can be deciphered from references to the mixing of vegetables, including cabbage, radishes, turnips, cucumber, and beets, which were given as rations to coolies during the construction of the Great China Wall in the third century B.C.[4] Vegetables in the Orient are often fermented in salt brines. The pickling of cucumbers probably originated in Southeast Asia.

1.5.1 Sauerkraut

Cabbage was a common vegetable in both Greek and Roman gardens. Artefacts from ancient Egypt depict use of cabbage as an offering to the gods. Greek doctors used cabbage as a general cure for illness.[4] Sauerkraut is prepared by fermenting shredded cabbage in salt solution. *Sauerkraut* is a German term meaning "sour cabbage"; this food has became popular in the United States and other European countries. At first the cabbage leaves were dressed with sour wine or vinegar. Later, the cabbage was broken or cut into pieces, packed into containers, and covered with sour juice from grapes or other fruits, sour wine, or vinegar. When the first acid liquids were replaced by salt and spontaneous fermentation resulted is not precisely known. Vaughn[40] speculated that the method used today was developed between 1550 to 1750 A.D.

Pederson[4] suggested that cabbage became the only ingredient used in preparation of sauerkraut in view of the health benefits ascribed to it by the Greeks and Romans and the plentiful supply of that vegetable in several areas of Europe. Related vegetables were included, probably cauliflower at about 1600 A.D., broccoli at about 1700 A.D., and Brussels sprouts at an earlier date.[4] Today, sauerkraut is an important industry

that makes use of the latest knowledge in microbiology and fermentation technology. *Leuconostoc mesenteroides* is the principal organism involved in sauerkraut fermentation, as it grows in vegetables more rapidly over a wide range of temperature and salt concentrations than any other lactic acid bacterium.[41] (See Chapter 14 for more details on the production and health properties of sauerkraut.)

1.5.2 Kimchi

Kimchi is the general name given to a group of acid fermented vegetable foods that have a long tradition in Korea. More specific names are used for these pickled vegetables, depending on the raw material, processing methods, season of the year, and locality.[42] Kimchi is a popular side dish served at every meal along with cooked rice and other dishes and is made primarily from cabbage or radish. (See Chapter 12 for more details on the production and health properties of kimchi.)

1.5.3 Pickled Vegetables

Pickled vegetables, made in households or small factories, have been popular in Egypt for centuries.[43] The vegetables pickled in Egypt include carrots, cucumbers, turnips, cauliflower, green and black olives, onions, and peppers. Pickled vegetables are used as appetizers and served with practically every meal. Homemade pickles made from fruits and vegetables are called *jeruk* in Malaysia; they have been popular since very early times. Cucumber is one of the oldest vegetables cultivated continuously by people. It is thought to have had its origin in India more than 3000 yr ago.[44] It is utilized both as a fresh vegetable and a pickled product.

1.5.4 Olives

Olives are one of the oldest fruit crops in the Mediterranean area. The exact date when olive fermentation started is not known. However, the more recent history of the table olive industry in California has been well documented.[44] Between 1870 and 1900, many varieties of olives were imported from the Mediterranean area. Olives were used for oil production in the Californian missions as early as 1780. The olive literature of California contains directions for pickling of ripe and green olives that were used in the home for many years; olive pickling became commercialized by 1900. The processes of pickling were standardized later on. Cruess[45] reports five processes for pickling olives in Mediterranean countries: Spanish green olive, French brine, dry salt, water, and Italian dried. In the first two processes, lye is used to destroy the bitter glucoside found in olives. Four types of fermented olives—California-ripe, brined Greek-type, Siciliano-type green, and Spanish-type green—are reported by Vaughn.[46] The extent of lye treatment, salting, and the period of fermentation varies with different types of olives, but the main organisms causing fermentation in all these varieties are *Lactobacillus plantarum*, *Lb. casei,* and *Leuconostoc mesenteroides.*[46] (See Chapter 15 for more details on the production and health properties of olives.)

1.6 FERMENTED FISH AND FISH PRODUCTS

Fermented fish products such as fish sauces, fish paste, or salted fish have been consumed since ancient times. Because of poor roads and other methods of transport, the provision of fresh fish to potential inland consumers was impossible, and this encouraged fermentation as a preservation technique. In Southeast Asian countries such as Thailand, Kampuchea, Malaysia, Philippines, and Indonesia, the use of fermentation as a preservation method for fish has been of great value since earliest times. In the countries of northern Europe, fermented fish products are used mainly as condiments, whereas in Southeast Asia, various fish products are regarded as staples.[47]

The earliest reported fermented fish sauce is *garum*, which is known to have been popular in the Roman era.[48] It is made from the viscera and blood of mackerel. Other fish sauces, for example, *botargue* and *ootarides*, were produced in Italy and Greece in the 19th century. Another sauce reported to be produced in ancient Greece was *aimeteon*, which was made from Tunny viscera and blood.[47] *Nuoc-mam* is a fish sauce prepared from small fish in Southeast Asia. The fish are fermented in earthenware containers in a high concentration of salt for several months. The clear amber liquid that rises is separated and consumed. *Shoittsuru* is the fermented fish of Japan sometimes referred to as fish soy; its origin may predate soy sauce.[4] *Burong dalag* is a blend of rice and the fish *dalag* prepared by fermentation in the Philippines.[4]

At present, a number of fermented fish sauces exist in the world. However, fish pastes are more popular than fish sauces, and are consumed as condiments. In general, the fermentation time for paste is shorter than for sauces. In southern India and Sri Lanka, pickled or Colombo curd fish have been known for many years. In this food, fish and salt in a 3:1 ratio are mixed in concrete tanks, dried tamarind fruit is added, and the mixture pickled.[47]

1.7 FERMENTED MEAT PRODUCTS

The *Sushrut Samhita*, an old Indian treatise written in about the third or fourth century A.D. based on the knowledge prevailing many hundreds of years ago, describes seven types of meat preparations. One of them is sour meat prepared using ghee (clarified butter), curd, rice gruel soured by fermentation, acid fruits, and pungent and aromatic ingredients. There are also indications of fermented meat products in the ancient Roman literature. These products originated in the Mediterranean region, where Romans added salt, sugar, and spices to ground meat and ripened it for periods of time to get a palatable product with a long shelf life. Ripening probably found favor due to the moderate temperature and frequent rainfall in these regions.[4]

Salting and drying of unground meat was the traditional way of meat preservation in Germany and other European countries. In Germany, the manufacture of fermented sausages commenced only some 150 yr ago, and most of the sausages are smoked, whereas in Mediterranean countries, France, Hungary, and Balkan countries, air-dried spicy sausages were predominant.[49]

The recorded history of sausage manufacture begins in the ninth century B.C., as established by mentions in Homer's *Odyssy*. However, it has been stated that the sausage was prepared and consumed by ancient Babylonians as far back as 1500 B.C. and the people of ancient China.[4] Grecian literature after Homer's time makes frequent mention of sausage or *oryae*. Fermented dry sausages probably had their origin in Italy about 250 yr ago.

The term *salami* might have originated from the city of Salamis, located on the east coast of Cyprus, that was destroyed in 449 B.C. It is also believed that sausage making was practiced in many areas of Europe during the Middle Ages.

Over time, some unique varieties of meat products developed in other regions of the world. All these fermentations are dependant on natural flora present in the raw materials, which decrease the pH and increase shelf life of the meat. After the successful use of starters in the cheese industry, the same cultures were tried for the fermentation of meat around 1940, but they did not proliferate in meat mixtures, probably because of their lack of tolerance to salt and nitrite.[50]

As with many other fermented foods, intensive research into the microbiology and the chemistry of sausage ripening was triggered when traditional empirical methods of manufacture no longer met the requirements of large-scale, consistent-quality, low-cost industrial production. It is therefore, not surprising that such research commenced in the United States in the 1930s, whereas in Europe the first systematic studies on the microbiology and chemistry of sausage ripening were published in the 1950s.[50] (See Chapter 10 for more details on the production and health properties of fermented meats.)

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2 Challenges Associated with the Development of Probiotic-Containing Functional Foods

Niamh Kearney, Catherine Stanton, Colette Desmond, Mairead Coakley, J. Kevin Collins, Gerald Fitzgerald, and R. Paul Ross

CONTENTS

2.1 INTRODUCTION

Probiotics are described as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host."[1] Examples of health benefits associated with the consumption of probiotics include a decrease in rotavirus shedding in infants,[2] reductions in antibiotic-associated diarrhea,[3] reduction in the incidence of childhood atopic eczema,[4,5] and management of inflammatory bowel diseases such as Crohn's disease.[6] Foods containing probiotics, such as fermented milks, yogurts, and cheese, fall within the functional food category, which includes any fresh or processed food claimed to have health-promoting and/or disease-preventing properties beyond the basic nutritional function of supplying nutrients. The area of probiotics, prebiotics, and synbiotics represent the largest segment of the functional food market in Europe, Japan, and Australia.

In order to exert health benefits on the host, probiotics must be able to grow in the human intestine, and, therefore, should possess the capability to survive passage through the gastrointestinal tract (GIT), which involves exposure to hydrochloric acid in the stomach and bile in the small intestine. *Lactobacillus* and *Bifidobacterium* species are ideal probiotic candidates for incorporation into foods for human consumption. These microorganisms are known inhabitants of the gastrointestinal tract (GIT) and share a number of common traits such as acid and bile tolerance, the ability to adhere to intestinal cells, and GRAS status (generally regarded as safe).[7] A major challenge associated with the application of probiotic cultures in the development of functional foods is the retention of viability during processing. Given that probiotics are generally of intestinal origin, many such strains of bacteria are unsuitable for growth in dairy-based media, and are inactivated upon exposure to high temperatures, acid, or oxygen during dairy and food processing. The survival of bifidobacteria during processing can be particularly challenging, as these are strictly anaerobic microorganisms with complex nutritional requirements. Maintaining the viability (minimum numbers of probiotic cultures present in the final product recommended to be 10^7 colony forming units [CFU] per milliliter or even higher)[8] and the activity of probiotic cultures in foods to the end of shelf life are two important criteria that must be fulfilled in order to provide efficacious probiotic food products.

Fermented dairy foods, including milk and yogurt, are among the most accepted food carriers for delivery of viable probiotic cultures to the human GIT. Because high levels of probiotics are recommended for efficacy of these products,[8,9] preparation of bulk cultures is required. However, because probiotics are normally of intestinal origin, these cultures exhibit poor growth rates in synthetic and milk-based media. Spray drying and freeze drying are useful means of introducing the probiotic culture into these food systems. The use of such approaches in preparing cultures may impair viability and probiotic functionality due to the extent of cell injury that may occur during these processes upon exposure to extreme heating and drying, or freezing and drying. Other approaches that have been used to improve the resistance of sensitive probiotic bacteria against adverse conditions during food processing, storage, and human ingestion include appropriate selection of acid-, bile-, and oxygen-resistant strains; stress adaptation; microencapsulation; incorporation of micronutrients and prebiotics; and genetic manipulation of probiotics,

all of which will be discussed in the following sections. This chapter will review developments in probiotic foods, with particular emphasis on the introduction of probiotic lactobacilli and bifidobacteria into foods for human consumption, and on approaches that have been tested for the enhancement of probiotic viability in food systems to the end of shelf life.

2.2 HISTORY OF CONSUMPTION OF FERMENTED DAIRY FOODS

The consumption of fermented milks containing bacterial cultures has long been associated with beneficial health effects, and probiotic cultures have had a long association with dairy food products. In 76 B.C., the Roman historian Plinio suggested the administration of fermented milk products for treating gastrointestinal infection.[10] The original observation of the positive role on health of some bacteria can be credited to the Russian scientist Metchnikoff. The works of Metchnikoff are regarded as the birth of probiotics.[11] In 1907, he suggested that the consumption of foods such as yogurt, kefir, and sour milk containing lactic acid bacteria (LAB) was associated with good health and longevity, and in his book *The Prolongation of Life* he reported that Bulgarian peasants who consumed large quantities of Bulgarian sour milk lived longer.[12] The Bulgarian sour milk contained the microorganism *Bulgarican bacillus*, which was later renamed *Lactobacillus bulgaricus*, and Metchnikoff reasoned that these bacteria eliminated putrefactive bacteria from the GIT.[13] Also around the beginning of the 20th century, Tissier, in parallel with Metchnikoff, proposed that bifidobacteria might be effective in preventing infections in infants, as they were the predominant component of the intestinal microflora of breast-fed infants.[14] In 1926, Henneberg proposed the use of an intestinal isolate, *Lactobacillus acidophilus*, to produce what he called "acidophilus-milch," or "reform yogurt."[15] This concept finally became a success in the 1980s in Germany and other Western European countries, and the *Lactobacillus* species used for the fermented product (referred to as "yogurt mild") were selected on the basis of their technological properties, and not their potential health benefits.[15]

Probiotic food constitutes a sizeable proportion of the functional food market,[16] and production continues to grow at an exponential rate. The market for fermented foods containing probiotics is most active in developed countries in Europe, Japan, Australia, and the United States.[16,17] Within Europe, the dairy sector is the most developed segment of the market, with probiotic yogurts and fermented milks, particularly in "daily dose" format, the most widely used. In 1997, these products accounted for 65% of the European functional foods market, valued at $889 million; followed by spreads, valued at $320 million and accounting for 23% of the market.[18] Some probiotic yogurt drinks and yogurts now also contain other bioactive ingredients such as plant stanols or sterols that lower cholesterol levels. Functional breakfast cereals and probiotic yogurt drinks and yogurts together accounted for more than three-quarters of the functional foods market, by value, in 2005–2006, with cholesterol-lowering margarines, soya milk, and cereal bars representing much of the balance. Probiotic dietary supplements have been slow to gain acceptance in Europe, but new applications are emerging all the time.

In Japan, bifidobacteria research began in the 1950s, and in 1971, the Morinaga Milk Industry Company developed the first bifidus product, which was a fermented milk containing *Bifidobacterium longum* and *Streptococcus thermophilus*.[14] Throughout the 1970s, technology was developed that was capable of delivering products containing viable bifidobacteria on a commercial basis, and probiotic consumption changed from being purely for therapeutic benefit to being more related to general health improvement.[14] The Morinaga Milk Industry Company launched a bifidus milk in Japan in 1977, and a bifidus yogurt in 1979, while Yakult launched a fluid yogurt called MilMil™ in Japan in 1978, containing *Bifidobacterium breve*, *Bifidobacterium bifidum,* and *Lb. acidophilus*.[14] Today, Yakult is sold in over 25 countries worldwide. However, in Japan, Yakult is not regarded as a functional food because the presence of probiotics in isolation from other functional ingredients does not carry functional food status.[19] (See Chapter 6 for more on products related to Yakult.) In 1991, functional foods were given legal status in Japan, where they are described as foods for specific human use (FOSHU).[20] A FOSHU is described as a food expected to have certain health benefits, and that has been licensed to bear a label stating to that effect.[21] Soft drinks in which dietary fiber and probiotics are the significant functional ingredients dominate the Japanese market.

In comparison to the European and Japanese markets, the U.S. dairy products market is currently underdeveloped, and dietary supplements are by far the most accepted product format.

After many years of popularity in the Japanese and European markets, and now in Australia and U.S., manufacturers of these products are venturing into new markets including the Arabian Gulf region, as evidenced by the variey of probiotics food products now available in the supermarkets and health food stores in these countries.[22]

2.3 DEFINITION OF PROBIOTICS

Although there is a long history of health claims concerning living microorganisms in food, the term *probiotic* appeared only in the 1960s, and since then a number of definitions have appeared in the literature. The term *probiotic,* which comes from the Greek meaning "for life," was originally used by Lilly and Stillwell[23] in 1965 to describe substances secreted by one microorganism that stimulate the growth of another.[24,25] It was later described by Parker[26] in 1974 as "animal feed supplements that have a beneficial effect on the host animal by affecting its gut flora." Fuller found this definition unsatisfactory as it did not exclude antibiotics, and redefined a probiotic as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance."[27] Fuller's definition was expanded to state that "a probiotic is a mono- or mixed culture of live microorganisms that, when applied to animal or man, affects the host beneficially by improving the properties of the indigenous microflora."[28] This definition stresses the importance of live microorganisms that occur in the mouth, gastrointestinal tract (GIT), upper respiratory, or urogenital tracts and improve the health status of both man and animal.[28]

In 1998, Guarner and Schaafsma[29] introduced the concept of consuming adequate numbers of probiotics to reach target sites in the body, and described them as "living organisms that, upon ingestion in certain numbers, exert health effects

beyond inherent general nutrition." Although specific numbers are not mentioned in the definition, it is thought that at least 10^9 CFU per day need to be ingested.

Salminen et al.[30] proposed that probiotics be defined as microbial cell preparations, or components of microbial cells, that have a beneficial effect on the health and well-being of the host. This definition emphasizes that probiotics can be either nonviable cells or parts of cells, because probiotics in these forms, as well as certain fermentation end-products and enzymes, have been shown to exert health benefits.[30] Here, the importance is underlined of understanding the specific functions of probiotics in the host. In 2001, a joint committee Food and Argriculture Organisation of the United Nations/World Health Organisation (FAO/WHO) expert consultation on health and nutritional properties of powder milk with live lactic acid bacteria redefined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host," again highlighting the importance of viability. The group recognized that probiotics should be capable of exerting health benefits on the host through their activity in the human body.[1] This definition is internationally recognized. Viability of probiotics in the final product is important, especially when it has been documented as one of the prerequisites for immune effects.[31]

Recently, probiotics have been defined as "living microorganisms that resist gastric, bile, and pancreatic secretions; attach to epithelial cells; and colonize the human intestine."[32] The definition of probiotics has changed from the original one of being a live active culture beneficially affecting the host by improving its intestinal microbial balance, to the current concept based on the specific effects of clearly defined strains. This focuses attention on demonstrated clinical effects, which may be mediated either through probiotic effects on the intestinal immune system or through modulation of the gut microbiota at specific locations.[33]

2.4 INTESTINAL MICROFLORA AND ECOLOGY OF THE GUT

The human intestinal tract is a nutrient-rich environment inhabited by up to 100 trillion (10^{14}) microbes. The vast majority reside in the colon, where densities approach 10^{11} to 10^{12} cells per milliliter, the highest recorded for any microbial habitat.[34] Colonization of germ-free infants starts immediately after birth; they acquire their microbiota initially from the vagina and feces of their mothers,[35] or from the environment (in a caesarean delivery), as well as by the diet, genetic background, and environment of the individual. The diverse microbial community of the human GIT encompasses both facultative anaerobic and obligate anaerobic microorganisms,[25] and is one of the preferred sources of potential probiotic microorganisms destined for human use.

The GIT microflora originate in the oral cavity where a complex microbiota exist,[36] including members of the *Prevotella*, *Porphyro*monas, *Peptostreptococcus*, *Bacteroides*, *Fusobacterium*, *Eubacterium*, and *Desulfovibrio* genera.[37] Bacteria experience large losses in viability in the stomach due to its strong acidic and peristaltic nature, and so only the most acid-resistant microorganisms survive.[25,38] Less than 10^3 cells per gram of mainly *Streptococcus*, *Staphylococcus*, and *Lactobacillus* species are found in the stomach.[38] In the small intestine, low pH, presence of bile,

and rapid transit time do not encourage the growth of bacteria. Gram-negative and anaerobic bacteria are predominant in the distal ileum, reaching levels of 10^5 and 10^7 cells per milliliter of contents,[39] which increase rapidly to 10^{11} to 10^{12} cells per gram of gut contents in the large intestine, as the flow of intestinal chyme slows upon entry into the colon.[40] Obligate anaerobic species of bacteria predominate the large intestine, and include both Gram-positive and Gram-negative bacteria.[41] The most numerically predominant are *Bacteroides* spp. and *Bifidobacterium* spp. The other major intestinal bacteria include clostridia, peptococci, streptococci, eubacteria, lactobacilli, peptostreptococci, ruminicocci, enterococci, colioforms, methanogens, dissimilatory sulfate-reducing bacteria, and acetogens.[13]

Traditional methods for determining microbial composition of the gut involved culturing appropriately diluted fecal samples on selective media. However, the selectivity of any medium is at best relative, and these methods are prone to both false-positive and false-negative results.[42] Various culture-independent methods have been developed; in particular, methods using the variable and conserved regions of the 16S rRNA have proved successful in characterizing the gut microbiota.[43] Sequencing of 16S rRNA genes has revealed that microbial diversity in the gut is far more extensive than previously described from studies of cultured microorganisms alone. In a study of human intestinal microbiota by Eckburg et al.,[44] bacterial and archaeal 16S rRNA sequences were derived from biopsies taken from six regions of the colon and one stool sample from each individual, and it was found that the human intestinal samples contained members of seven divisions of bacteria, namely, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria*, which together with divisions derived from previous studies (*Spirochaeates*, *Vadin*BE97),[45] brought the total number to nine. Fluorescent in situ hybridization (FISH), which uses a specific fluorescent oligonucleotide probe binding to rRNA in whole cells, has been used to enumerate bacteria in complex ecosystems. For example, using a *Bifidobacterium* probe (Bif164-probe), bifidobacteria between 10^9 and 10^{10} per gram dry weight of adult feces could be detected, and *Lactobacillus* numbers of 10^7 to 10^8 per gram dry weight were estimated using the Lab158 *Lactobacillus*-enterococci targeted probe.[46] Enumeration of fluorescent microbes can be done microscopically by visual counting[47] or by flow cytometry.[48] DNA arrays may be used for the analysis of microbial diversity by rapidly analyzing RNA abundance or DNA homology of genes.[46]

Vaughan and co-workers stressed the importance of fingerprinting techniques, such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) and terminal-restriction fragment length polymorphisms (T-RFLP) in the study of microbial diversity and community behavior over time in the GIT.[49] DGGE analysis of GIT samples has indicated that the total microbiota of each individual had a unique pattern, reflecting their differences in composition, suggesting that they are partly dependent on the host genotype.[50] Zoetandal and colleagues showed greater similarities between the gut microbial communities of monozygotic twins than between monozygotic twins and their unrelated marital partners, using fingerprinting methods. The results were interpreted as either reflecting a genotypic effect, or similarities between twins' microbial communities that were likely acquired by transmission from their shared mother.[51]

Lozupone and Knight[52] introduced a new method called UniFrac for computing differences between microbial communities based on phylogenetic information. UniFrac is applied to published 16S rRNA gene libraries and measures the phylogenetic distance between sets of taxa in a phylogenetic tree. Ley et al.[53] used the UniFrac method to test the effects of kinship and genotype on diversity. The 16S rRNA sequences from cecal microbiota of genetically obese ob/ob mice, lean ob/+ mice, and wild-type siblings, and their ob/+ mothers, all fed on the same polysaccharide-rich diet were analyzed. The results revealed that regardless of their ob genotype, the mothers and their offspring shared cecal microbiotas with similar community membership. Obesity was associated with a shift in the relevant abundance of the specific taxa present, such that there was an increased ratio of *Firmicutes* to *Bacteroidetes* in obese mice compared to lean mice.

The influence of intestinal bacteria on human health can be considered harmful, beneficial, or neutral. *Bifidobacterium* and *Lactobacillus* species are beneficial microorganisms and can contribute to digestion, immune stimulation, and inhibition of pathogens. *Bacteroides*, *Escherichia*, *Clostridium,* and *Proteus* species are examples of potentially harmful bacteria found in the GIT, as they are capable of producing harmful substances including amines, indole, hydrogen sulfide, and phenols from food components.[14] Harmful bacteria in the intestine have been linked to a number of clinical disorders such as cancer, inflammatory disease, ulcerative colitis, and also an increase in the host's susceptibility to infection by enteropathogens such as *Salmonella*, *Campylobacter*, *Escherichia*, and *Listeria*. It is important that the correct balance of bacteria be maintained in order to allow the intestine to operate optimally.[13] In order to understand the interaction of the microbiota with the host, as well as microbe-microbe interactions in the intestine, there is an urgent need for genome sequences of commensals. The number of beneficial food-related microorganisms for which genome sequence data are available is increasing,[54] and additional genome statistics have been published by Klaenhammer et al.[55]

One approach to sequencing genomes of commensals is the construction of metagenomic libraries, consisting of large, cloned DNA fragments in the absence of culturing.[56] A human gut microbiome initiative has been proposed that will deliver deep draft whole genome sequences for 100 species representing the bacterial divisions found in our distal gut.[57] A number of studies have used gnotobiotics (animals raised under germfree conditions) for colonizing at varying points in their life cycle by a single microbe, or complex collections of microbes. The development of communities has been investigated, and the impact determined that different members have on community function and host biology.[58] Although these models have been available for 50 yr, genomic and powerful computational tools for assessing diversity in our gut microbiota, the gene content of the microbiome, and the metabolome encrypted by this collection of microbial genes have only been developed in the last 5 yr.[53,59,60,61] Examples of model organisms include: *Mus musculus* (common mouse), as the mouse and human microbiota are similar at the division level, with *Firmicutes* and *Bacteroidetes* dominating,[53] and zebrafish, as they are transparent until adulthood, allowing visualization of microbes in their native gut habitats in real time.[58] In a recent reciprocal transplantation experiment where the gut microbiota of a common mouse was introduced into a germ-free zebrafish, and vice versa, the recipient

gut sculpted the community composition to resemble its own native community.[58] In a study by Samuel and Gordon,[60] a mutualistic relationship between two members of the colon—a prominent archaeon, *Methanobrevibacter smithii*, and *Bacteroides thetaiotaomicron*—was revealed. *M. smithii* regulates the specificity of polysaccharide fermentation by directing *B. thetaiotaomicron* to ferment fructans influencing the amount of calories deposited in the fat stores. As a result, *B. thetaiotaomicron* obtains more energy, which allows a larger population to be supported, whereas *M. smithii* obtains formate from the fermentation, which it uses for methanogenesis, and its population also expands. Also *B. thetaiotaomicron–M. smithii* co-colonization produces a significant increase in host adiposity compared with monoassociated animals, as *M. smithii* regulates the specificity of polysaccharide fermentation (especially fructans) and, therefore, influences the amount of calories deposited in the fat stores. Although this is an oversimplified model, it indicates a means for controlling obesity in humans by enhancing the ability to extract energy from polysaccharides.

Defining the human gut microbiota and microbiome will provide a better understanding of the relationship of the host with the microbial community, and should help in the prevention of diseases such as atopy, colon cancer, diarrheal diseases, and inflammatory bowel diseases.

2.5 DESIRABLE PROBIOTIC CHARACTERISTICS

Microbes from many different genera are being used as probiotics, including *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Bacillus*, *Escherichia*, *Enterococcus,* and *Saccharomyces*. Lactobacilli are the most commonly used probiotics in food, whereas bifidobacteria are used less, as they are sensitive to oxygen and have more strict growth requirements, making them technologically more unsuitable for use. With the exception of propionibacteria and enterococci, the other species mentioned are not usually used in fermented food products, but as probiotics in dietary supplements or in capsules, powders, etc.[62] Ideally, a microorganism should meet a number of predefined criteria in order to be considered as probiotic (see Table 2.1).

The microbes administered should be safe, have GRAS status, and also have a long history of safe use in foods.[63] All probiotic strains should have nonpathogenic properties, and ideally should exhibit tolerance to antimicrobial substances, but should not be able to transmit such resistance to other bacteria.

Adherent probiotic strains are desirable because they have a greater chance of becoming established in the GIT, thus enhancing their probiotic effect.[8] Adhesion to the intestinal mucosa is considered important for immune modulation (the intestine being the largest immune organ of the body), and for pathogen exclusion by stimulating their removal from the infected intestinal tract.[64] Two human *Lactobacillus* strains were examined for adherence properties in a study by Fernández, Boris, and Barbés.[65] Both strains adhered to Caco-2 cells, through glycoproteins in *Lactobacillus gasseri*, and through carbohydrates in *Lb. acidophilus*, and both strains were able to inhibit certain enteropathogens, including *Salmonella*, *Listeria*, and *Campylobacter* without disturbing the normal microbiota. They also found in this study that the *Lb. gasseri* that strongly attached to the intestinal Caco-2 cells inhibited the attachment of *Escherichia coli* 0111 under the condition of exclusion. Ability to

TABLE 2.1
List of Desirable Properties of Probiotic Microorganisms for Incorporation into Fermented Food Products

1. Human origin
2. GRAS status
3. History of safe use in food
4. Documented health benefits
5. Antimutagenic and anticarcinogenic properties
6. Nonpathogenic
7. Tolerance to antimicrobial substances yet inability to tolerate other bacteria
8. Adherence to intestinal mucosa
9. Ability to reduce pathogen adhesion to surfaces
10. Antimicrobial activity against potentially pathogenic bacteria
11. Immunostimulation without proinflammatory effect
12. Acid tolerance
13. Human gastric juice tolerance
14. Bile tolerance
15. Phage resistance
16. Oxygen and heat tolerance
17. Desired metabolic activity
18. Ability to grow in milk
19. Good sensory properties
20. Retain viability and stability during food processing, storage, and following consumption

survive passage through the GIT is a requirement in order to confer health benefits to the host. Acid tolerance, tolerance to human gastric juice, and bile tolerance should all be established by using in vitro methods.

Probiotic microorganisms should also be technologically suitable for incorporation into food products, such that they retain both viability and efficacy in that food product (to a commercial scale) prior to and following consumption. Probiotics should be capable of surviving industrial applications (e.g., common dairy processing methods using pharmaceutical manufacturing protocols), of thriving in the product to the end of shelf life,[66,67] and of having an acceptable taste throughout the storage time. Above all, probiotic food products must demonstrate efficacy in controlled and validated clinical trials to prove that the probiotic characteristics were not altered or lost during manufacturing.

2.6 ISOLATION AND ENUMERATION OF PROBIOTIC BACTERIA

The isolation and enumeration of probiotic strains, such as bifidobacteria and lactobacilli from the mammalian intestine or the carrier food product, can pose a significant challenge in itself. In particular, these microorgansims can be very fastidious, oxygen-sensitive, and difficult to grow in laboratory media. In addition, the differential enumeration of probiotic and starter bacteria in products such as fermented milks, yogurts, and cheeses, can often be very difficult, due to the presence of

multiple species of LAB. As the efficient isolation and enumeration of probiotics is a critical step in the development of functional foods, the advances in the formulation of media for bifidobacteria and lactobacilli, and the use of culture-independent molecular tools for the quantification of these probiotic strains, will be reviewed.

The choice of selective media (providing preferred nutrients, antibiotics, vitamins, etc.) and incubation conditions (suitable temperature, pH, and redox potential) can electively select and isolate a bacterial group of interest[68] (see Table 2.2). Extensive literature exists on the culture media suitable for the isolation, detection, and enumeration of bifidobacteria,[69,70] however, so far no medium has been developed that is fully selective for bifidobacteria, and at the same time, has no effect

TABLE 2.2
Examples of Selective Culture Media That Have Been Used for the Enumeration of Bifidobacteria and Lactobacilli

Bacterial species	Agar medium	Reference
Bifidobacterium spp.	YN-6	77
Bifidobacterium spp.	BIM-25	78
Bifidobacterium spp.	MRS + Dic	81
Bifidobacterium spp.	TPY + Dic	81
Bifidobacterium spp.	BL–OG	85
Bifidobacterium spp.	RAF 5.1	89
Bifidobacterium spp. (from chicken caecal samples)	TOS–AM50	91
Bifidobacterium spp.	NPLN–Agar	93, 76
Bifidobacterium spp.	MRS–LP	93
*Bifidobac*terium spp.	MRS–NPLN	93, 161
B. bifidum	Cheese whey-based medium supplemented with *N*-acetylglucosamine and yeast extract in the presence of sodium thioglycolate	87
B. bifidum	BSM	90
B. longum	BSM	90
B. thermophilum	BSM	90
Lactobacillus acidophilus	MRS agar containing gluconate, ribose, sorbitol, salicin, fructose, or mannitol	161
Lb. acidophilus	MRS–clindamycin	166
Lb. acidophilus	Na–salicin	166
Lb. acidophilus	Bile medium	101
Lb. acidophilus	Medium based on X-gluc	102
Lb. rhamnosus	MRS–AC	93
Lb. paracasei (from cheese)		
Lb. rhamnosus	LC	93
Lb. paracasei (from yogurt)		
Lb. casei	LC	161

on their growth.[71] There is no single selective medium suitable for all species of bifidobacteria.[72] Several selective media have been developed for the enumeration of bifidobacteria.[73–82] Bifidobacteria media often contain special growth factors, substances that lower the redox potential (cysteine, cysteine hydrochloride, cystine, ascorbic acid, sodium sulfite, liver extract), and antimicrobial substances that inhibit the growth of other bacteria (e.g., lactobacilli and other LAB, propionibacteria, and *Actinomyces* sp.).[69,83] An inexpensive whey-based medium supplemented with *N*-acetylglucosamine and yeast extract in the presence of sodium thioglycolate has been developed for *B. bifidum* ATCC 11863, promoting good growth of this strain at 37°C.[84]

Recent advances have been made in the development of appropriate selective media for members of the genus *Bifidobacterium*. In 1997, Rada[85] reported that bifidobacteria were resistant to the antibiotic mupirocin, whereas this agent inhibits several gram-positive bacteria including other closely related LAB. In a review by Roy,[86] it was stated that when de Man, Rogosa, and Sharpe (MRS) medium was supplemented with cysteine hydrochloride, it became highly elective for a wide range of *Bifidobacterium* species. In a study by Simpson et al.,[87] MRS was supplemented with both cysteine hydrochloride and mupirocin (termed *Bifidobacterium* selective medium [BSM]), and the resulting medium was found to be elective for bifidobacteria but inhibitory to a wide range of nonbifidobacteria strains commonly included in probiotic animal feed. Thitaram et al.[88] reported that the addition of mupirocin and glacial acetic acid to transoligosaccharide propionate agar medium (TOS) did not inhibit the growth of bifidobacteria, but inhibited three *Lactobacillus* strains and one *Streptococcus* strain. These results are consistent with the results from the previous data,[86,89] which suggests that the antibiotic mupirocin can be used to distinguish *Bifidobacterium* spp. from *Lactobacillus* spp.—usually difficult due to their shared biochemical properties and habitats. NPLN medium (containing the antibiotics neomycin sulfate, paromomycin sulfate, and nalidixic acid, and the reducing agent lithium chloride), and MRS-LP (supplementation of MRS with L-cysteine HCL, lithium chloride, and sodium propionate) were found to be suitable for the selective enumeration of commercial probiotic *Bifidobacterium* strains found in yogurt and cheese also containing the starter cultures, *S. thermophilus* and *Lb. delbrueckii* subsp. Bulgaricus.[90] It was also concluded that the yogurt starter cultures *S. thermophilus* and *Lb. delbrueckii* subsp. Bulgaricus could be optimally enumerated on M17 medium and MRS 5.2, respectively.

The media used for isolating and enumerating lactobacilli depend on the type of sample, the specificity required, and, to some extent, the characteristics of the particular *Lactobacillus* strain being cultured. For most lactobacilli, various requirements for essential nutrients are met when the medium contains fermentable carbohydrates, peptone, meat, and yeast extracts. Supplementation with tomato juice, manganese, acetate, and oleic acid esters, especially Tween 80, are stimulatory or essential for most species. A widely used selection medium that includes these compounds is MRS, as mentioned previously.[91] Lactobacilli adapted to particular substrates may require special growth factors, for example, MRS supplemented with maltose, raffinose, or melobiose in place of dextrose is used for the enumeration of *Lb. acidophilus* in yogurt.[92] Because starter yogurt bacteria generally cannot utilize,

and hence grow on, a wide range of carbohydrates, unlike *Lb. acidophilus*, a simple differential medium can be used containing a single sugar (such as ribose, fructose, glucose, sorbitol, salicin, or mannitol) suitable for growth of *Lb. acidophilus* but not the other yogurt bacteria.[93] This approach has also been used to enumerate lactobacilli in Swiss[94] and Mozzarella[95] cheese varieties.

Other media that have been described for the enumeration of *Lb. acidophilus* include lactobacillus selection agar (LBS),[96] cellobiose esculin agar,[97] bile medium,[98] and agar medium based on X-gluc.[99] In a recent article by Van de Casteele,[90] the preferred medium for the selective enumeration of two commercial strains of *Lb. acidophilus* was MRS-clindamycin. For the enumeration of probiotic cultures *Lb. paracasei* and *Lb. rhamnosus*, LC medium (proposed by Ravula and Shah[100]) for yogurt products and MRS-AC medium (pH is adjusted to 5.2 with acetic acid) for cheese products were recommended.[90] The product matrix, the target probiotic, and the diversity of the background flora in the product are major considerations when choosing the selective culture medium.[90]

Classical cultivation methods for isolating and enumerating bacteria fail to provide an insight into bacterial components that are unculturable in the laboratory due to an inability to reproduce their metabolic and physiological requirements in vitro.[101] Genetics-based probing strategies are applicable for monitoring the bacterial composition of mixed LAB populations,[33,47,73,102,103] effective methods used being fluorescent in situ hybridization (FISH) and microchip assays. FISH is a culture-independent technique, using a specific fluorescent oligonucleotide probe binding to rRNA in whole cells to detect, identify, and enumerate bacteria. Hybridized cells are enumerated by epifluorescence microscopy or flow cytometry.[47,49] In microchip assays, labelled oligonucleotide probes are bound to the microchip and denatured DNA samples are applied.[104] These methods have been used to monitor the bacterial populations in fermented foods and in fecal samples. Another method for the quantitative detection of bacterial groups in a sample, independent of culturing techniques, is that of real-time polymerase chain reaction (PCR). Universal primers are based on conserved regions of housekeeping genes in all bacteria, whereas specific primers are based on regions conserved only within a specific group of bacteria. PCR product yield can be measured after each cycle, as real-time PCR incorporates fluorescently labelled probes for detection.[105]

Culture-independent molecular tools (such as those mentioned above) for the quantification of probiotics in commercial products have only recently been developed and the instrumentation required for these techniques are expensive. Therefore, many manufacturers still rely on conventional cultivation methods.

2.7 CULTIVATION OF PROBIOTICS FOR INCORPORATION INTO FUNCTIONAL FOODS

Although the most important characteristics of probiotic bacteria are their positive effects on host health, the extent of probiotic growth and survival in milk-based media and during product manufacture, and shelf life can be important considerations for selection of strains for food applications. Because the ability to culture the probiotic of interest to high cell density in a suitable medium for food applications is

an essential prerequisite to their incorporation into foods, the topic of cultivation of these strains will be dealt with in detail in this chapter. Some probiotic strains have demonstrated the ability to grow in the food product after manufacture, for example, human-derived *Lb. paracasei* in Cheddar cheese,[106] thus allowing lower levels to be used during the manufacturing process.

2.7.1 Culturing of Bifidobacteria

Bifidobacteria are generally known to be nutritionally fastidious microorganisms, requiring certain amino acids and vitamins for their growth.[107] The growth of bifidobacteria in culture media is often related to the presence of various growth factors.[108] Bifidobacteria are generally difficult to propagate as they are not acid tolerant, and cannot grow in a medium with a high oxidative potential.[109,110] Reducing agents such as cysteine, cysteine-hydrochloride, and ascorbic acid are often added to growth media.[109] Synthetic media such as MRS and tryptone phytone yeast (TPY) broth are too complex and expensive for generating large quantities of bifidobacteria cultures for commercial applications.[111] Also, the addition of bifidobacteria grown in synthetic media to dairy products such as yogurt and ice cream can contribute to off-flavor unless they are extensively washed, and furthermore may be in breach of regulations for adding bacterial cultures to dairy products.[111,112] Thus, the potentially most useful media for the delivery of probiotic bifidobacteria to the human GIT are milk and yogurt.[113] Moreover, bifidobacteria can be protected by milk proteins during digestion, allowing better delivery to the colon.[114] Therefore, a milk-based medium is appropriate for producing an inoculum for a quality dairy product while maintaining the texture of the product.[111]

However, bifidobacteria are often difficult to propagate in bovine milk because of its deficiency in necessary growth factors.[107,111,112,115] Bifidobacteria cells have been shown to change their morphology from typical branched, bifurcated Y-form cells in MRS to a variable morphology in milk, which has been attributed to a lack of certain nutrients or anaerobic conditions.[111] Whereas milk contains many of the essential nutrients necessary for the growth of bifidobacteria, these may not be at optimal concentrations, and are often in a form inaccessible to the microorganism.[116] For example, there is a lack of available nitrogen in milk for bifidobacteria because of the low concentrations of free amino acids and peptides, despite its relatively high content of casein (inaccessible to the microorganism).[108,116]

In 1993, Murti et al.[117] suggested that bifidobacteria were able to grow more extensively in soy than in cows' milk, and in a recent study by Farnworth et al.[118] it was suggested that the human-derived *Bifidobacterium* RBL 00079 strain is a good candidate for incorporation into soy-based products because, after a 12 h fermentation with yogurt starter bacteria, the soy beverage yogurt had approximately four times more *Bifidobacterium* RBL 00079 than cows' milk yogurt. In other cases, however, milk constituents have been shown to be stimulatory to probiotic strains. For example, whey permeate (a by-product of the cheese industry, consisting mainly of lactose, minerals, and nitrogen compounds) was shown to increase bifidobacteria growth during pH-controlled batch culturing when used as a supplement in MRS medium.[119]

A variety of approaches have been used to improve the growth of bifidobacteria in milk-based media, which include the addition of growth factors.[108] These growth factors, referred to as *bifidus factors*, are thought to be present in the intestines of breast-fed infants, and are considered to be responsible for the predominance of bifidobacteria in the intestines of an infant.[108] A variety of complex oligosaccharides (termed *prebiotics*), many of which are found naturally in human milk, are also thought to be responsible for promoting the growth of bifidobacteria.[120] Studies have indicated that consumption of these complex carbohydrates can result in significant increases in bifidobacteria numbers in the human gut microflora and in feces.[121–123] The definition of a prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health."[124] Oligosaccharides, such as inulin and transgalactooligosaccharides, are used as bifidogenic oligosaccharides in the food industry.

The term *synbiotic* is used to describe the combination of a probiotic and a prebiotic, and is defined as "a mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/or by activating the metabolism of one or a number of health promoting bacteria."[125–127] Foods such as chicory, garlic, onion, Jerusalem artichoke, and asparagus all contain fructooligosaccharides, and these have been associated with improvements in the human gut, including increased lactobacilli, bifidobacteria, and short-chain fatty acid levels, as well as decreased clostridia, fusobacteria, bacteroides, and pH levels.[128] Prebiotics have also been suggested to have a beneficial effect on reducing blood lipids, although clinical evidence so far has been limited.[128]

In addition to oligosaccharides and carbohydrates, other biologically complex materials such as bovine casein digest, yeast extract, amino sugars, and peptides have been examined in efforts to improve the growth of bifidobacteria.[108,113,115] Poch and Bezkorovainy demonstrated that yeast extract and bovine casein digest promoted the growth of bifidobacteria in TPY media.[113] Klaver et al. reported that 15 out of 17 *Bifidobacterium* strains tested grew poorly in milk, which was attributed to lack of proteolytic activity in these strains, whereas addition of casitone (casein hydrolysate) or a mixture of amino acids to the milk-based medium resulted in good growth of most of the strains.[108] Another approach has involved the coculturing of proteolytic species, such as lactobacilli with bifidobacteria, which has resulted in the enhanced availability of sufficient nitrogenous compounds, leading to improved growth of bifidobacteria in milk.[108] For example, *B. lactis* demonstrated enhanced growth when co-cultured with *Lb. acidophilus* in milk.[116] Bifidobacteria species have also shown variable growth in media, as studies by Desjardins et al. have indicated that bifidobacteria of infant origin grow much better in milk than those of adult origin;[115] further, Poch and Bezkorovainy indicated that bifidobacteria species of infant origin grow better than those of adult origin in the presence of growth factors.[113]

Cell immobilization is a method of retaining microorganisms in a specific location of a fermentation system in order to reach high cell concentrations.[129] Cell entrapment in a food-grade porous matrix is an immobilization technique used for food applications. The immobilized cells are incubated in a nutritive medium resulting in

high cell density. Biofilm-type cell growth within the gel beads results in a high rate of cell release into the fermentation medium due to pressure from cell expansion, collisions, and shearing forces in the bioreactor.[129] An example of this system is the immobilization of *B. infantis* in κ-carrageenan/locust bean gum gel beads; these were used to continuously ferment skim milk supplemented with 1% yeast extract. A maximum cell volumetric productivity of approximately 1×10^{12} CFU/L/h was achieved in the cultured milk.[130]

2.7.2 Culturing of Lactobacilli

Lactobacilli are extremely fastidious, adapted to complex organic substrates, and for energy, they require carbohydrate and carbon sources, as well as amino acids, nucleotides, and vitamins.[131] The composition of milk satisfies some of the growth requirements of lactobacilli, containing more than 87% water, ~4.7% lactose, ~3.8% fat, ~3.3% protein, ~0.2% citrate, and ~0.6% minerals.[15] Typically, lactobacilli can be cultivated quite successfully in milk, and will reach maximum numbers after 24 h incubation at 37°C.[132,133] Lactobacilli grown in milk can usually reach levels of up to 10^8 or 10^9 CFU/mL, by which time they will also have entered the stationary phase.[132] During the fermentation of milk with lactobacilli, the pH is typically in the range of 3.9 to 4.4. Thus, in some cases, it is the acidity of the final fermentate that can prove inhibitory to lactobacilli when cultured in milk-based media, whereas it does not affect the survival of the aciduric or acid-tolerant species of lactobacilli.[131] At this point, fermentation is usually stopped by cooling and/or neutralization, which will prevent acid injury[132] during subsequent processing or storage.

In cases where the probiotic does not grow well in milk, the level of inoculum can be increased to overcome the shortfall in numbers, or the milk can be fortified with various additives to promote growth of the culture. Additives such as tomato juice,[134,135] casein peptone,[134] whey protein,[136] sucrose,[137] papaya pulp,[138] manganese and magnesium ions,[139] simple fermentable sugars,[140] and a combination of casitone and fructose[141] have all been used to promote the growth of lactobacilli in milk. In a study by Saxena et al., supplementation of milk with a combination of casitone and fructose enhanced viable numbers of *Lb. acidophilus* by 1.5- to 2.0-fold during 21 d of storage. These supplements effectively reduced the generation time for all *Lb. acidophilus* strains tested, and enhanced acid production and sugar utilization when compared with growth and metabolism of the control culture.[141] In a similar study by Rana et al., maximum growth of *Lb. acidophilus* was obtained in skim milk supplemented with 0.5% yeast extract and 1.0% glucose, among various whey and skim milk preparations.[142] Continuous neutralization of the medium during fermentation to the initial pH (6.5) at periodic intervals (8 h) resulted in a further increase in cell numbers.[142]

Not all strains of lactobacilli perform similarly in milk during growth and storage, indicating that performance of strains should be evaluated individually prior to commercial use. For example, in a study by Sanders et al., the performance of six commercially available lactobacilli in fluid milk was compared during storage at 4°C and 10°C for 21 d, and during frozen storage at –20°C for 6 weeks. The cultures tested remained stable in fluid milk with less than a tenfold decline in numbers;

however, during frozen storage, performance of the cultures varied, with no significant change in viability for four of the six strains, whereas the numbers of two of the strains were reduced by more than tenfold following 6 weeks of frozen storage.[143]

If probiotic lactobacilli are to be added as adjunct cultures to fermented dairy products such as yogurt and cheese, it must be considered that living bacteria will interact with their environment. The chemical makeup of the dairy product is, therefore, an essential element when considering the metabolic activities of the probiotic.[15] This means that essential variables for the propagation of live microorganisms in milk and milk products are the type and quantities of available carbohydrates, and the degree of hydrolysis of milk proteins and lipids.[144,145] On the other hand, the proteolytic, lipolytic, and saccharolytic properties of probiotics in milk-based products would be potentially important for further degradation of proteins, lipids, and complex carbohydrates, leading to changes in the flavor of the dairy product. From a commercial point of view, it is essential that the flavor and texture of the probiotic fermented product remains appealing to the consumer.

2.8 PHYSIOLOGICAL FACTORS AFFECTING GROWTH AND SURVIVAL OF PROBIOTICS IN FUNCTIONAL FOODS

A number of physiological traits have been identified that make the incorporation of probiotic lactobacilli and bifidobacteria into dairy foods difficult, and methods are being sought to overcome such constraints. This section examines some of those most important characteristics of probiotic microorganisms, such as their acid, bile, and oxygen stress tolerance.

Essential determinants in the choice of a suitable probiotic *Lactobacillus* strain for commercial use are the ability to survive transit through the small intestine, and bile tolerance.[146] The terminal ileum and colon have proven to be the sites of colonization for intestinal lactobacilli; however, little data is available on the resistance of potential probiotic lactobacilli to small intestinal secretions.[146] Lactobacilli of intestinal origin appear to be more bile resistant than those of fermented food origin.[147] An estimate of the numbers of *Lb. acidophilus* cells in a fermented milk capable of surviving intestinal transit was reported by Marteau et al. to be 1.3 to 1.5% of an oral inoculum.[148] Interestingly, lactobacilli have shown strain variation in their resistance to bile salts, a trait that is considered important for the selection of probiotic lactobacilli. Many lactobacilli are able to deconjugate bile acids,[149,150] using the enzyme bile salt hydrolase, although the significance of this activity in vivo is not completely understood. *Lactobacillus plantarum* is used in dairy, meat, and vegetable fermentations, as it is a robust strain capable of surviving passage through the stomach, can reach the ileum in high numbers, and is detectable in the colon.[151] The genetic responses of a sequenced *Lb. plantarum* strain, aimed at the identification of proteins important for bile salt resistance, were described by Bron et al.[152] Thirty-one genes induced by bile were detected in this strain. The genes had cell membrane-associated functions (possible exporters of bile), cell wall-associated functions (possible defense mechanisms), functions involved in redox reactions, and regulatory functions. In other studies, it was found that general stress proteins (such as molecular chaperones and heat shock proteins) are induced by bile stress. It has

been observed in several bacteria, including *Listeria monocytogenes* and *B. adolescentis*, that the pretreatment of cells with high temperatures or detergents (inducing heat-shock proteins) can offer cross-protection against bile.[153,154]

Acid tolerance is important to survive passage through the GIT, and also for probiotic survival in fermented foods.[8] Lactobacilli, particularly those of intestinal origin, are considered intrinsically resistant to acid environments; however, the level of tolerance is strain-dependent and pH-dependent. Even at pH ≥ 2.0, *Lb. acidophilus* is able to maintain cytoplasmic pH at values near neutrality.[155] Upregulation of genes involved in stress protection, such as F_0F_1-ATPase (an important element in the response and tolerance to low pH in *Lb. acidophilus*),[156] can produce dramatic changes in culture performance. For example, acid adaptation of *Lb. acidophilus* has been successfully used to enhance survival of the culture in normally lethal acid conditions and in yogurt.[157] The presence of glucose in in vitro simulated gastric juice was shown to enhance the survival of *Lb. rhamnosus* GG from 6.4 to 8 $\log_{10}$ CFU/mL.[158] It was determined in this study that glucose provided the ATP required by F_0F_1-$ATP_{ase,}$ permitting optimal proton (H^+) extrusion, thereby maintaining pH homeostasis in acidic conditions.

Because the intestinal tract is considered to be the natural environment of many probiotic bacteria, the oxygen content and redox potential of their growth medium must be considered.[15] Oxygen can easily dissolve in milk. Thus, viability in fermented dairy foods is influenced by oxygen content in the product in addition to oxygen permeation through the package. Dave and Shah[159] showed that survival of *Lb. acidophilus* in yogurt was directly affected by the dissolved oxygen content, which was found to be higher in yogurts made in plastic containers than in glass. Thus, it may be important to store the products in glass containers or to increase the thickness of the packaging materials.[157] Also, if ascorbic acid is added, it acts as an oxygen scavenger, decreasing the redox potential and supporting the viability of lactobacilli.[159]

Interestingly, two antioxidative strains of lactobacilli, hesitantly identified as *Lb. fermentum*, were isolated from the intestinal microflora of a healthy child.[160] Survival time of these strains in the presence of reactive oxygen species was significantly increased, compared to that of a nonoxidative strain. The antioxidative capacity of *Lb. casei* KCTC 3260, isolated from a milk product, was reported to be caused by high iron and copper chelating activity, instead of superoxide dismutase (SOD) production.[161] Such resistance to oxidative stress may enhance the survival of these potential probiotic lactobacilli in both the intestinal microbial ecosystem and under exogenous oxidative stress conditions. Also, understanding the molecular mechanisms governing the resistance of these lactobacilli to oxidative stress could potentially lead to the development of other aerotolerant probiotic lactobacilli.

Similar to lactobacilli, bifidobacteria also show considerable strain variation in their resistance to acid and bile stress.[162] Selection of probiotic bifidobacteria is sometimes limited by their intrinsic inability to survive harsh conditions in the gut and the acid conditions of fermented foods. The conventional view is that the acid tolerance of bifidobacteria is weak; however, in a study by Matsumoto, Ohishi, and Benno, it was reported that strains of *B. lactis* and *B. animalis* were stable at pH 3 to 5 for 3 h compared to nonacid-tolerant strains.[163] The pH of gastric juice has to be around 3 to 4 for the digestion of food,[164] and therefore *B. lactis* and *B. animalis*

have sufficient acid tolerance to reach the intestinal tract after their oral administration. These authors also found that under acidic conditions the H^+-ATPase activity of the nonacid-tolerant strains decreased, whereas the H^+-ATPase activity of the acid-tolerant strains rapidly increased, suggesting that the nonacid-tolerant strains were damaged in the acidic environment, and subsequently were unable to discharge H^+ in order to maintain a constant intracellular pH.[163] In a study by Clark and Martin, it was reported that *B. longum* could survive bile concentrations as high as 4.0%.[165] A similar result was reported in a study by Guerin, Vuillemard, and Subirade, where *B. bifidum* free cells survived well after 3 h of incubation at 37°C in 2% and 4% bile.[166] Further, they demonstrated that *B. bifidum* cells immobilized in gel beads with a double membrane coating, enhanced the cell resistance to bile. Microencapsulation of sensitive bifidobacteria in protein or polysaccharide gel beads has been reported in many studies to provide protection during simulated gastrointestinal transit.[167,168]

As with lactobacilli, environmental adaptation of bifidobacteria may prove to be an important survival mechanism during exposure to a variety of stresses. The ability of *Bifidobacterium* spp. to adapt to acid stress, and the general resistance of acid-adapted cells to other environmental stresses (including bile salts, H_2O_2, and cold storage), were investigated by Hee et al.[169] Acid adaptation of *B. breve* ATCC 15700 (pH 5.2 for 2 h) was found to enhance survival of the culture 100-fold at pH 2.0 for 60 min, compared to the survival of an unadapted control culture. Furthermore, acid-adapted cells were also better able to survive exposure to normally lethal conditions of H_2O_2 (1000 ppm for 60 min) and cold storage (4°C for 7 d). Similarly, bile salt adaptation of *B. adolescentis* NCC251 generated homologous protection against normally lethal concentrations of bile salts, in addition to stimulating cross-protection against freeze thawing cycles and lethal heat stress.[154] A possible relationship between cell surface hydrophobicity (CSH) and this tolerance to environmental stresses in *Bifidobacterium* spp. has been suggested.[170] CSH was determined using bacterial adherence to hydrocarbons assay (BATH) for seven bifidobacteria strains, where it was found that strains with the highest CSH demonstrated significantly more resistance to stresses such as bile salt, H_2O_2, heat, cold storage, and acid.

Because bifidobacteria are anaerobic, oxygen toxicity is also an important consideration. In a study by Lin and Chang, both intact cells and intracellular cell-free extracts of *B. longum* demonstrated antioxidative activity.[171] Ahn Jun Bae et al. examined the effect of oxygen stress on *B. longum*, and found that in the presence of oxygen, the lag phase became extended and cell growth was limited.[172] Morphology during oxygen stress was also altered, with the *Bifidobacterium* cells becoming longer in size, and the formation of nodes on the surface of the cells due to incomplete cell division was also observed. Cellular fatty acid profiles changed, such that the carbon chain was shortened and dimethyl acetals originating from plasmalogen were reduced, whereas cyclopropane fatty acids were increased. This group also identified a 35.5 kD protein, Osp, which was upregulated in an oxygen tolerant *Bifidobacterium* strain, and it was considered that the protein may have a role in defense against oxygen stress.

Encapsulation techniques, namely extrusion and emulsion, to encapsulate probiotics for their use in fermented food products have been investigated to protect the bacteria in the environment of the food, and to improve their survival levels. The

minimum level of viable probiotic bacteria in a food product should be $\geq 10^7$ CFU per milliliter or gram of a product at the time of consumption.[173] Yogurt is one of the main probiotic carriers, yet poor viability of probiotic bacteria, particularly bifidobacteria, has been reported in yogurt[174,175] due to the effects of hydrogen peroxide, dissolved oxygen, and acidity. Gel entrapment of probiotic cultures using natural biopolymers, such as calcium alginate, κ-carrageenan, and gellan gum, has been used most extensively. Enhanced cell protection and survival in products, and ultimately in the human intestine using the immobilized cell technology have been described by several authors.[129,166,168,173,176,178]

Two-stage fermentation is another method that has been used to improve the survival of probiotic cultures. Yogurt starter cultures, which are essential in yogurt production, produce unfavorable substances such as hydrogen peroxide and acids during fermentation that have inhibitory effects on probiotics. One way to overcome this is to perform the initial fermentation with probiotic cultures, ending with fermentation using yogurt cultures such as *S. thermophilus* and *Lb. delbrueckii* subsp. Bulgaricus.[179] An increased number of probiotic counts has been reported in products prepared by the two-step fermentation process.

2.9 CHALLENGES ASSOCIATED WITH THE DEVELOPMENT OF DRIED PROBIOTIC CULTURES

Food products or supplements containing viable probiotic strains are frequently supplied to the market in a lyophilized form.[180] The preservation of microorganisms by desiccation has been used for decades. Probiotic cultures are typically supplied in dried form for use in traditional food and as starter cultures. The object of producing dried preparations of probiotic cultures is to enable long-term storage, while preserving cell viability, as well as for convenience in handling, marketing, and consumption.[132] Freeze drying and spray drying are the most frequently used drying methods.[181] This section will focus on the freeze drying and spray drying of probiotic lactobacilli and bifidobacteria, and the factors that need to be considered to achieve high levels of viability of these cultures, including cell growth phases and concentration, growth media, protective agents, stress induction, rehydration, and storage conditions.

2.9.1 Freeze Drying

Freeze drying is the preferred method for preservation of lactic acid starter bacteria and for culture collections worldwide. The process involves the removal of water from the product by sublimation and desorbtion. There are three main phases:

1. *Freezing:* The mobile water of the product is frozen.
2. *Primary drying:* Frozen moisture is removed by sublimation of the ice crystals to water vapor (achieved under a high vacuum).
3. *Secondary drying:* Temperature is increased to desorb bound water, such as water of crystallization, until the residual water content falls to the range required for optimum product stability.

Loss of viability during freeze drying is associated with stress that is induced by temperature changes, phase changes, and drying, a combination of which tend to damage cell membranes and proteins. The process of freezing and the rate of freezing can be detrimental to the viability of the bacterial cell after drying.[182] Also, freeze drying is a lengthy process, therefore more expensive than other drying methods. When applied to the preservation of bacterial cultures such as lactobacilli[183] and bifidobacteria,[184] much of their activity is typically lost after a few weeks of storage at room temperature.

2.9.2 Spray Drying

Spray drying is the most common process used in the dairy industry, as it produces a higher number of viable bacteria at relatively low cost (compared to freeze drying),[9] and the resulting powders are dry, stable, and easily transported.[183] Spray drying involves the transformation of feed from fluid into a dried particulate form by spraying the feed into a hot drying medium. The feed solution is atomized and introduced to the drying chamber along with hot air. The mixture of hot air and atomized feed moves towards the air exhaust of the drying chamber; the time taken for this to happen is called the *residence time*. During this residence time, the feed droplets lose moisture to hot air, and the resultant dry powder falls onto a conical portion of the drying chamber, slides down through a rotary valve located at the bottom of the chamber, and is collected in a collection bag/bottle.

The *outlet temperature* is the temperature at which the product leaves the drying chamber, and is said to have a major effect on the viability of the culture. Desmond et al. reported that higher stability was achieved during storage of spray-dried powder produced at a lower outlet air temperature.[185] However, it has also been reported that if the air outlet temperature is too low, the residual moisture content of the powder will exceed 4%, the level required for prolonged storage life, stability, and spoilage prevention.[186,187] Several studies have used the spray drying process for preservation of probiotic lactobacilli and bifidobacteria, including, *Lb. paracasei*,[187,188] *Lb. rhamnosus* GG,[189] *Lb. curvatus* and *Lb.* sp. 8Z,[190] *Lb. acidophilus*,[132] *Lb. bulgaricus,*[191,192] *Lb. helveticus*,[183] *Lb. salivarius*,[187] *B. ruminantium*,[193] and *B. longum*.[194]

Problems associated with spray drying are common, such as, low survival rates during drying, low stability under storage, and difficulties in rehydrating the product.[190] During spray drying of cells, low survival rates may arise due to dehydration and high temperature, leading to injury or death of bacterial cells.[195–198] Previous reports have shown that the destruction of bacteria during heat stress and spray drying cannot be ascribed only to a thermal effect, but, also to a nonthermal effect caused by loss of bound water at the cell surface.[199] Teixeira et al. and Daemen and Vanderstege found that probiotic lactobacilli showed increased sensitivity to lyzozyme and NaCl (indicators of cell wall and cell membrane damage) following spray drying.[191,199] Other possible sites in the cell where damage may occur as a result of spray drying include DNA[200] and ribosomes.[201]

The optimal growth phase of bacteria for survival of the drying processes is largely strain dependent. However, a number of studies have suggested that stationary phase cells appear to give the highest recovery after drying and during storage.

Stationary phase cells of *Lactobacillus rhamnosus* GG were found to be most suitable for the spray drying process, as a recovery of 31 to 50% was achieved, in comparison to early log phase cells, which exhibited 14% survival. Lag phase cells showed the highest susceptibility, with only 2% survival.[189] Similar results were reported by Teixeira et al., where higher numbers of viable cells were obtained by spray drying *Lb. bulgaricus* in their stationary phase.[191] Prasad et al.[202] reported that the storage stability of *Lb. rhamnosus* HN001, which was heat-shocked after the stationary phase, was superior to that of the culture that was heat-shocked after the log phase. This suggests that the stationary phase induces various physiological states within the cells, similar to starvation conditions and glucose depletion, that trigger multiple stress responses to allow survival of the cell population.[202]

There are a few reports outlining the optimal cell concentration required for viability after spray drying or freeze drying. A common principle was that the higher the initial cell concentration, the longer the shelf life of the product, when the optimal initial concentration was related to the protective medium used during drying.[203] In other words, a lower initial cell concentration is required for optimal freeze-spray drying recovery, when increased concentrations of protective additives are used within the protective medium. To overcome inactivation during drying and poor stability during storage, studies have concentrated on the use of protective additives and the content of the growth media used. Such studies included the incorporation of thermoprotectants such as trehalose,[204] nonfat milk solids and/or adonitol,[189,205] and growth-promoting factors, including various probiotic/prebiotic combinations[185,189,206,207] and granular starch.[208]

To improve the viability of *Lb. paracasei* NFBC 338, this culture was grown in a mixture of reconstituted skim milk (RSM) and gum acacia (GA) prior to spray drying. The results of this study indicated that the incorporation of GA significantly increased the protection of the probiotic culture during drying, storage, and when exposed to porcine gastric juice, compared to the control culture grown in milk powder alone.[185] The presence of the prebiotics polydextrose and inulin did not enhance the viability of *Lb. rhamnosus* GG during spray drying or powder storage.[189] However, high-viability synbiotic powder products were developed, which may be useful as functional food ingredients.

Skim milk has been shown to be a good carrier for bifidobacteria, as *B. longum* achieved about 82.6% survival after spray drying with skim milk. In addition, bifidobacteria showed the highest survival after drying with an outlet air temperature of 50°C.[209] Simpson et al. also found skimmed milk to be a suitable carrier for *Bifidobacterium* species, and the addition of GA had no significant effect on survival or viability.[210] Cryoprotectants can be added during the growth of lactobacilli and bifidobacteria, or prior to freeze drying. These include nonfat milk solids, serum, trehalose, glycerol, betaine, sucrose, glucose, fructose, mannose, lactose, and polymers such as dextran and polyethylene.[211] Many studies suggest that the use of trehalose enables higher survival of microorganisms during freezing and drying.[212–214] It has been suggested that the most efficient dessication-protective agents are a mixture of proteins and sugars.[215] This is supported in a study by Desmond et al., which showed that survival of *Lb. paracasei* increased up to 1000-fold, when 10% GA was added to 10% RSM.[185]

Encapsulation, as a protection mechanism, allows the active core ingredient, or substrate, to be separated from its environment by a protective film. This separation occurs until the release of the functional ingredient is desired. In the case of probiotics, this would be in the jejunum and ileum in the human body.[209] Encapsulation offers protection for live cells from extremes of heat or moisture, such as those encountered during drying and storage. Selmer-Olsen et al. found that encapsulating lactobacilli in calcium alginate beads, improved their heat tolerance.[205] Subsequently, this technology was used to prolong the viability during storage of a spray-dried *B. ruminatum*.[193] In this study, the spray coating process for the production of starch-encapsulated bifidobacteria was optimized; however, the strain was not protected against adverse environmental conditions, such as acid shock or storage in two dried food preparations. In a study by Lian et al., four *Bifidobacterium* strains were successfully spray-dried and encapsulated in gum acacia, gelatin, and soluble starch.[194] Survival of these probiotic bacteria varied with strains and was highly dependent on the carriers used (Figure 2.1).

Control of the resistance of probiotics to stresses (such as heat, oxygen, and acid stress) may have potential practical benefits in industrial fermentation processes in which bacteria with enhanced stress tolerance are required. After a nonlethal heat shock, bacteria are able to tolerate a second heat stress higher in intensity due to heat-induced thermotolerance.[216] Teixeira et al.[217] and Gouesbet and Boyaval[218] reported that heat adaptation increased the thermotolerance of lactobacilli. Similarly, a heat-adapted probiotic, *Lb. paracasei* NFBC 338, exhibited greater thermotolerance (survival at a lethal temperature of 60°C), than controls in MRS and RSM, and during spraying (Figure 2.2a), viability of the adapted culture was enhanced 18-fold.[188] Bacterial survival of a specific stress can also be improved by pretreatment of cells to a sublethal heterologous condition such as moderate temperature, low pH, or moderate osmolarity. The positive effect of suboptimal growth temperature (25°C) on resistance of *Lb. acidophilus* to environmental stresses such as freezing, heating, osmotic stress, and exposure to ethanol, peroxide, or acid was reported by Lorca and de Valdez.[219] In addition, Gouesbet

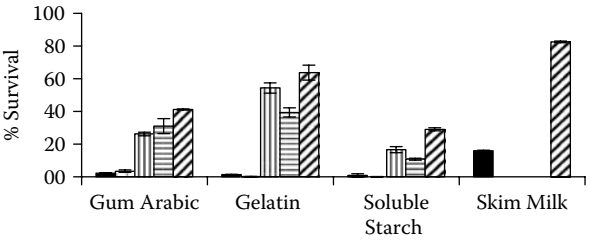

FIGURE 2.1 Survival of bifidobacteria after spray drying with various carriers, where the outlet temperature during drying was 50ºC. The five strains tested were *B. infantis* CCRC 14633 (■), *B. infantis* CCRC 14661 (▫), *B. longum* ATCC 15708 (▥), *B. longum* CCRC 14634 (▤), and *B. longum* B6 (▨). The survival of *B. infantis* CCRC 14661, *B. longum* ATCC 15708, and *B. longum* CCRC 14634 was not determined using skim milk as the carrier material. (Adapted from Lian, W.C., Hsiao, H.C., and Chou, C.C., Survival of bifidobacteria after spray-drying, *Int. J. Food Microbiol.*, 74, 79–86, 2002. With permission.)

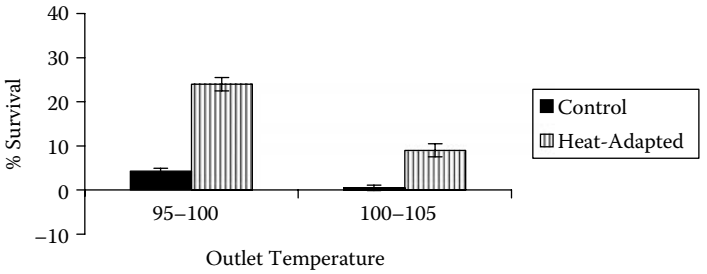

FIGURE 2.2A Percentage survival (CFU/g) of control and heat-adapted (52°C × 15 min) *Lb. paracasei* NFBC 338 during spray drying at outlet temperatures of 95–100°C and 100–105°C.

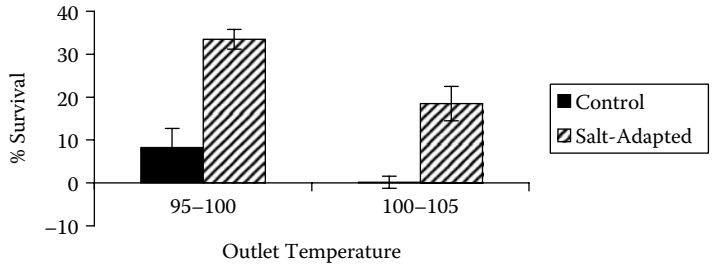

FIGURE 2.2B Percentage survival (CFU/g) of control and salt-adapted (0.3 M NaCl × 30 min) *Lb. paracasei* NFBC 338 during spray drying at outlet temperatures of 95–100°C and 100–105°C. (From Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., and Ross, R.P., Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying, *Int. Dairy J.*, 11, 801–808, 2001. With permission.)

and Boyaval[218] and Desmond et al.[188] demonstrated the acquisition of cross-stress tolerance to heat by exposure to mild osmotic stress. The effect of salt adaptation (0.3 M NaCl for 30 min) on the heat resistance of *Lb. paracasei* NFBC 338 during spray drying (Figure 2.2b) was also investigated, and it was found that there was a 16-fold higher viability of the salt-adapted culture compared to the viability of an untreated control culture dried under the same conditions.[188]

Advances in genomics and proteomics have made both lactobacilli and, to a lesser extent, bifidobacteria more amenable to genetic manipulation by increasing their viability when exposed to stressful environments. For example, overexpression of the heat-shock protein chaperones, GroES and GroEL, in *Lb. paracasei* NFBC 338 improved its ability to withstand thermal stress, but it did not perform as well as the heat-adapted parent culture.[220] This is expected however, as the heat-shock response involves a consort of proteins including DnaK, DnaJ, and GrpE; tagatose-6-phosphate-aldolase; glyceraldehyde-3-phosphate dehydrogenase; and trios phosphate isomerase.[202] Interestingly, the GroESL-overproducing strain also exhibited increased solvent tolerance, and showed itself capable of growing in the presence of butanol (0.5% v/v) for over 5 h, whereas the viability of the parent strain declined.[220] It was found in *B. longum* (an oxygen-tolerant strain) that a protein,

Osp, was upregulated in response to an oxygen stress,[178] and in *Lb. acidophilus*, genes involved in the acid stress response, such as F_1F_0-ATPase, were upregulated in response to a low pH.[162] These genes may provide suitable targets for research on improving oxygen tolerance and acid tolerance in functional food development, and in the intestinal microbial ecosystem.

The final critical step in the revival of cells after drying is rehydration. Rehydration conditions and procedures have a significant influence on the recovery and viability of dried cells.[221] Variations can be found between strains and species that make it impossible to use a universal rehydration medium for freeze-dried cells.[222] It has been proposed that the same rehydration solution used for cryopreservation increases viability, as a high recovery of *Candida sake* cells from a freeze-dried sample was achieved when 10% skimmed milk (used as a protectant) was used as a rehydration medium.[223] This idea had already been proposed by Ray et al., who reported that using a rehydration fluid that provides a high osmotic pressure to the freeze-dried cells provided a degree of control to the rehydration process (i.e., preventing osmotic shock).[224] These authors also found that temperature influenced the recovery of freeze-dried cells, as rehydration at 15 to 25°C produced the highest numbers of recovered cells compared to those at 35°C and 45°C, where the cell recovery was lower but the growth more rapid.[224] Regarding spray-dried cultures, only small or insignificant differences in the recovery of cells were found;[185,225] however, it has been reported that rehydration temperature has an influence on recovery. The viability of *Lb. delbrueckii* subsp. Bulgaricus increased linearly with temperature between 4°C and 50°C.[225]

The methods of storage and the packaging used also influence the shelf life of any dried probiotic products, and the most common factors to avoid are oxygen, moisture, light, microbial contamination, and high temperatures.[215] The storage stability of freeze-dried cultures is higher at lower temperatures, and under an oxygen-free atmosphere,[226,227] and the storage stability of spray-dried cultures is also inversely related to temperature.[185,228] During storage, membrane lipid oxidation is a detrimental factor to stability. The change in the degree of unsaturation of lipids strongly affects the passive permeability of the membrane,[229] and the change in lipid composition of the cell membrane was found to increase with time.[230] To prevent lipid oxidation, the addition of antioxidant agents to scavenge free radicals, such as ascorbic acid or monosodium glutamate, has been used. These agents had a protective effect on *Lb. bulgaricus* cells at low temperatures (4°C), but as the temperature was increased (20°C), the death rate of the culture became higher in the presence of these compounds than in the control.[225] Therefore, these compounds can act as both antioxidants and prooxidants, and the prooxidant activity seems to be temperature-dependent. Protective agents added to the media prior to the drying processes can also protect the cultures during the storage period.[185,189] Packaging materials must prevent exposure to light, moisture, and oxygen. The viability of *S. thermophilus* and *Lb. bulgaricus* was compared after storage in air and after storage in nitrogen under vacuum, and it was found that storage within glass vials sealed under vacuum or nitrogen gas was superior to storage in air.[226] Other studies reported that laminated pouches were better than glass for storage of *S. thermophilus* and *B. longum*.[231]

2.10 PROBIOTIC PRODUCT DEVELOPMENT

Fermented foods are the most popular food delivery system for probiotic organisms,[232] with yogurt being the most common product. Other fermented products used for delivery of probiotics include soft-, semi-hard, and hard cheese, ice cream, and frozen fermented dairy desserts. The most commonly used LAB considered as potential probiotics include lactobacilli and bifidobacteria.[233] From a food processing perspective, it is desirable that such strains be suitable for large-scale industrial production by being capable of withstanding the processing conditions described above, such as freeze drying or spray drying. Bifidobacteria are extremely fastidious; they are generally sensitive to oxygen and, therefore, pose particular challenges for incorporation into food products. Process modifications, which limit oxygen stress, and the use of growth factors to ensure cultivation to high numbers for inoculation purposes, are therefore desirable when working with these organisms. From a cultivation point of view, a *Bifidobacterium* strain should ideally be acid- and oxygen-tolerant, and should rapidly grow and acidify milk so as to reduce the incubation time necessary, thus limiting the possibility of contamination.[107,115]

Incorporation of lactobacilli into the food chain can also be difficult. An important technological reason for the use of dairy products as carriers of lactobacilli is that many of them have already been optimized to some extent for survival of live fermentation microorganisms. Thus, the existing technologies can be readily adapted to allow the incorporation of probiotic lactobacilli.[15] There are, however, problems associated with the incorporation of lactobacilli into milk-based products. Some of the biggest drawbacks include the poor temperature, salt, oxygen, acid, and bile tolerance of many species. Solutions to some of these problems include selection of acid- and bile-resistant strains, use of oxygen-impermeable containers, two-step fermentation, microencapsulation, stress adaptation, incorporation of nutrients such as peptides and complex carbohydrates,[157] and overexpression of genes involved in bacterial survival.[220]

Functional foods (foods containing probiotics, which claim to have a positive effect on health) have gained popularity and acceptance worldwide as a number of these products are available commercially, and the range of such products continues to expand. In the sections below, physiological constraints associated with probiotic cultures, and the technological challenges associated with the development of some fermented dairy products incorporating these probiotic strains—in particular, probiotic lactobacilli and bifidobacteria—are described.

2.10.1 Yogurt and Fermented Milk Drinks

Several health benefits have been reported for traditional yogurts,[234–236] making them an obvious choice as carriers for probiotic bacteria. *S. thermophilus* and *Lb. delbrueckii* subsp. Bulgaricus are generally used as the starter cultures for the manufacture of yogurt. Probiotic bacteria grow slowly in milk, hence yogurt starter cultures are added to speed up the fermentation process, and the probiotic cultures such as *Lb. acidophilus*, *Bifidobacteriu*m spp., and *Lb. casei*, are subsequently added as adjunct cultures.[237] When probiotic and starter cultures are both present during the fermentation stage it is important to use compatible and suitable blends of

probiotic/starter cultures.[238] In some cases, the starter cultures may produce inhibitory substances such as hydrogen peroxide or lactic acid, adversely affecting probiotic viability.[157,239] In other cases, the starter cultures may enhance the growth and survival of probiotics by producing growth-promoting factors or by reducing the oxygen content in the milk.[157,159,239]

There has been a significant increase in the production of "bio-yogurts," which contain *Lb. acidophilus* and *Bifidobacterium* spp. (known as AB-cultures)[240] *Lb. acidophilus*, *Bifidobacterium* spp., and *Lb. casei* (known as ABC-cultures),[241] in addition to the traditional yogurt starter cultures, *S. thermophilus* and *Lb. bulgaricus*. For the manufacture of these probiotic yogurts, it is particularly attractive that conventional yogurt processing procedures can be applied with the probiotic bacteria added prior to fermentation along with the yogurt starter cultures, or after fermentation to the cooled product before packaging.[241] The methods used to manufacture stirred yogurt and drink yogurt, in particular, are well suited to the addition of probiotics after fermentation.[15]

The viability of probiotic bacteria in yogurt and fermented milk drinks can be unstable, depending on a number of factors, including the types of strains used and the production method; the interaction between the bacterial species present; the chemical composition of the fermentation medium; milk solids content; the inoculum size; fermentation time; final acidity of the product; availability of nutrients, growth promoters, and inhibitors; the concentration of sugars; dissolved oxygen content (particularly for *Bifidobacterium* species); and storage temperature of the fermented dairy product.[157,240,242,243–245] Live cultures of probiotic lactobacilli and bifidobacteria have been reported to remain viable in yogurt at levels of $\geq 10^6$ CFU/g during refrigerated storage.[246,247]

The effect of refrigeration on the viability of lactobacilli in fermented milk and yogurt was examined by Nighswonger et al., where it was found that three out of five strains showed no significant loss of viability during storage.[243] Similarly, in a number of commercially available probiotic yogurts, viable numbers of lactobacilli varied greatly during refrigerated storage; the majority contained viable counts greater than 10^5/g even at the end of shelf life (about 2 or 3 weeks in most cases).[175] After 20 h of fermentation of goat milk with *B. animalis* and *Lb. acidophilus*, maximum viable counts of 10^7 CFU/mL and 10^8 CFU/mL were reached, respectively, and during refrigerated storage both strains exhibited good survival levels with viable numbers remaining essentially constant throughout the experiments.[248]

Microencapsulation has applications in yogurt products. In the encapsulated form, probiotics are protected from bacteriophage and harsh environments, thereby improving the manufacture of fermented dairy products, as bacteria can maintain their characteristics, have higher stability during storage, and higher productivity than free cells (i.e., nonencapsulated cells). In a study by Sultana et al., microencapsulation of *Lb. acidophilus* in an alginate–starch mix was found to protect the culture from acid stress, and to enhance viabilty of the culture in yogurt by 0.5 log over an 8-week period.[188] The number of *B. breve* R070 cells entrapped in whey protein microcapsules were significantly higher than the number of free cells after 28 d in yogurt stored at 4°C, and after sequential exposure to simulated gastric and intestinal juices.[168] Microencapsulation in κ-carrageenan was used to preserve the viablility of

bifidobacteria in set yogurt for 30 d during refrigerated storage.[173] However, in this case the product was adversely affected, which underlines the need to maintain or to improve the sensory characteristics of a product when reformulating processing conditions.

Bifidobacteria are unable to grow in milk.[108] This is related to the low concentration of free amino acids and small peptides in milk, which are insufficient to support the growth of *Bifidobacterium* spp. Synergistic and growth-promoting reactions between *Lb. acidophilus* and *B. bifidum* in milk are known to occur, providing the necessary growth stimulants for bifidobacteria.[249] The starter culture *Lb. delbrueckii* subsp. Bulgaricus produces essential amino acids due to its efficient proteolytic activity.[250] However, this strain also produces lactic acid during refrigerated storage, and if this happens it causes a loss in viability of the probiotic bacteria. *S. thermophilus* does not inhibit the growth of probiotic organisms; indeed, it may stimulate their growth by the consumption of oxygen. The addition of casein or whey protein hydrolysates, yeast extract, glucose, and vitamins have also been reported to enhance the growth of *Lb. acidophilus* and *Bifidobacterium* spp. in milk.[251,252] Prebiotics are included in numerous probiotic products so as to improve the growth of bifidobacteria in the intestine. The term *synbiotic*s is used when referring to the use of probiotics and prebiotics in combination.[125] Such prebiotics in bio-yogurts have the potential to increase bifidobacteria levels not only in the colon, but also during the shelf life of the product.[208] The rupture of yogurt bacteria can also enhance the viability of probiotic bacteria in milk, as β-galactosidase is released, breaking down the lactose in milk to galactose and glucose, which can then be utilized by the lactobacilli and bifidobacteria strains in the yogurt.[247]

The production of probiotic yogurts generates the need to selectively enumerate both *Bifidobacterium* spp. and probiotic lactobacilli in the initial product after manufacture, and in the product during refrigerated storage. Therefore, in order to assess probiotic viability, there exists a need for simple and reliable methods for routine enumeration of specific probiotic cultures.[157,246] Media used for the differential enumeration of probiotic cultures must take into consideration the type of food, the species or strains to isolate and to enumerate, as well as the nature of the competing genera.[253] Therefore, individual selective media cannot be applied in all situations, and they should be evaluated for the specific strain of interest in a given situation.[253]

Culture media for the enumeration of probiotic bacteria in yogurt can essentially be divided into four main groups:

1. General media that will give an overall total colony count without distiguishing different genera or species, e.g., MRS medium[91]
2. Media for the enumeration of yogurt culture organisms, e.g., Lee's agar, reinforced clostridial agar adjusted to pH 5.3,[157] and M17 agar
3. Media used to selectively grow each probiotic genus, e.g., NA–salicin,[162] MRS–clindamycin,[92] bile medium and media based on X-gluc for the enumeration of *Lb. acidophilus*, LC agar[254] or B–MRS agar[255] for the selective enumeration of *Lb. casei*, and NNLP agar[256] or LP–MRS agar for isolating *Bifidobacterium* sp. (In a study by Nebra et al., the recovery of oxygen-stressed bifidobacteria was increased by the addition of mixtures of

reducing agents including L-cysteine, sodium pyruvate, and sodium thioglycolate and by preincubation for 4 h at 37°C[257])
4. Media that allow the enumeration of all four bacterial types found in yogurts with visually distinguishable colonies on the same plate, e.g., TPPYPB agar[73]

In cases where the concentration of the starter and probiotic cultures are in the same quantitative range, a subtraction method can be used where the number of colonies counted on the selective medium for the probiotic culture is subtracted from the total count of colonies on the medium supporting the growth of both.[90] Molecular tools for the enumeration of probiotics in commercial products are also available, and have already been discussed in parts 3 and 4 of this chapter.

2.10.2 Probiotic Cheese

Probiotic microorganisms must survive the extended storage period required for cheese ripening (many cheese varieties require periods greater than 6 months), as well as the entire shelf life of the cheese, in order to exert their health benefits on the consumer. The manufacture of probiotic cheese necessitates that either the strains grow to high numbers during ripening, or that they are incorporated during manufacture at high levels, and survive the cheese ripening period. During cheese making or maturation probiotic microorganisms must not produce metabolites that would cause defects, and they should not interfere with the activity of other essential microorganisms in the cheese.

In a study by Gardiner et al., the probiotic *Enterococcus faecium* (which has the ability to relieve irritable bowel syndrome) survived to high numbers (4×10^8 CFU/g) in Cheddar cheese during ripening at 8°C for 15 months.[258] In addition, the cheese was particularly suitable for the passage of probiotic bacteria through the GIT, when compared to fermented milk such as yogurt, because of lower acidity and the existence of a complex cheese matrix of protein and fat providing protection. Probiotic cheese with human-derived *Lb. paracasei* have been manufactured without having an effect on cheese composition.[67,106] *Lb. paracasei* NFBC 338 and NFBC 364 grew to 2.9×10^8 CFU/g in mature cheese in 3 months, and maintained the numbers for up to 200 d.[67] A number of other cheese varieties have been investigated as carriers of probiotic microorganisms, including white-brined,[259] goat,[107] Crescenza,[260] cottage,[261] Kariesh,[262] Canestrato Pugliese,[263] fresco,[255] Tallaga,[264] fresh cheese,[265] Ras,[266] and soft.[267]

2.10.3 Frozen Dairy Products

Ice cream, frozen yogurts, and frozen desserts have potential as carriers of probiotic organisms, but the effect of freeze stress on viability during manufacture and extended storage must be taken into consideration. Alterations to cell membrane permeability, and intracellular dehydration caused by ice crystal formation that may rupture cells, are likely causes of microbial inactivation during freezing.[268] Microencapsulation can be used to reduce cell injury or cell loss in these products. In almost all cases, natural biopolymers such as calcium alginate, carrageenan, gellan gum, and chitosan are favored by researchers for gel entrapment of functional dairy

products. For example, in a study by Ravula and Shah, the survival of sodium alginate encapsulated *Lb. acidophilus* in fermented frozen dairy desserts was enhanced by 2 logs when compared with the control (free) cells.[269] Frozen dairy products such as ice cream contain several natural substances with cryoprotective properties, including casein, sucrose, and fat.[270] However, in some cases, additional protection is required to improve the survival of cells during freezing.

The effect of pH[269,271] on probiotic survival and the addition of cryoprotectants such as sucrose[269] and glycerol[270] have also been examined, in an attempt to maintain viability during frozen storage. *Lb. bulgaricus* cells entrapped in beads of calcium alginate survived in continuously frozen ice milk (90% survival), which was much better than cells that were not entrapped (40% survival). Addition of entrapped lactobacilli had no measurable effect on the sensory characteristics of the milk.[272] In a recent study, survival of freeze-dried *Lb. bulgaricus* was enhanced during storage at −20°C over 10 months, when cells had been grown in the presence of fructose, lactose or mannose, or when glucose, fructose, monosodium glutamate or sorbitol was added to the drying medium.[273] Trehalose, a disaccharide of glucose, has also been found to be effective at protecting bacterial cells during freezing.[213]

A Biogarde® ice cream was marketed in Germany in the mid-1980s that was reported to contain 10^8 CFU/g of *Lb. acidophilus*, 10^7 CFU/g of *B. bifidum,* and also *S. thermophilus*.[126] A *B. bifidum* strain was incorporated into the ice cream initially at 2.5×10^8 CFU/mL, and cell numbers of 1×10^7 CFU/mL were maintained for 17 weeks of storage at −20°C.[271] *B. lactis* Bb-12 was also incorporated, and it survived at levels greater than 10^6 CFU/g for 52 weeks of frozen storage without significantly affecting flavor.[270] In addition, frozen storage has been shown to have little effect on the survival of lactobacilli.[274] *Lb. acidophilus* has been found to survive between 10^6 to 10^8 CFU per gram or milliliter in ice cream,[270,271,275] frozen yogurt,[274,276] and other frozen dairy desserts.[256] As with many stress conditions, the ability of lactobacilli to withstand frozen storage is strain dependent.[275] In an attempt to examine the effects of short-term frozen storage on lactobacilli, Holocomb et al. reported that frozen storage at −5°C for 6 h had no adverse effect on the bile salt sensitivity of the organism.[276] The ability of lactobacilli to withstand even longer periods of freezing has also been reported[256,270,271,275] with probiotic viability maintained at ~10^6 CFU per gram or milliliter, for up to 52 weeks at −20°C.

2.10.4 Nondairy Products

Active cultures may be used to add function to foods that are not milk-based, such as mayonnaise,[277] fruit drinks,[278] cereals,[279] and meat.[280] Mayonnaise was manufactured with *B. bifidum* and *B. infantis* as encapsulated cells, which survived to 1×10^5 CFU/g and 1×10^4 CFU/g respectively for 12 weeks.[277] Whereas these bifidobacteria survived only at low levels, their addition to mayonnaise was responsible for reducing the total bacterial count, inhibiting the growth of yeasts and molds for up to 10 d, and also improving sensory properties. *Lb. plantarum* is typically associated with fermented foods of plant origin. Johansson et al. used *Lb. plantarum* in a drink that was made with rose hips and oats, and subsequently demonstrated positive health benefits of this probiotic drink through human-feeding trials.[281] In 1994, a probiotic

functional food (named Proviva) was launched in Sweden that did not contain milk or milk constituents. The probiotic *Lb. plantarum* 299v was used to ferment oatmeal gruel that was mixed in a fruit drink; the consumer product containing $\approx 5 \times 10^{10}$ CFU of *L. plantarum* per liter. This functional food is marketed as being the world's first nondairy probiotic drink.[282]

Dried preparations of probiotics are of particular interest for the manufacture of infant formulae.[283] The gut microflora of breast-fed infants differs from that of formula-fed infants,[284] and therefore there is interest in the development of formula, enriched with probiotic microorganisms as well as prebiotics, for the stimulation of the infant gut microflora. Halpin-Dohnalek et al. reported that the use of three freeze-dried preparations of *Lb. reuteri, Lb. acidophilus*, and *B. bifidum* in infant formula was effective in preventing diarrhea in infants when 10^8 to 10^9 CFU of the three organisms were consumed per day.[285] Many infant formulae include dried prebiotics such as inulin. It was reported that consumption of inulin increased the content of *Bifidobacterium* and *Lactobacillus* in the feces of formula-fed babies, without affecting the number of bacteroides or the total anaerobic count.[286] Other prebiotics used in infant formulae include oligosaccharides[287] and lactulose.[288]

A number of pharmaceutical preparations containing bifidobacteria either alone or in combination with other probiotic strains are available worldwide.[289] Such products include Bifidogène® (*Bifidobacterium* sp.), Synerlac® (*B. bifidum*, *Lb. acidophilus,* and *Lb. delbrueckii* subsp. Bulgaricus), and Lyobifidus® (*B. bifidum*), produced in France, and Eugalan®, Euga-Lein®, and Lactopriv® all containing *Bifidobacterium* sp., produced in Germany.[289] A list of commercially marketed formulations of probiotics are reviewed, with reference to the reliability, utility, and safety of these products.[290] In a study by Reid et al., 64 healthy women were given daily oral capsules of *Lb. rhamnosus* GR-1 and *Lb. fermentum* RC-14 for 60 d. The study demonstrated that this probiotic combination can be taken orally on a daily basis for 2 months without any side effects. The therapy resulted in a significant improvement in the vaginal flora in term of increased lactobacilli presence, and decreased yeast and coliforms, thereby reducing the risk of infections in healthy women as well as those prone to urogenital disease.[290]

Fruit juice has been suggested as a good medium for probiotic ingredients,[291] as it is considered a health product, and has wide consumer appeal. Juices fortified with the probiotic microorganisms *Lb. rhamnosus* GG, *Lb. casei Imunitass*, or *Lb. paracasei* NFBC 43338 exhibited good shelf life and acceptable sensory properties after 7 d of storage. The study revealed that repeated exposure to probiotic-containing orange juices enhanced consumer acceptance and liking for their sensory characteristics.[292]

Soy-based probiotic products are desirable in cases where consumers are allergic to cows' milk, and also as a vegetarian alternative. Studies have indicated that soy is a good substrate for probiotic bacteria but not for the traditional yogurt starter *Lb. delbrueckii* subsp. Bulgaricus.[293] This suggests that some probiotic bacteria could better compete with yogurt cultures in a soy-based substrate.[118] Alpro has dominated the European market since it first started producing soya milk in the 1980s, and it has launched a range of soy-based products, including probiotic dairy-free "yogurt," under the Alpro and Provamel brands.

2.11 CONCLUSIONS

Probiotic research has expanded rapidly over the past few years. One of the many reasons for this is the heightened awareness of their clinically proven health-promoting effects in humans, and hence the growing interest in the incorporation of probiotic microorganisms into food products. The probiotic strain chosen should lend itself to food processing, through efficient large-scale cultivation, concentration, and incorporation into food products, for it to be successful as a candidate functional food component. Also, it is crucial that the probiotic strain and stability of the desirable characteristics be maintained during processing, storage, and delivery of the final product. In this chapter, we have attempted to outline some of the problems that are encountered during the development of efficacious functional foods containing viable probiotic strains, and some novel approaches to improve the survival of probiotics during processing in food systems and following ingestion.

The mechanisms by which functional microbes and ingredients affect human gut health are still largely unknown. The knowledge acquired by genomics on the genetics and physiology of a probiotic strain can be used for strain improvement. For example, it may be possible to eliminate an undesirable trait from a strain by simple mutagenesis. Alternatively, the use of recombinant DNA technology may allow the production of strains with exactly the correct combination of properties. Such improved understanding may offer solutions to many of the challenges that currently hamper the commercial development of functional foods containing particular probiotic strains by allowing the preconditioning of strains to be used for industrial fermentation.

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3 The Properties of *Enterococcus faecium* and the Fermented Milk Product—Gaio®

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CONTENTS

3.1 INTRODUCTION

Many fermented foods are produced throughout the world. Fermentation is a process that transforms the starting material into a product that may have enhanced nutritional and/or organoleptic characteristics. With the advent of probiotics, many researchers have analyzed the microflora in traditional fermented foods in attempts to find foods that contain bacteria that may be beneficial to health, metabolism, and disease resistance. In a few cases, an opposite approach has been taken. Based on studies testing individual bacteria in animals and humans, new products have been developed that include these bacteria, thereby creating new probiotic foods. (See Chapter 6 on LcS for such an example.)

Early studies on *Enterococcus faecium* and its effects against diarrhea and, more importantly, on cholesterol metabolism showed that *E. faecium* might be an ideal candidate to include in a fermented milk probiotic product. Gaio (which contains both *E. faecium* and *Streptococcus thermophilus*) was developed and is now distributed in at least two European countries. This chapter reviews studies where *E. faecium* and Gaio were tested for their effects on serum cholesterol, diarrhea, and mutagens.

3.2 SERUM CHOLESTEROL AND CARDIOVASCULAR DISEASE

3.2.1 Background

Coronary artery disease is one of the most frequent causes of morbidity and mortality all over the world.[1] In the United States, it accounts for fully one-half of the nearly one million deaths each year from cardiovascular disease and is the leading cause of death for both genders.[2] Each year, about 1.5 million Americans suffer acute myocardial infarction, and almost all myocardial infarctions are due to atherosclerosis of the coronary arteries.[3] It is known that individuals with some conditions, designated as risk factors, have a higher chance in prematurely developing this disease. Among the risk factors, hypercholesterolemia is one of the most important. A continuous and graded positive relation was demonstrated between serum total cholesterol level and coronary artery disease mortality in the more than 350,000 men screened for the Multiple Risk Factor Intervention Trial (MRFIT).[4] However, the numbers of people with very high serum cholesterol levels are not expressive, so they do not account for a large number of cases of symptomatic coronary disease. The vast majority of these cases are individuals presenting cholesterol considered to be in the normal (average) range or with a slight increase.[5,6] According to the National Cholesterol Education Program—Adult Treatment Panel III,[7] the optimal levels in a human blood lipid profile are total cholesterol below 200 mg/dL, low-density lipoprotein (LDL) cholesterol below 100 mg/dL, high-density lipoprotein (HDL) cholesterol above 40 mg/dL and triglycerides below 150 mg/dL. Such patients, in general, also have other risk factors, including smoking, hypertension, diabetes mellitus, sedentary life, weight excess, and psychosocial stress, because of the modern lifestyle typical in western industrialized countries.

It was demonstrated that healthy people presenting serum cholesterol levels within the normal range have a reduction in the risk of future cardiovascular events when their cholesterol levels are decreased.[8] One metaanalysis performed showed

that for a 10% cholesterol reduction, the mortality risk due to cardiovascular disease is decreased by 15%, and total mortality risk is decreased by 11%.[9] Consequently, any dietary intervention that could help to decrease serum cholesterol levels, particularly in people who do not have highly elevated levels, probably will be helpful in the prevention of coronary heart disease.

3.2.2 Cardiovascular Disease and Probiotics

Modulation of the microbial community (approximately 10^{14} bacterial cells/g) in the gut by probiotics and prebiotic foodstuffs has been considered as an important opportunity to positively influence human health.[10–12] Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improve health.[11] Probiotics are defined as live microbial food ingredients that are beneficial to health.[13]

The question of whether lactic acid bacteria (LAB) can be beneficial to health was raised a long time ago. Metchnikoff[14] was the first to show an interest in LAB and their possible beneficial effects on health. He claimed that lactobacilli from fermented milk were able to prolong life through a reduced formation of toxins in the gut. Mann and Spoerry[15] described a hypocholesterolemic effect of fermented milk in the natives of the African Masai tribe. These authors proposed that dietary habits were the explanation for the low incidence of ischemic heart disease among these people, because they traditionally had a high intake of meat, milk, and fermented milk. This meant that they also were consuming large quantities of saturated fat and cholesterol. After these seminal observations, many studies addressed this topic, as has been reviewed.[16–19] Until now, no clear answer has been available, and even more difficult is the identification of a possible biochemical mechanism that can explain how this improvement of health is really achieved. One possibility is that milk and fermented milk products have an effect on the metabolism of serum lipids and lipoproteins, resulting in a better serum lipid profile. Not all studies have come to the same conclusion, as discussed by Eichholzer and Stähelin[20] in an extensive review of this subject, and more recently by the Mission Scientifique de Syndifrais[21] and by St-Onge et al.[22] A controversy exists because milk and milk products have been shown to be atherogenic due to their concentration of saturated fat and cholesterol,[18] but they also possibly play a role in the prevention of coronary heart disease. There appear to be scientific reports to support both effects. Other benefits of milk products such as yogurt have been reported, including beneficial intestinal flora changes, better immunological response, production of antibiotic substances, improved calcium absorption, prevention of osteoporosis, cataract prevention, and prolongation of life,[16,17,19] that would justify increased consumption of milk products.

A group at Kiev University (unpublished results) demonstrated a significant hypocholesterolemic effect utilizing a milk product fermented with bacteria isolated from Abkhasia in the Caucasus. This region has a reputation for the longevity of its people, and it is known that fermented milk is a major part of the traditional diet of this population, suggesting a link between these two observations.

The studies analyzed in this chapter utilizes a yogurt-like product, similar to the one used in the study of Kiev University, but produced in Denmark. The product Causido® (Gaio) was constituted as a fermented (probiotic) dairy product, containing a bacterial culture (*E. faecium, S. thermophilus*) consumed for different periods of time.

3.3 *ENTEROCOCCUS FAECIUM*

3.3.1 Characteristics and Occurrence

Enterococci are included in the broad category of LAB.[23] Enterococci are Gram-positive, nonsporeforming, catalase-negative, oxidase-negative, coccus-shaped bacteria, and occur singly, in pairs, or in chains.[24,25] The identification of the members of the genus *Enterococcus* using traditional classification tests is difficult, because there are no phenotypic characteristics that can be used to distinguish them from other closely related bacteria. Enterococci are generally considered as being hardy because they survive in a wide range of temperatures, pH levels, saline solutions, and environments, such as are found in the human gastrointestinal tract.[26] An important distinguishing characteristic (from a human health perspective) of various enterococci is their ability to resist many antibiotics.[27]

Enterococci occur on plants and in the feces of animals and human. *Enterococcus faecalis* and *E. faecium* are the two most common species found in human gastrointestinal tracts. *E. faecalis* counts can reach 10^5 to 10^7 colony-forming units (CFU)/g, while *E. faecium* levels of 10^4 to10^5 CFU/g are found.[27,28] Levels of these two species can vary among individuals; diet and other factors are believed to alter the proportions of *Enterococcus* species. *E. faecalis* has been isolated in feces of neonates, but *E. faecium* has not.[28,29]

3.3.2 Antibiotic Resistance

Many authors have presented criteria that should be used when choosing potential probiotic bacteria.[30–32] Above all, it is agreed that any microorganism that is intentionally added to a food should be generally recognized as safe (GRAS). For example, the microorganism should not be pathogenic, should not produce toxins or metabolites that could adversely affect the health and metabolism of the host, and should not negatively impact on bacterial populations already resident in the host. A large number of fermented food products that contain LAB are now sold because they have a long history of being safe. Within the LAB, enterococci and streptococci have been identified as causes for concern.[30] With the introduction of a fermented milk product on the market that contains *E. faecium*, the question of antibiotic resistance has been raised.[33]

Plasmids are genetic elements independent of the cell chromosome. Over time, some plasmids have developed genes that make them resistant to selected antibiotics. Plasmids are easily transferred between cells, and this allows the efficient spread between bacteria of resistance to an antibiotic. Antibiotics originally were substances produced by fungal microorganisms to eliminate competition from bacteria for survival reasons. However, the number of antibiotics now available and

their widespread use in prescription drugs and for general disinfection purposes has raised concerns about the potential for the development of antibiotic resistance. The introduction of probiotic foods into the diet raises the possibility of the ingested probiotic becoming antibiotic-resistant from related intestinal bacteria that already have acquired resistance, or more seriously, the reverse—a probiotic product containing antibiotic-resistant bacteria that pass antimicrobial resistance genes or genes that encode for virulence factors onto resident bacteria.

Most strains of enterococci are resistant to tetracycline, erythromycin, clindamycin, chloramphenicol, and sulfonamides.[27] This, together with the fact that the incidence of infections attributed to enterococci appears to be increasing and along with the difficulty in treating such infections, places these organisms as important human pathogens of concern. The hospital environment is one where enterococcal contamination has received much attention. However, because vancomycin-resistant enterococci (VRE), in particular, have been identified in a wide variety of farm animals and birds, it is not clear whether the food is a major vector in the transfer of VRE.[34]

With respect to Gaio, Lund et al.[35] were able to show that when Gaio was taken together with vancomycin, the total number of enterococci decreased at day 10 of the feeding trial, but by 3 weeks after the cessation of the experiment, enterococci numbers were 100 times those counted at the beginning of the experiment. Subjects having received only the Gaio had no such increase in enterococci numbers, indicating that the antibiotic treatment may have given the ingested probiotic bacteria in the Gaio an advantage due to reduced colonization resistance. No major overgrowth of VRE occurred in any subjects, including those receiving the vancomycin treatment. However, some subjects (20%) were transient carriers of VRE. Resistant strains isolated in subjects were not associated with the consumption of Gaio, whereas pulse-field gel electrophoresis analysis showed that the strains of *E. faecium* found in Gaio, and VRE fecal samples were different. Lund et al. were able to state that "no resistance against vancomycin emerged in intestinal enterococci, and *E. faecium* from the Gaio product was not found to acquire vancomycin resistance during the study period."

In a companion paper that used some of the samples from their 2000 study,[35] Lund et al.[36] used genotypic and phenotypic analyses to confirm that *E. faecium* found in Gaio can survive GI tract transit, and that during the consumption period of the experiment, Gaio-derived *E. faecium* became the dominant part of the total *E. faecium* in the gut. However, the Gaio-derived *E. faecium* was not found in fecal samples of subjects receiving both the Gaio and vancomycin, and it did not persist 3 weeks after consumption was stopped.

Adams[37] pointed out that there have been no reports of infection contracted as a result of consumption of foods containing enterococci. However, the Lactic Acid Bacteria Industrial Platform recommended that enterococci should not be used in foods unless there was a demonstrable (possibly health) benefit.[38]

3.4 PRODUCTION OF GAIO

Gaio was first produced by the Danish dairy corporation MD Foods A/S established in Aarhus in Denmark. Recently, MD Foods merged with Arla Foods, and the product is only produced by Arla Foods and consumed in Denmark and Sweden.

The production of Gaio uses a fermentation of milk at a temperature of 37°C. The level of starter inoculated is approximately 5×10^{12} CFU/1000 L of milk. The fermentation time is approximately 9 h, to a final pH of 4.5. The final product is very viscous and has a mild, slightly acid taste. The product is sold in plastic containers of 500 g as "natural" and with different fruit flavors. The product is distributed and sold refrigerated.

The original Ukrainian bacterial culture (Causido) is used to produce Gaio. This culture contains one human species of *E. faecium* and two strains of *S. thermophilus*. The CFUs of the fresh product are 10^5 to 10^9/mL for *E. faecium* and 5 to 20×10^8/mL for *S. thermophilus*. One hundred grams of the product has an energy content of 240 kJ and contains 4.9 g of protein, 5.4 g of carbohydrate, and 1.5 g of fat (66% as milk fat and 33% as soybean fat). The cholesterol content is about 5 mg for every 100 g of the product. Vitamins E and C are added in accordance with the original Ukrainian recipe, giving final concentrations of 0.5 mg and 10 mg, respectively, per 100 g.

3.5 CHOLESTEROL EXPERIMENTS

3.5.1 Early Work

The observation that the Masai people had low serum cholesterol levels in spite of their high dietary cholesterol intakes[15] initiated interest in the beneficial effects of fermented milk on cholesterol metabolism, because the Masai consumed large quantities of fermented cows' milk. Several studies were then carried out, with mixed results, in animal models (rat, rabbit) and humans to investigate the properties of milk fermented with a variety of bacteria on cholesterol metabolism.[39–41]

Zacconi et al.[42] used axenic mice to show that when mice were fed with a hypercholesterolemic diet for 60 d, animals reared in sterile boxes had higher serum cholesterol levels than those dosed with various bacteria. Although the quantity of bacteria given to each animal was not indicated, it was evident that the largest reductions in serum cholesterol levels occurred in mice given *E. faecium* (females: –16.9%; males: –7.8%) and *Lb. acidophilus* (females: –11.4%; males: –5.3%). Zaconni et al. also found that the reductions were greatest in the female mice, and that the animals receiving the *E. faecium* were healthier than the axenic mice.

3.5.2 Studies with *Enterococcus faecium*

Mikeš et al.[43] carried out a human-feeding trial in which subjects were given lyophilized *E. faecium* M-74 (5×10^9/d) in capsules for 6 weeks. The mean number of *E. faecium* in fecal samples generally rose and plateaued during the period of dosing, and then fell slowly during the following 5 weeks. However, large individual differences in the numbers of *E. faecium* recovered in fecal samples were noted. Fecal ß-D-glucuronidase activity was measured during the experiment, and it was found that activity decreased during the dosing period and remained low 5 weeks after the dosing with *E. faecium* was stopped. The serum LDL and total serum cholesterol rose during the initial weeks of the study; both parameters dropped significantly (compared to values obtained before the study) 14 d after the consumption of the

bacteria was discontinued. Conversely, serum HDL values rose after the bacteria treatment stopped. There were no changes in other blood parameters.

To better understand how the bacteria might be altering cholesterol metabolism, the metabolic activation of neutrophils was measured, because it is known that neutrophils are capable of producing reactive oxygen intermediates that can oxidize lipoproteins and thereby contribute to atherosclerosis. Mikeš et al. found that during the time when LDL and total cholesterol were lowered, stimulated neutrophils from subjects had increased ability to reduce 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and to produce superoxide. This finding was consistent with an earlier observation that *E. faecium* was able to stimulate human peripheral neutrophils resulting in the production of reactive oxygen intermediates in vitro.[44] The observed reciprocal correlation between cholesterol levels and neutrophil INT reductase activity and superoxide production raised the possibility that the *E. faecium* might be affecting some oxidative process, which in turn reduces cholesterol levels.

In a recent human trial (double blind, placebo controlled) that lasted over 1 yr, Hlivak et al.[45] reported that daily consumption of capsules containing *E. faecium* M-74 (2×10^9 CFU/capsule) and selenium (30 μg/capsule, organically bound) produced a gradual decline in serum total cholesterol that was significantly different from the day 0 value after 3 weeks, and that persisted 4 weeks after the cessation of treatment. The total serum cholesterol for the placebo group also had a gradual, but not statistically significant decrease over the course of the experiment. LDL values declined in a pattern similar to total cholesterol; the *E. faecium* group had significantly lower LDL values one month after the experiment compared to day 0 values. HDL and triglyceride values did not change in the placebo group, but the *E. faecium* group had significantly reduced HDL after 12 weeks. This latter effect was attributed to activation of inflammation processes that can reduce the size of HDL particles. In a companion paper, Hlivak et al.[46] presented data from this same clinical trial to explain how the consumption of *E. faecium* M-74 may help prevent and treat cardiovascular disease. Compared to day 0, subjects receiving daily capsules of *E. faecium* M-74 had significantly reduced serum levels of the cell adhesion molecule sICAM-1, and significantly decreased expression levels of the surface adhesion molecules CD11b on lymphocytes, and CD31 and CD54 on granulocytes. Paradoxically, significant increases were also measured for the expression of some other surface adhesion molecules. Hlivak et al.[46] stated that studies at the molecular and genetic level could be used to explain and predict atherosclerosis.

3.5.3 Studies with *Streptococcus thermophilus*

S. thermophilus and *Lactobacillus bulgaricus* are the two bacteria added to milk to produce yogurt. The effect, if any, of S. *thermophilus* on serum cholesterol is not clear because most feeding trials with animals and humans have tested *S. thermophilus* in combination with other LAB. For example, Akalin et al.[47] reported a study where they fed mice a chow diet and yogurt containing either *S. thermophilus* and *Lb. delbrueckii* ssp. Bulgaricus or *S. thermophilus* and *Lb. acidophilus*. The fresh yogurt had 10^7 lactobacilli/mL. The number of *S. thermophilus* in the yogurt was not measured. The animals eating the yogurts had lower serum cholesterol levels and LDL cholesterol

(significantly lower in the case of *S. thermophilus* and *Lb. acidophilus)* than control mice that ate chow. Serum triglycerides did not appear to be affected. These authors concluded that the effects on cholesterol metabolism were attributable to the *Lb. acidophilus* in the yogurt and that the *S. thermophilus* had no effect.

Kawase et al.[48] showed that *Lactobacillus casei* TM0409, *S. thermophilus TMC1543,* and whey protein concentrate had a synergistic effect on lowering serum cholesterol in rats. They then used the two bacteria to produce a fermented milk that contained 6.1×10^8 *Lb. casei*/mL and 2.6×10^7 *S. thermophilus*/mL and fed it to male human volunteers for 8 weeks (200 mL, twice a day). HDL cholesterol was significantly increased after 4 weeks, and the increase continued until week 8. Total serum cholesterol was lower, but not statistically lower, in the group receiving the fermented milk compared to the group receiving a placebo. No mechanism was proposed to explain the hypocholestrolemic effect of the fermented milk.

3.5.4 Human Trials with Gaio

The first study to utilize a milk product fermented with bacteria isolated from Abkhasia was carried out by Sarkisov at Kiev University in Ukraine. However, the results were not published. These authors showed a cholesterol-lowering effect of approximately 39 mg/dL, an increment in HDL cholesterol, and a reduction in triglycerides after 4 weeks of dietary supplementation with the new product in a very heterogeneous group of males and females.[49]

Four studies in which the effect of Gaio consumption on blood lipid profiles was evaluated can be found in the literature. The first published trial to test the effects of Gaio (a product practically identical with the Ukrainian one, though produced in Denmark), on LDL cholesterol level was performed by Agerbaek et al.[50] They studied 58 male volunteers of Danish descent, all 44 yr old, with normal cholesterol fasting values. All were selected from a cohort examined in 1989 and again in 1990 in a study of the prevalence of risk factors for coronary heart disease at the University Hospital of Aarhus. They were selected on the basis of having had normal fasting values of serum cholesterol (5.0 – 6.5 mmol/L) and triglycerides (< 5.0 mmol/L) at both examinations, no history of cardiovascular, cerebrovascular, or metabolic disease; normal weight (body mass index [BMI] < 27.5) with a stable weight; alcohol consumption < 315 g/week; and normal blood pressure (< 150/95 mmHg). During the intervention period, the subjects maintained their habitual diets supplemented with 200 mL/d of either Gaio or a similar placebo product (chemically fermented). Fasting blood samples were drawn initially and after 3 and 6 weeks and analyzed for plasma values of total cholesterol, HDL cholesterol, and triglycerides. LDL cholesterol was estimated by the Friedewald formula [LDL cholesterol = total cholesterol – HDL cholesterol – VLDL cholesterol (triglycerides/5)].[51] After 6 weeks, the consumption of Gaio produced an average reduction of 6% in total serum cholesterol levels, completely ascribed to a decrease in LDL cholesterol level of 10%, whereas HDL cholesterol and triglycerides were unchanged. The authors stated that although the findings were promising, the results could not be extrapolated to other human subjects who were not included in their study. They suggested further studies to determine the potential effects of the new fermented milk product on lipoproteins

among women, the elderly, and in subjects with manifest hypercholesterolemia. They also stated that the potential beneficial effect for middle-aged men would be true only if the cholesterol-lowering effect persisted for longer periods than the 6 weeks investigated in their study. This is based in the findings of prevention studies using drugs. Those studies showed that improvement in vascular risk only begins to appear after 1 to 2 yr of drug use and cholesterol reduction.[9]

Following this line of reasoning, Richelsen et al.[52] studied the consumption of Gaio for a longer period of time in a randomized, double-blind, and placebo-controlled trial that included 87 nonobese and normocholesterolemic females and males, aged 50 to 70 yr. The volunteers were recruited through announcement in the local newspaper. Before inclusion in the experiment, blood samples were drawn to eliminate subjects with liver disease, diabetes, kidney disease, anaemia, and hypercholesterolemia (total cholesterol > 8.0 mmol/L). Inclusion criteria were healthy men and women aged 50 to 70 yr (the women were all postmenopausal), body mass index < 32 kg/m^2, and no medication influencing plasma lipids. Participants were instructed not to change their ordinary diet, alcohol intake, level of physical exercise, and tobacco consumption during the study period. They consumed 200 mL of either the fermented milk product (Gaio) or a placebo (chemically fermented). The study showed a rapid reduction in LDL cholesterol level by about 8% after 1 month, but after 6 months, although the effect remained, the reduction in serum LDL cholesterol was similar to the reduction observed in the placebo group. The authors reasoned that after 1 month of the placebo use there was a gradual fall in total and LDL cholesterol in both genders, making the interpretation of the results less clear-cut. Thus, after 6 months the levels of total cholesterol and LDL cholesterol were significantly lower than initial values in both groups, but the reduction and the absolute values were not different in the two groups (treatment versus placebo). They speculated that the placebo product itself, without the bacterial culture but chemically fermented with an organic acid (gluconic acid-delta-lactone) containing 1.5% fat, could have cholesterol-lowering effects or, alternatively, that the seasonal variation in subjects' lipid levels[53] could explain the results. The authors also suggested that some people participating in the trial could be "responders," whereas others could be "nonresponders," but the basis for this biological (genetic) phenomenon is still unknown. Another possibility suggested by the authors was that the absence of effect could be due to the lack of statistical power to detect a 4 to 5% reduction in blood cholesterol due to the small number of subjects included in the study.

Bertolami et al.[54] tested the effect of Gaio in a group of patients with primary hypercholesterolemia (11 men and 21 women, 36 to 65 yr old) who had not shown a significant improvement in LDL cholesterol level after dietetic modifications alone (Phase I of the American Heart Association–National Cholesterol Education Program [NCEP] Adult Treatment Panel II). A prospective, randomized, double-blind, 8-week crossover design, controlled by placebo (a chemically fermented milk) was used. After initial clinical and laboratory analysis, the patients began to consume 200 g daily of Gaio or the placebo in a randomized and double-blind manner. Seventeen patients started the trial using the active product and fifteen began with placebo. After 8 weeks, blood was collected again for lipid profile evaluation and the crossover was made (those consuming Gaio changed to placebo and vice versa).

After an additional 8-week period, blood was collected for the last lipid profile determination. The results showed that Gaio was able to significantly reduce total cholesterol by 5.3% and LDL cholesterol by 6.15% in these hypercholesterolemic subjects, compared with the placebo product. Like Richelsen et al.,[52] Bertolami et al. also suggested the possibility of different patient responses ("responders" and "non-responders") to the use of Gaio and the placebo product (see Tables 3.1 and 3.2).[54]

TABLE 3.1
Media and Standard Deviation During Each Phase of the Gaio Feeding Study

	mg/dL(lipid profile) or kg		
Parameters measured	**Phase 1**	**Phase 2**	**Phase 3**
	(Diet only)	(Diet + placebo)	(Diet + Gaio)
Total cholesterol	248.47 (± 26.75)	249.09 (± 28.45)	235.75 (± 35.03)[a,b]
Triglycerides	119.16 (± 49.28)	118.38 (± 39.47)	116.66 (± 38.79)
HDL cholesterol	52.38 (± 14.00)	56.91 (± 15.96) [c]	54.41 (± 14.97)
LDL cholesterol	172.22 (± 21.17)	168.59 (± 24.18)	158.00 (± 31.04)[d,e]
Weight	66.55 (± 10.57)	66.05 (± 10.37) [f]	65.97 (± 10.60)[g]

Note: Significant difference between phases: [a] Comparison between averages observed after phases 1 (diet only) and 3 (active product) – $P = 0.012$; [b] Comparison between averages observed after phases 2 (placebo) and 3 (active product) – $P = 0.004$; [c] Comparison between averages observed after phases 1 (diet only) and 2 (placebo) – $P = 0.001$; [d] Comparison between averages observed after phases 1 (diet only) and 3 (active product) – $P = 0.002$; [e] Comparison between averages observed after phases 2 (placebo) and 3 (active product) – $P = 0.012$; [f] Comparison between averages observed after phases 1 (diet only) and 2 (placebo) – $P = 0.026$. [g] Comparison between averages observed after phases 1 (diet only) and 3 (active product) – $P = 0.014$.

Source: From Bertolami, M.C., Faludi, A.A., and Batlouni, M., *Eur. J. Clin. Nutr.* 53, 97–101, 1999. With permission.

TABLE 3.2
Percent Changes of Total Cholesterol and LDL Cholesterol Presented for Each Study Phase of the Gaio Feeding Study

Cholesterol	**Phase comparison**	**Mean (SD)**	**Maximum decrease**	**Maximum increase**
Total cholesterol	D × P	+ 0.39 (6.82)	– 11.38	+ 13.45
	D × Y	– 5.04 (10.44)	– 24.26	+ 23.10
	P × Y	– 5.30 (9.38)	– 23.40	+ 18.77
LDL cholesterol	D × P	– 1.90 (9.39)	– 19.87	+ 16.02
	D × Y	– 8.29 (13.29)	– 37.69	+ 23.78
	P × Y	– 6.15 (12.73)	– 30.00	+ 18.65

Note: D = diet only; P = diet plus placebo; Y = diet plus yogurt; SD = standard deviation.

Source: From Bertolami, M.C., Faludi, A.A., and Batlouni, M., *Eur. J. Clin. Nutr.* 53, 97–101, 1999. With permission.

Agerholm-Larsen[55] studied the effects of Gaio in 70 overweight and obese subjects in a randomized, double-blind, placebo- and compliance-controlled, parallel protocol. The study was designed as a five-armed parallel study in which Gaio was compared with two other fermented milk products, a chemically acidified milk product with an organic acid (delta-acid-lactone) instead of a living bacterial culture, and an inert placebo pill. One comparison milk product was fermented with two strains of *S. thermophilus* (~ 1×10^7 CFU/mL) and two strains of *Lb. acidophilus* (~ 2×10^7 CFU/mL), and the other was fermented with two strains of *S. thermophilus* (~ 8×10^8 CFU/mL) and one strain of *Lb. rhamnosus* (~ 2×10^8 CFU/mL). The protocol involved, besides lipid level evaluation, the determination of fibrinogen and C-reactive protein because these two acute-phase proteins are also implicated as risk factors for coronary artery disease in healthy men.[56,57] The authors planned to offer an increased amount of fermented milk product (450 mL daily) to obtain a greater effect on LDL cholesterol. The 20 men and 50 women participating were healthy, weight-stable, overweight and obese ($25.0 < \text{BMI} < 37.5$ kg/m^2), 18 to 55 yr old. Participants were instructed not to change their habitual diet, level of physical exercise, tobacco and alcohol habits, or their body weight during the study period. Compliance was tested at home every second week (weeks 2, 4, 6, and 8) by analyzing sample yogurt bags labelled with ^{13}C-acetate by a nondispersive infrared spectroscopy method. After 4 weeks of consuming 450 mL of Gaio a day, there was no decrease in LDL cholesterol levels. After 8 weeks these levels decreased significantly by 8.4%, and fibrinogen increased also significantly compared to the placebo group, whereas C-reactive protein did not change. The authors expected to see differences in lipids after 4 weeks as demonstrated in previous comparable studies but found none. Although they had no obvious explanation for the lack of reduction in LDL cholesterol levels after 4 weeks, they speculated that a possible mechanism behind this finding could perhaps be the small number of subjects in each group.

According to the authors, it is likely that there is some between-subject variability in intestinal colonization of the active bacteria that could influence these results. As fibrinogen is an acute-phase protein, the authors speculated that the increased fibrinogen concentration found in the Gaio group could be attributed to immunostimulation. They also suggested that it was not possible to exclude the idea that a transient colonic inflammation caused by the bacterial strains in Gaio was the reason for the increase in fibrinogen in subjects consuming this fermented milk product. However, the lack of any increase in C-reactive protein does not support this possibility.

3.6 METAANALYSIS OF CHOLESTEROL EXPERIMENTS

In a published metaanalysis, Agerholm-Larsen et al.[58] questioned the conflicting results pertaining to this product's efficacy in reducing plasma cholesterol. They analyzed six studies conducted with Gaio involving 425 subjects of both genders and different initial cholesterol levels, concluding that five studies showed a small beneficial short-term effect of 6 to 10% on serum LDL cholesterol (Sarkisov et al.,[49] Agerbaek et al.,[50] Richelsen et al.,[52] Bertolami et al.,[54] Agerholm-Larsen et al.[55]). However, the long-term effect was inconclusive (Richelsen et al.[52]), and one study failed to demonstrate any effect at all (Sessions et al.[59]). The authors pointed out

TABLE 3.3
Human Studies of the Effect of Gaio on Total and LDL Cholesterol

Study	Study group	Percentage decrease in total cholesterol	Percentage decrease in LDL cholesterol
Sarkisov (unpublished results, 1991)	Hypercholesterolaemic	15.87	16.85
Agerbaek et al. (1995)	Normocholesterolaemic	6.08	9.77
Richelsen et al. (1996)	Normocholesterolaemic	3.51 (not significant)	5.96 (not significant)
Sessions et al. (1998)	Hypercholesterolaemic	0.17 (not significant)	3.6 (not significant)
Bertolami et al. (1999)	Hypercholesterolaemic	5.3	6.15
Agerholm-Larsen et al. (2000)	Normo-cholesterolaemic	5.14	6.64

that a metaanalysis suffers from unpublished data material because negative studies often remain unpublished, leading to publication bias. However, they confirmed that they had no knowledge of other unpublished material on the Causido culture. As a conclusion, they suggested that the metaanalysis based on the five controlled study interventions showed that the intake of the fermented milk product (Gaio) produced a statistically significant and clinically important reduction in plasma cholesterol. They found a reduction of 5% in LDL cholesterol, which is considered large enough to have a beneficial effect on risk factors for coronary heart disease.[9] However, they emphasized that long-term studies on Causido are required to document whether a sustained effect on the blood lipids occurs.

A summary of the results of the studies evaluating the effects of consumption of Gaio on plasma lipid profiles is shown in Table 3.3. Studies are listed by author, type of population enrolled (normo- or hypercholesterolemic), and the percent changes of total cholesterol and LDL cholesterol comparing active treatment versus placebo (only the study from Sarkisov was open and did not involve a placebo control).

3.7 MECHANISM OF ACTION

The reason for the observed hypocholesterolemic effect in subjects consuming Gaio is not fully understood at this time. It has been proposed that the cholesterol-lowering effect is related to the bacterial culture in the product.[50] A potential explanation given by Agerholm-Larsen et al.[55] is that there is an association between the gut microflora and cholesterol absorption in the small intestine. The intestinal bacteria can bind bile acids to cholesterol, resulting in the excretion of bile acid-cholesterol complexes in the feces. Decreased bile acid recycling through the enterohepatic circulation would result in cholesterol uptake from the circulation into the liver for de novo synthesis of bile acids. Another possible explanation was provided by St-Onge et al.[22] High numbers of bacteria in products such as yogurt when consumed will ensure passage of sufficient numbers of bacteria into the intestine to exert effects on metabolism. Because

the bacteria contained in fermented milk products are consumed with macronutrients that alter the stomach's pH (buffering effect), bacterial survival is increased, and the bacteria pass into the small intestine and then into the large intestine. Once in the large intestine, the bacteria ferment indigestible carbohydrates and produce short-chain fatty acids. The relative proportions of this production are likely to alter cholesterol synthesis. Gaio contains *S. thermophilus* and *E. faecium*. In vitro studies have shown that *S. thermophilus* is acid sensitive and cannot survive the passage through to the small intestine. However, *E. faecium* is known to have good bile tolerance. Consequently, Agerholm-Larsen et al. suggest that *E. faecium* is the bacterial strain with the cholesterol-lowering effect.[55]

Rossi et al.[60] showed that *E. faecium* has the ability to remove and assimilate cholesterol. Using a MRS medium supplemented with bile salts (Oxgall), 5 of 14 strains of *E. faecium* tested were able to reduce the concentration of suspended cholesterol (100 mg/L) by 30% or more, but only when bile salts were present in the MRS broth. There was an appearance of cholesterol in the bacterial cells that paralleled the decease of cholesterol in the MRS broth. Only 1 of 5 stains of *Lb. acidophilus* tested was able to reduce cholesterol concentrations. Using a similar protocol, the group then showed that *E. faecium* plus *Lb. jugurti* (1:1) not only reduced cholesterol by 43%, but could also be used to produce a fermented soymilk product with acceptable physiochemical and sensory attributes.[61]

3.8 OTHER PROPERTIES OF *ENTEROCOCCUS FAECIUM*

3.8.1 Treatment of Diarrhea

One of the most obvious applications of probiotics is to restore the intestinal microflora population during diarrhea. A variety of LAB, including *Lb. rhamnosus* GG, *Lb. reuteri*, *Streptococcus boulardii*, and *E. faecium*, have been tested for their ability to reduce the severity or duration of various diarrheas.[62] A group (78 people) of sufferers of acute diarrhea were found to have a lower frequency of diarrhea after 7 d of treatment with *E. faecium,* compared to those receiving a placebo.[63] Results were not as positive in a 3 d study involving patients suffering from diarrhea due to infection with *Vibrio cholerae* (114 subjects) and enterotoxigenic *Escherichia coli* (41 subjects). In this study, there was no difference in the duration of the diarrhea between the placebo group and those receiving the *E. faecium*.[64]

3.8.2 Antimutagenicity

Milk fermented with *E. faecium* has been investigated for its antimutagenic properties. Belicová et al.[65] used an ether extract of milk that had been fermented with *E. faecium* to carry out a variety of tests. They reported that their milk extract showed a dose-dependent inhibition of mutagenesis induced by chemical and physical mutagens. Using both *Salmonella typhimurium* TA97 and TA100, they showed that the fermented milk extract (4 uL/plate) could reduce UV-irradiation damage by 72% and 55% (for TA97 and TA100, respectively). A 10 uL dose of extract produced about a 30% reduction in the mutagenicity of nitrovine; the same

dose reduced 5-nitro-2-furylacrylic acid mutagenicity by up to 25% and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) mutagenicity by 28%.

Earlier, Ebringer et al.[44] showed that viable *E. faecium* M-74 showed significant antimutagenic effects on nitrovin and 2-aminofluorene mutagenicity (Ames test) but heat-treated nonviable cells did not. They also reported that *E. faecium* cells themselves showed no immunostimulatory activity, but mixtures of *E. faecium* and phagocytes significantly stimulate mean INT-reductase activities. Ebringer et al. concluded that *E. faecium* contained factors that could reduce the effect of the mutagens they tested and that heat stable proteins might be responsible.

3.8.3 Cheese Making

Enterococci bacteria are commonly used in the production of raw ewes' and goats' milk cheeses. They contribute to proteolysis, lipolysis, and citrate breakdown, and thereby influence ripened cheese taste and flavor. *E. faecium* has been shown to be a good starter adjunct in the production of Cheddar cheese. Gardiner et al.[66] showed that 9 months after adding 2×10^7 CFU/mL (0.1%) *E. faecium* PR88 to a commercial lactococcal starter, 3×10^8 CFU/g were viable. Cheese with the added bacteria was found to have increased proteolysis and higher levels of some odor-active compounds. The Cheddar cheese containing the *E. faecium* was judged (by a commercial grader) to be ripening faster and had a better flavor than the control cheese. This study confirmed previous reports about the positive effects of enterococci on ripening and flavor development in Cheddar cheese.[67–69]

Enterococci bacteria have also been found on beef, poultry, and pig carcasses; cooked pork; and vegetables, especially olives. Their presence does not necessarily imply contamination by fecal material.[70]

3.9 CONCLUSIONS

After analyzing the results of published studies results using Gaio as a possible option to obtain reduction in total and LDL cholesterol levels, it can be concluded that more studies are needed to ascertain:

- Whether the effects of prolonged consumption of Gaio on blood lipid profiles are the same as those observed during shorter periods of consumption, and
- Whether the final beneficial effect will lead to an improvement in coronary heart disease prevention with fewer events and fewer deaths due to this worldwide health problem of modern societies.

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4 Kefir—A Fermented Milk Product

Edward R. Farnworth and Isabelle Mainville

CONTENTS

4.1 INTRODUCTION

Kefir is a beverage produced by the action of lactic acid bacteria (LAB), yeasts, and acetic acid bacteria on milk. This complex mixture of microorganisms produces a distinctive fermented milk product with unique properties.

The first fermented foods may have been produced by accident. However, fermentation of foods such as milk became a widespread method of preservation before refrigeration was introduced or preservation procedures such as canning and pasteurization were developed and used to extend shelf life. It would appear from the oral tradition of kefir that fermentation of milk in skin bags as a way of preserving milk led to the production of the first kefir grains and started the long tradition of producing kefir. Kefir has been produced using milk from cows, ewes, goats, and buffalo[1–3] and has been sold in Europe under a variety of names including *kephir*, *kiaphur*, *kefyr*, *képhir*, *kéfer*, *knapon*, *kepi*, and *kippe*.[4] (See Chapter 1 for more details on the history of kefir.)

Traditional home production of kefir has been joined by commercial production in many countries, and this has helped to increase the consumption of kefir and to promote its reputation as being good for health. Kefir is at the same time a functional food and a probiotic, and there is growing evidence that this unique fermented milk product may indeed be helpful in many disease or infection conditions.

Early reports on kefir quote original research that was carried out in the former Soviet Union.[2,5] The composition of this unique product has not been fully described, and it is evident that more research is required on the microbiology of kefir before the product can be fully understood from a scientific point of view.[6,7] Consumer acceptance may be slow because of the unique taste of kefir. The reported health properties of kefir and the desire of consumers to consume probiotic products may open new markets.[8]

4.2 KEFIR GRAINS

Kefir is produced by adding either a starter culture called *kefir grains* directly or a percolate of the grains to milk. Kefir grains are a mass of several different bacteria and yeasts imbedded in a complex matrix of protein and carbohydrate. The microorganisms in the kefir grains ferment the milk, and the grains can be recovered at the end of the fermentation process. The grains have been described as resembling elastic small florets similar to cauliflower in shape, yellow or white in color, and 20 to 30 mm in size.[9,10] Figure 4.1 is a photograph of kefir grains. A crude analysis of the grains shows that they are a mass of bacteria, yeasts, polysaccharides, and proteins with a chemical composition of 890 to 900 g/kg water, 2 g/kg lipid, 30 g/kg protein, 60 g/kg sugars, and 7 g/kg ash.[3] A study of the proteins in kefir grains using SDS-PAGE on acrylamide gels indicated that the major grain proteins had a higher molecular weight than milk proteins, indicating that they were not proteolysis products.[11]

4.2.1 The Microorganisms in Kefir Grains and Kefir

The bacteria, yeasts, polysaccharides, and proteins in kefir grains added to milk produce kefir. Usually there is no pasteurization step after fermentation, and therefore

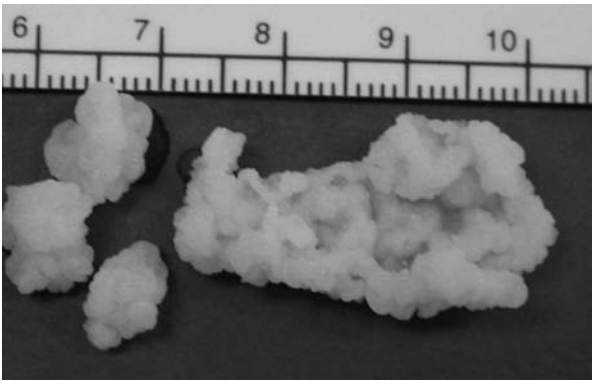

FIGURE 4.1 Kefir grains (scale in cm).

live bacteria and yeasts are found in the final product. In order to understand the fermentation process better and also to evaluate the health benefits of eating kefir, the microbiological profile of it has been studied by many researchers. The isolation and identification of microorganisms in kefir grains and kefir depend on the choice of suitable growth media, and more recently, sophisticated methods of identification have been used. It should be noted here that the identity of several microorganisms found in kefir has been revised over time as more definitive methods of classification have been used. In some cases, the nomenclature assigned to various bacterial species has also changed. The names of microorganisms used in this chapter are the names used in the original scientific articles cited in the text.

Three main genera make up the bacterial population of kefir: lactobacilli, lactococci, and leuconostoc. A fourth genus, acetobacter, is also often mentioned, but its presence is not reported by all research teams studying kefir. Some teams even pose the questions as to whether acetobacter could be a contaminant.[12] Studies in our laboratory did demonstrate the presence of acetobacter in kefir, but the relatively low numbers could explain why other teams could have missed them.[13] A suitable selective medium for this genus is essential for its isolation.

Studies on the selection of a suitable selective growth medium for the lactobacillus species are numerous, but the media are often not suitable for the growth of some *Lactobacillus* species found in kefir or kefir grains.[14–16] Fujisawa et al.[17] observed that *Lactobacillus kefiranofaciens* grew on KPL agar at 30°C, but not on BL and MRS agar. Kojima et al.[18] found that Rogosa medium in 100% cheese-whey solution (Rogosa-CW) gave the best results for the isolation and cultivation of lactobacilli from kefir grains. Farnworth and Mainville[13] compared MRS, KPL, and Rogosa-CW to the lactic acid whey medium (LAW)[19] and found LAW to have the best recovery and growth rate for the lactobacilli present in kefir and kefir grains. This was especially true for the *Lb. kefirgranum* isolates.

Many studies have been carried out to identify the various bacteria and yeasts in kefir grains and in the final product. [20–23] Early studies revealed that many of the bacteria isolated are closely related and therefore difficult to isolate and identify.[20] As noted by Koroleva,[24] studying and monitoring kefir grains is difficult because when the various microorganisms are separated as pure cultures, they do not grow in milk,

or have decreased biochemical activity. Because of this, kefir has been cited as an example of symbiosis;[25] the growth and survival of individual strains are dependent on the presence of others. Toba[26] indicated several possible metabolic products that might contribute to the symbiotic relationship in kefir grains.

The growth of *Lactobacillus kefir* was enhanced when *Candida kefir* was added, either before or simultaneously, to the milk to be fermented.[27] Growth of the yeast, however, was not stimulated by the presence of the bacteria. When the two organisms were cultivated together, the amounts of lactic acid, glycerol, and ethanol produced were increased. The growth of several bacteria isolated from kefir grains is improved when yeast extract is added to the growth medium,[13] indicating that the yeasts found in kefir grains are essential to maintain the integrity and viability of the microflora population. Vitamins, amino acids, and other essential growth factors for bacteria are produced by yeasts, whereas bacterial metabolic endproducts are used as energy sources by yeasts.[28] Figure 4.2 is an electron micrograph of kefir grains showing bacteria surrounding a yeast cell; the close proximity presumably indicates some sort of physical and chemical interaction.

The symbiosis found in the kefir grain microorganism population allows the grains to maintain uniformity so that throughout the year the microbiological profile of kefir grains and the kefir drink remain stable in spite of variations in milk quality and the potential presence of antibiotics and other inhibiting substances.[29] The microorganism profile of the final product does not necessarily parallel that of the grains because of conditions (pH and other) during the fermentation process. Also, the location of the microorganisms in the grains may be a factor. Yeasts are generally found in the interior of the grains, whereas the lactococci are found on the exterior.

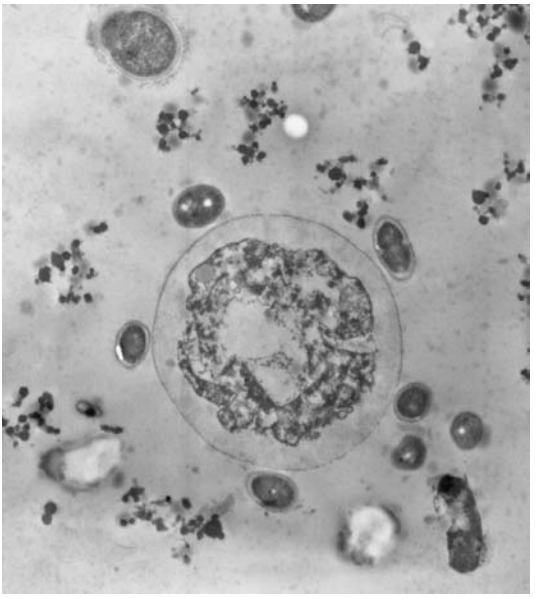

FIGURE 4.2 Electron micrograph of kefir showing symbiosis between bacteria and yeast. (Photo courtesy of D. Montpetit, Agriculture and Agri-Food Canada.)

Therefore, the number of yeasts found in the final product is lower than those counted in the grains themselves, whereas lactococci are numerous in the final drink. The complex microbiological composition of kefir grains produces kefir. Therefore, unlike with yogurt, the drink kefir cannot be used as a starter to produce more kefir.

Initially, traditional selective media were used to isolate and identify individual bacterial strains. Recent studies dealing with the microflora identification of kefir grains and kefir drink show the importance of using the tools of molecular biology to clearly characterize the bacteria and yeasts present in these types of products.[30–33] Earlier reports on the composition of kefir lists many different types of LAB, yeasts, and acetic acid bacteria.[33–42] These identifications were based on phenotypic characteristics, which are often not sufficient to identify an organism to the species level.

Bacteria such as *Lb. kefir* and *Lb. brevis*, which are phenotypically very similar, are good examples to demonstrate the need to use molecular tools for identification. Some researchers have reported that they are able to differentiate the two species based on the fermentation of sucrose, trehalose, and xylose,[43] but results were not always obvious because some strains of *Lb. brevis* will not ferment one or more of these sugars.[44] Table 4.1 summarizes the most recent studies published on the identification of kefir microflora. Studies performed by Angulo et al.,[12] Rohm et al.,[45] Pintado et al.,[43] Lin et al.,[46] Simova et al.,[39] and Witthuhn et al.,[41,42] are based on phenotypical traits of the strains isolated. Bosch et al.[40] used whole-cell protein pattern by SDS-PAGE to identify their kefir isolates, whereas papers published by Takizawa et al.,[30] Wyder and Puhan,[31] Wyder et al.,[32] and Mainville et al.,[33] used restriction fragment length polymorphism (RFLP), DNA–DNA hybridization, and other molecular tools to characterize the strains they have isolated. One would not expect the list of bacteria and yeasts composing the grains to be very extensive, nor should it vary significantly from one part of the world to another if good care, similar growth conditions, and proper sanitary conditions are maintained. One could even assume that if contaminant species were to come in contact with the kefir population, it would probably not survive, or its growth would be inhibited due to the production of compounds such as bacteriocins by the symbiotic flora of kefir. However, over time and under different growing conditions, kefir grains may change their microbial makeup and fermentation properties.[47]

Most of the microbiological studies done on kefir and kefir grains have centered on the identification of the constituent bacteria. In many fermented milk products, yeasts are not desirable; they cause spoilage because the low pH provides a selective environment for their growth.[44] For kefir, yeasts play a key role in the fermentation process and even though the number of yeasts in the final drink is less than in the grains,[48] it is important to maintain the balance of bacteria to yeasts. Yeasts contribute to the unique characteristics of kefir. Rosi[20] was one of the first researchers to study the yeasts in kefir grains using electron microscopy. She found that the yeasts tended to be located in the center and along the peripheral channels of the grains. Using morphological and physiological characteristics, DNA-based composition, and electrophoresis patterns, Iwasawa et al.[49] identified *Torulopsis holmii* in commercial kefir grains. Engel et al.[21] in their survey of commercial and home-produced kefir, found both lactose-fermenting and nonlactose-fermenting yeasts in most products, although some commercial products called "kefir" contained no yeasts. Most

TABLE 4.1
Microorganisms Identified in Kefir and Kefir Grains

Lactic acid bacteria	Yeasts	Others	Sources	References
Lb. kefiranum, *Lb. kefiranofaciens,* *Lb. kefir,* *Lb. parakefir*	N.D.	N.D.	Christian Hansen (Copenhagen, Denmark); kefir grains	30
Lb. brevis, *Lb. viridescens,* *Lb. kefir,* *Lb. fermentum,* *Lb. rhamnosus,* *Lb. casei* ssp. *tolerans,* *Lb. casei* ssp. *pseudoplantarum,* *Lb. acidophilus,* *Lb. gasseri,* *L. lactis* ssp. *lactis,* *S. salivarius* ssp. *thermophilus,* *Leuconostoc* spp.	*Torulaspora delbrueckii,* *Saccharomyces cerevisiae,* *S. unisporus,* *Candida kefyr,* *C. holmii,* *C. friedrichii,* *Kluyveromyces lactis,* *Pichia fermentans*	*Pediococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Acetobacter* spp., *Escherichia coli*	Eight sources from northwest region of Spain; kefir grains	12
N.D.	*Kluyveromyces marxianus,* *Pichia fermentans,* *Saccharomyces cerevisiae,* *S. dairensis*	N.D.	Nineteen sources from Austria; kefir grains and kefir drinks	45
Lb. helveticus, *Leuconostoc mesenteroides*	*K. marxianus,* *Pichia fermentans*	N.D.	Taiwan; kefir grains	46
N.D.	*K. marxianus,* *Candida kefyr,* *C. colliculosa,* *Torulaspora delbrueckii,* *Brettanomyces anomalus,* *Saccharomyces unisporus,* *S. turicensis*	N.D.	Five sources of different origin (Toni AG, Zürich, Switzerland; Biolacta-Texrl, Poland; private sources); kefir grains and kefir drinks	31,32
Lb. kefir, *L. lactis* ssp. *lactis*	*Saccharomyces unisporus*	N.D.	Portugal; kefir grains	43
Lb. delbruekii ssp. *bulgaricus,* *Lb. helveticus,* *Lb. casei* ssp. *pseudoplantarum,* *Lb. brevis,* *L. lactis* ssp. *lactis,* *S. thermophilus*	*K. marxianus var. lactis,* *S. cerevisiae,* *C. inconspicua,* *C. maris*	N.D.	Bulgaria; kefir grains	39

Lactic acid bacteria	Yeasts	Others	Sources	References
Lb. fermentum, *Lb. brevis*, *Lb. delbruekii* ssp. *delbruekii*, *Lb. curvatus*, *L. lactis* ssp. *lactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, *Leuconostoc lactis*, *Leuconostoc mesenteroides var. dextranicum*	*Geotricum candidum*, *Zygosaccharomyces* sp., *Cryptococcus humicolus*, *Clambica*, *C. krusei*, *C. kefyr*, *C. lipolytica*, *S. cerevisiae*, *C. holmii*	N.D.	Eight sources from South Africa; kefir grains (traditional and mass cultivation)	41,42
Lb. kefir, *Lb. parakefir*, *Lb. plantarum*	N.D.	N.D.	Four sources from Argentina; kefir grains	40
Lb. kefir, *Lb. kefiranofaciens*, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *cremoiris*	*Candida kefyr*, *Saccharomyces exiguus*	*Acetobacter aceti*	Russia; kefir grains and kefir drink	13,33

Note: N.D.—not determined.

often cited are *Candida kefyr*, *Saccharomyces exiguus*, *Brettanomyces anomalus, Kluyveromyces lactis* var. lactis, *S. cerevisiae*, and *S. unisporus*.[50]

The stability and composition ratio of the microorganisms in kefir between production and consumption are important to consumers who are eating kefir as a probiotic for its health-promoting properties. Figure 4.3 shows the changes in the total number of lactococci, lactobacilli, and yeasts in commercial kefir that had been stored at 4°C for 42 d.[51] The patterns for the three categories of microorganisms studied were similar in natural (unflavored) or strawberry (15% by weight) flavored kefir. Numbers of lactococci declined by almost 2 log units by the end of 42 d. Even with this decline during storage, the number of microorganisms in the drink remained high enough for the product to be considered a probiotic.

4.2.2 Kefiran

Early observations of the structure of kefir grains noted that some of the bacteria were encapsulated by a polysaccharide.[34,37] La Riviére et al.[52] carried out the first studies of the polysaccharide and gave it the name *kefiran*. Kooiman[53] was able to show that the water-soluble polysaccharide consisted of approximately equal proportions of glucose and galactose. The chemical structure proposed is shown in Figure 4.4.

This structure was modified by Mukai et al.,[54] who used nuclear magnetic resonance (NMR) to show that 6-*O*-substituted galactose may also exist in the structure

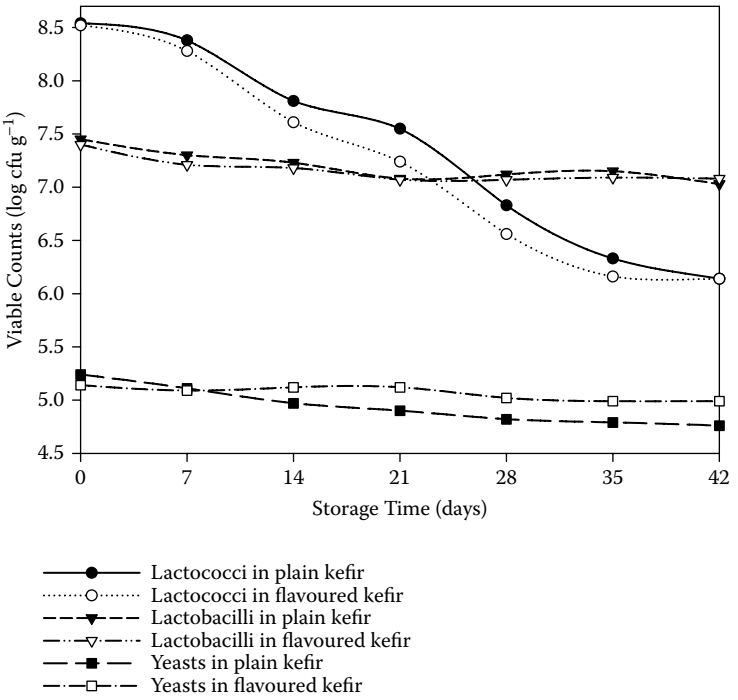

FIGURE 4.3 Changes in numbers of lactococci, lactobacilli, and yeasts during the storage of kefir.

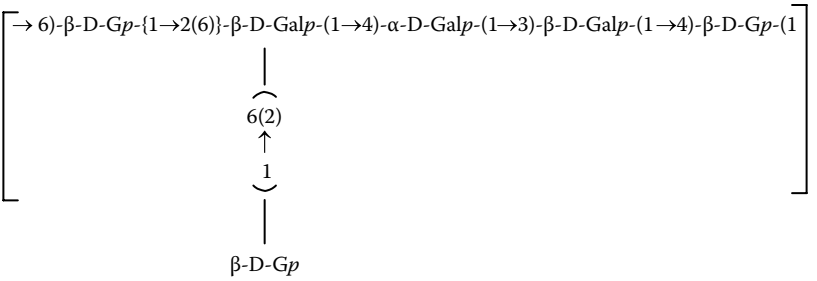

FIGURE 4.4 Chemical structure of kefiran. (From Kooiman, P., *Carbohyd. Res.*, 7, 200–211, 1968. With permission.)

of kefiran. However, to date, no complete structural analysis with NMR confirmation has been published for kefiran.

It appears that kefir grains are the only source of kefiran, but it is not known whether more than one polysaccharide or various isomers of kefiran exists in kefir grains or kefir. Koroleva[24] quotes Russian references that claim kefir grains can contain up to 34% polysaccharide, whereas the final drink contains 0.2 to 0.7% polysaccharide.

The bacteria that produce kefiran have been the subject of additional research. La Riviére et al.[52] originally suggested that *Lb. brevis* was the bacterium producing

kefiran. In 1983, Kandler and Kunath[55] identified *Lb. kefir* as the bacteria responsible for it. Toba et al.[56] isolated a polysaccharide-producing bacterium, which they named *Lactobacillus kefiranofaciens*.[15] *Lactobacillus* sp. KPB-167B was published by Yokoi et al.,[57] and *Lactobacillus kefirgranum* and *Lactobacillus parakefir* were reported by Takizawa et al.,[30] as the responsible organisms. Yokoi et al.[58] were able to isolate five different polysaccharide-producing bacteria of the genus lactobacillus from commercially available kefir grains. Yokoi et al. stated this list of bacteria producing kefiran indicates the complexity of the taxonomic relationships of the bacteria isolated from kefir, and they implied that other bacteria found in kefir grains may be capable of producing kefiran-like polysaccharides, especially kefir grains originating from different geographical locations.

Toba's group tried to produce a new fermented milk drink using the kefiran-producing *Lb. kefiranofaciens* they had isolated from kefir grains, but the product had a ropey consistency and scored low on consumer acceptability criteria.[59] In spite of this, Toba et al.[59] stated that the health benefits of kefiran might be great enough to convince people to consume the product.

Several groups have tried to find media and growth conditions that would optimize the production of kefiran. Toba et al.[56,60] were able to produce 80 mg polysaccharide/L of media using a whey-based growth medium that had increased glucose and added trypticase peptone compared to their standard whey-based medium. They also reported that the viscosity of the fermented drink was directly proportional to the polysaccharide content, and they used viscosity measures to gauge the efficiency of their different growth media.

The five different bacteria isolated by Yokoi et al.[58] were capable of producing 273 to 406 mg of total (supernatant + capsule) polysaccharide/L of media. Again they used a whey-based growth medium. Using this idea, Rimada and Abraham[61] showed how kefir grains can be used to produce exopolysacccharide from deproteinized whey. They were able to acidify the whey and reduce the lactose content of this abundant diary industry by-product. Yokoi and Watanabe[62] reported yields of 2.04 g polysaccharide/L of medium after 4 d of culturing, using a modified de Man, Rogusa, Sharp Lactose (MRSL) medium that contained 10% lactose. Micheli et al.[63] have been able to batch produce large quantities (2 g/L) of kefiran from a slime-forming rod-shaped *Lactobacillus* isolated from grains obtained from a local dairy in Italy, using the medium reported by Yokoi and Watanabe.[62] More recently, Cheirsilp et al.[64] used a fed-batch addition of fresh medium with increased yeast extract content to achieve a final kefiran concentration of 5.41 g/L at 87 h, using a mixed culture of *Lb. kefiranofaciens* and *S. cerevisiae*.

It is not clear how many different bacteria synthesis kefiran or kefiran-like polysaccharides. Although kefiran is found in kefir grains, few reports have been published where the concentration of kefiran in the final drink has been reported.[24] Kefiran may be contributing to the texture of kefir.

4.2.3 Electron Microscopy of Kefir Grains

Initially, light microscopy and later, transmission electron microscopy and scanning electron microscopy have been used extensively to study and describe the structure and mycology of kefir grains.[9,10,20,36,46,65–70] Figure 4.5 is the scanning electron

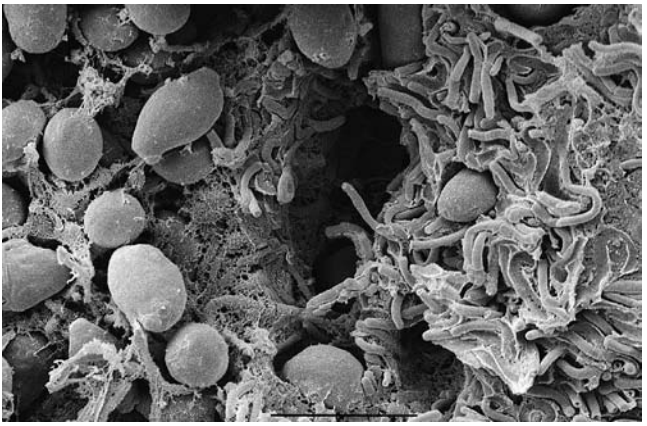

FIGURE 4.5 Electron micrograph of kefir grains showing bacteria and yeasts in carbohydrate or protein matrix. Magnification X 2555, bar indicates 10 μm. (Photo courtesy of M. Kalab, Agriculture and Agri-Food Canada.)

micrograph of kefir grains obtained from the Moscow Dairy Institute that shows the matrix of bacteria, yeast, polysaccharide, and protein. Molska et al.[66] used electron micrographs to estimate the composition of kefir grains. They reported that their commercial grains from Poland contained 66% bacilli, 16% streptococci, and 18% yeasts. The microbiological population appears to be different on the surface of the grains than in the interior.[10] This is due in part to differences in the pH throughout the grain; the interior has been reported to have a very low pH that inhibits the growth of lactococci.[69] Generally, long rod-shaped bacteria predominate on the surface, whereas yeasts are concentrated in the interior of the grain.[46,65] This difference is important because the surface microorganisms are the ones that probably have the greatest impact on the fermentation process that produces kefir. Toba et al.[67] observed differences in the type of bacteria on the surfaces of propagable and nonpropagable grains and concluded that long rod-shaped bacteria with filamentous appendages were necessary for kefir grains to function. However, a large variation in the bacterial population occurs between grains and even within the same grain. Lin et al.[46] concluded that place of origin of the grains alone may explain the differences that have been reported in various electron microscopy studies.

4.2.4 Maintaining Viable Kefir Grains

A study of methods to preserve kefir grains showed that grains stored at −20°C for 120 d had the same microflora profile and produced kefir with the same rheological characteristics, acidity, and carbon dioxide content as kefir produced from nonstored grains. Grains stored at 4°C and then used to inoculate milk produced inferior quality kefir.[71] Lyophilizing kefir grains has also been used as a way of producing starter cultures for industrial production.[72] However, a study of the properties of kefir grains that had been lyophilized and then stored for 1–3 months in packages made from films with different water and oxygen permeability showed that the rehydrated grains were slower and less able to lower pH, produced less acidity, had decreased

lactose utilization, and a lower lactic acid production than fresh grains. The bacterial profile of the rehydrated grains was also different than the fresh grains.[73] This same group compared freezing, refrigerating, air-drying, and lyophilization to preserve kefir grains. They concluded after 10 months of storage that freezing and refrigeration preserved acidification activity, whereas air-drying and lyophilization increased lag phase and resulted in a lower initial rate of pH decrease.[74]

Traditionally, kefir grains have been replicated in cows' milk. However, Abraham and de Antoni[11] have shown that the same grains that grow in cows' milk can grow and replicate in soybean milk. When soybean milk was used, the resulting grains are more compact and smaller in size, and have a yellowish color—most likely due to the protein. When the grains are added to the soybean milk, the pH is lower after 30 h than it is in cows' milk. After 20 subcultures, the soybean-grown grains had a higher protein content and lower polysaccharide content than the milk-grown grains, although the microbial profiles of the two grains were similar.

4.2.5 Other Uses of Kefir Grains and Kefir

Plessas et al.[75] used the mixed culture contained in kefir grains as a bread starter with lean dough that produced bread with increased shelf life, and acceptable taste. Kefir itself has been added as a coculture in feta-type cheese starter, with acceptable organoleptic results.[76] The cheese industry by-product whey has been shown to be a good starting material for kefiran production when treated with kefir grains.[77] Alternatively, whey can be used to produce kefir single-cell protein biomass on an economic scale for animal feed.[78] A wide variety of commercially valuable esters and alcohols have also been produced using kefir yeast cells.[79,80]

4.3 COMMERCIAL KEFIR PRODUCTION

4.3.1 Size of Production

Kefir is a relatively new commercial product in North America, and this, combined with the fact that the majority of kefir is produced and consumed in Eastern Europe, has made gathering of statistics on kefir production and per capita consumption difficult.[81] In addition, many data-gathering organizations do not differentiate between the various types of fermented (processed) milk products, and so kefir is often included with yogurt, buttermilk, and traditional Russian dairy products such as smetana, tvarog, blinchiki, and pelmeni. A sharp decrease in milk production and a substantial fall in the spending capacity of consumers led to a significant decrease in the consumption of milk and milk products in the Ukraine and many eastern European nations.[82] However, the importance of kefir in the Russian diet is demonstrated by the fact that in 1992 it was included on the list of regulated commodities together with such other staples as milk, sugar, salt, and bread.[83] The industrial production of kefir has been large enough that Germany, Austria, Brazil, France, Luxembourg, Norway, Switzerland, Czechoslovakia, and Israel have regulations defining the composition and method of production of kefir.[84,85] In the United States, California has regulations concerning kefir milk.[86] Table 4.2 is a listing of production levels of kefir from various countries.

TABLE 4.2
Commercial Production of Kefir in Various Countries

Country	Year	Production	Comment	Reference
USSR	1940	15,900 tn		3
USSR	1975	254,000 tn		3
Sweden	1985	17,000 tn		3
Poland	1982	22.5 million L		72
Poland	1988	35.3 million L		72
Poland	1997	255 million L	Kefir + buttermilk + flavored milk	87
Poland	1998	276 million L	Kefir + buttermilk + flavored milk	87
Poland	1999	327 million L	Kefir + buttermilk + flavored milk	87
Hungary	1996	116,337 tn	Fermented or acidified dairy products	88
Hungary	1997	119,255 tn	Fermented or acidified dairy products	88
Hungary	1998	138,986 tn	Fermented or acidified dairy products	88

4.3.2 Methods of Production

Home production of kefir is practiced in many countries, and scientific studies have often used samples gathered from homes. The traditional method of kefir making is performed by adding kefir grains directly, as a starter, to pasteurized, cooled milk. In home production, fermentation temperature and time are not rigidly controlled. The final product cannot be used to inoculate new milk to produce kefir because the original balance of microorganisms in the grains has been disrupted; kefir grains are essential to the process.[89] Recently, Yuksekdag, Z.N. et al.[90] studied the acid production, hydrogen peroxide production, proteolytic activity, and acetaldehyde production of 21 strains of *Lactobacillus* species isolated from Turkish kefir, as a way of understanding the role bacteria in kefir grains play in the fermentation process.

The properties of kefir (chemical, physical, and organoleptic) were initially difficult to duplicate in large-scale productions. Some processes were developed where no grains were used, but the quality of the product was much different from that of kefir fermented with grains.

Large-scale production of kefir has been hindered by the problems involved in reproducing the kefir grains and producing a consistent product. However, production of kefir on an industrial scale is common in many European countries, and patents have been taken out in several countries describing the process.[91–94] Several variations exist in the protocol used in the commercial production of kefir. Initially, the set method was used to produce kefir. In this procedure, inoculated milk was filled into bottles, fermented at a controlled temperature until a strong coagulum is formed, and then cooled. However, the kefir produced was of low quality compared to that produced on a smaller scale using traditional methods. Today, kefir is produced by a stirred method where fermentation, coagulum formation, agitation, ripening, and cooling all occur in one vessel.[24] The composition (chemical, organoleptic, microbiological characteristics) of the final product depends on the type of milk used, the source of grains, the preparation of the mother culture (often produced by coarsely

sieving the grains and using the percolate), the length of fermentation, the inclusion of a cooling step, and the inclusion of a maturation step.

A major difference in processing involves the choice of using either kefir grains or a kefir grain-free extract as the mother culture. Traditional artisan production of kefir involved inoculating milk with a quantity of grains (2–10%), and allowing the fermentation to proceed for approximately 24 h, to a predetermined pH or until a desired taste or texture is obtained. Fermentation is carried out at 20 to 25°C. A maturation step, carried out at 8 to 10°C for 15 to 20 h, is often added. The grains are then sieved out and can be used for a new fermentation or preserved (1–7 d) in fresh milk. Leaving the grains in the final product results in excessive acid production and inferior taste.[86,95,96]

For the production of "Russian style" kefir, a grain-free inoculum or mother culture is prepared by carrying out traditional kefir fermentation, sieving the finished product, and using the percolate to inoculate the milk. About 1 to 3% mother culture is added to pasteurized milk. When lyophilized starter cultures are used, the mother culture is prepared by adding 1 g of lyophilized kefir grain starter to 3 L of milk.[72] Koroleva[97,98] has suggested that the fermentation step should be followed by a slow cooling step (to a temperature of 8–10°C) and then a ripening (maturing) step that allows microorganisms to grow and taste and aroma to develop. Korovkina et al.[99] showed that as the temperature of the fermentation step increased (from 19°C to 28°C), the pH of the kefir produced dropped, and the viscosity increased. There was little change in the amount of CO_2 and ethanol produced.

A third step, to produce what has been termed "industrial kefir," involves taking some of the Russian-style kefir and adding it (2–3%) to a new source of milk, and again carrying out a fermentation (8–20 h at 20–22°C) and maturation (12 h to 7 d at 8 to 16°C) steps.[96]

Koroleva[97,98] reported the effect of various variables on the production process and the composition of starters (mother culture) used to produce Russian-style kefir. As the level of inoculum (as kefir grains) increased, the duration of the fermentation process became shorter. However, the changes to the microbiological profile (decreased levels of heterofermentative lactic acid streptococci and yeasts) were judged not to be favorable. The number of major groups of microorganisms increased as the quantity of kefir grains inoculated into the milk decreased. The pH of the starter was also dependent on the quantity of inoculum used: a starter pH of 3.6 to 3.8 was found using a ratio of 1:10 (starter to milk), and a pH of 4.4 to 4.6 with a ratio of 1:30 or 1:50. Garrote et al.[100] also showed that a drop in pH during fermentation was greater when the amount of grains added was increased, and that the ratio of grains to milk affected the viscosity of the final product. The apparent viscosity of kefir produced with 10 g grains/L of milk was highest; adding less or more grains per liter of milk reduced the viscosity. The numbers of yeasts and lactobacilli did not change in the final product produced with different ratios of grains to milk, but the number of lactococci decreased as the amount of grains added increased.

When starters were produced at 25°C, the numbers of homofermentive bacteria (particularly lactobacilli) were favored compared to starters prepared at 18 to 20°C. Higher temperature fermentation resulted in lower pH, which inhibited the growth of both homofermentive and heterofermentive lactic acid streptococci and yeasts.

Agitation of the fermentation mix had the benefits of preventing the growth of molds on the starter surface and promoting the even distribution of microorganisms and metabolites. A tenfold increase in homofermentative lactic acid streptococci and yeasts followed agitation, but there was no effect on the numbers of heterofermentative lactic acid streptococci, thermophilic lactobacilli, and acetic acid bacteria or any changes in the quantities of volatile fatty acids in the finished starter. In some facilities, kefir grains are washed with water once a week. Data from Koroleva[97] showed that washing reduced the numbers of the main groups of starter microorganisms. When the washed grains were then used in the production process, fermentation times were increased, and the final product had poor taste and consistency.

Various schematics have been published detailing the methods for producing kefir on an industrial scale.[8,96,101,102] Rossi and Gobbetti[103] outlined the details of a continuous process for producing kefir using a multistarter made up of bacteria and yeast isolated from kefir grains. Kefir was produced over a 30-day period; it was lower in viscosity and lacked some of the volatiles normally found in kefir.

Pettersson et al.[104,105] listed several reasons for the need for a simpler starter culture to replace traditional kefir grains in the production process, including easier handling, uniformity of starter activity, reduced risk of infection and changes in the starter, final product uniformity, uniform yeast content, and improved storage characteristics. They developed a freeze-dried starter containing 75% homofermentative streptococci, 24% citric-fermenting streptococci, 0.5% lactobacilli, and 0.1% yeasts that produced a kefir with characteristics (pH, lactic acid production, and viscosity) similar to those of kefir produced with traditional grains and that had higher organoleptic scores (yeast taste, overall aroma) than the kefir produced from grains. This freeze-dried starter was also found to produce a more stable bacterial population in prepared kefir than traditionally regenerated kefir grains did.

Other attempts have been made to produce a less complex starter culture that could replace kefir grains in the production of kefir.[106,107] By using two fermentation steps—*Lb. bulgaricus, Lb. acidophilus, Streptococcus thermophilus, Streptococcus lactis,* and *Leuconostoc*—followed by *Saccharomyces cerevisiae*, products were produced, but they had different characteristics (pH, viscosity, titratable acidity, and viable yeast counts) than the control kefir product. More recently, Rossi and Gobbetti[103] combined four lactic acid bacteria and two lactose-negative yeasts all isolated from kefir grains to produce a multistarter that allowed them to produce kefir using a continuous process.

No one to date has been successful in producing kefir grains themselves, and therefore new kefir grains can only be obtained by the propagation of existing grains. Few sources of kefir grains exist today, and although kefir drink can be found in many countries, grains are not commercially available. It has not been possible to establish whether all existing grains have a common origin. Koroleva[108] cautioned that any attempt to replace kefir grains by pure microorganism cultures would not be effective because the unique composition of kefir grains that has evolved over time cannot be replicated or replaced. Any symbiotic relationships between bacteria and yeasts that have developed over time would be difficult to replicate.

It is evident that the characteristics of kefir—chemical, microbiological, and sensory properties—eaten by the consumer are influenced by the starting material,

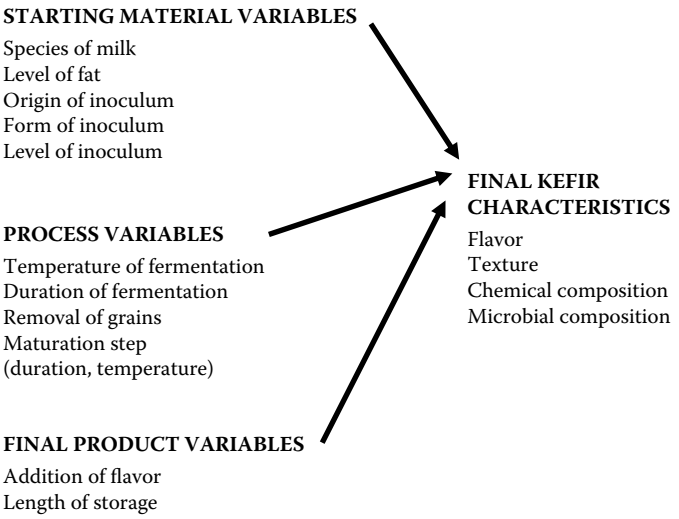

FIGURE 4.6 Processing factors affecting the characteristics of kefir.

various process variables, and manipulations of the final product. Figure 4.6 summarizes the factors that impact on the properties of the final product.

4.4 COMPOSITION OF KEFIR

The reported microbiological and chemical composition of kefir varies widely because the fermentation products in the final product are greatly influenced by the source of kefir grains used during fabrication. In addition, different types of milk (various species, various levels of fat) and different production methods (commercial, artisan) can be used. Generally, the pH of kefir is between 4.2 and 4.6. The ingredients most commonly measured as indicators of quality are CO_2, protein, lipid (fat), lactose, ethanol, and lactic acid. Table 4.3 is a summary of the composition of kefir reported in the literature.

4.4.1 Carbon Dioxide Content

Yeasts and some heterofermentive lactic acid bacteria are responsible for the production of the CO_2 gas in kefir. The CO_2 content increases during fermentation as the pH drops. If the fermentation is carried out for longer than 24 h, CO_2 production plateaus after 48 h.[70] The gas production leads to "fine flake" coagulum formation and also imparts a sparkling mouth feel to kefir.[24] The presence of the gas bubbles in the drink has prompted some to refer to kefir as the "champagne of fermented milk drinks."

Early research on yeasts in kefir showed that yeast isolated from commercial kefir grains was able to ferment glucose, galactose, mannose, and sucrose, but not lactose. Fructose was only fermented after an inductive period.[47] The amount of CO_2 produced appeared to be dependant on the presence of constituent enzymes that Iwasawa et al. thought might be unique to this yeast. Clementi et al.[48] studied the production of CO_2 by two yeasts as a way to better understand the factors that control

TABLE 4.3
Chemical Composition of Various Kefirs

CO_2		**Reference**
Polish commercial kefir	24.74% (v/v)	94
Grain fermented, 24 h	1.33 g/L	48
Grain free, 24 h	0.65 g/L	48
Protein		
Taiwanese kefir from grains (cow's milk)	3.16–3.18%	70
Polish commercial kefir		93
Fat		
Taiwanese kefir from grains (cow's milk)	3.07–3.17%	62,70
Lactose		
Traditional kefir	2.5%	107
Eastern European stir type	3.7–3.8%	107
Irish kefir from grains (diluted milk)	1.8%	69
Taiwanese kefir from grains	2.81–3.13%	70
Ethanol		
Polish commercial kefir	0.021–0.029%	93
Traditional kefir	0.5–1.5%	107
Eastern European commercial stir type	0.02–0.114%	107
Irish kefir from grains (diluted milk)	0.04%	69
Taiwanese kefir from grains	0.17–0.25%	70
Lactic Acid		
Traditional kefir	0.7–1%, 50% as L(+) isomer	107
Eastern European commercial stir type	0.7–0.8%	107
Irish kefir from grains (diluted milk)	0.5%	69

the CO_2 content of kefir, because they felt that even with the possibility of producing a kefir from different starters (not grains), the CO_2 content had to be maintained. They were able to show that in 2.5 h, immobilized yeast cells added to lactic acid fermented milk could produce CO_2 levels comparable to those of traditional kefir that had been fermented for 24 h.

The production of CO_2 during the fermentation step and continuing on in the finished product presents a unique packaging problem that can cause bulging of containers and leakage of contents.[102,110] In some countries, kefir has been either produced or sold in glass bottles. However, polyethylene foil packs covered with aluminum foil or plastic containers with an aluminum foil cover are more flexible containers that can accommodate the gas produced after packaging.

4.4.2 Fat Content

The fat content of kefir can be altered based on the type of milk fermented. In the former Soviet Union, kefirs have been sold that vary from fat free up to 3.2% fat.[111]

In her studies of various fermented milk products, Alm[112] found only minor differences in the fat content and composition (mono-, di-, and triglycerides; free fatty acids; and steroids) of kefir compared to the starting milk. The fact that free fatty acids were found in all the fermented milks analyzed (including kefir) indicated a disruption of the fat molecules in milk during fermentation, and Alm[112] speculated that this could result in an increase in the digestibility of milk fat in fermented products compared to other sources of fat. The lipid soluble coenzyme CoQ10 has been found in kefir.[113]

4.4.3 Lactose or Lactic Acid Content

The lactose found in milk is degraded to lactic acid during fermentation; this causes the pH to drop and the milk to thicken. As much as 30% of the milk lactose is broken down during fermentation.[70] β-galactosidase is the enzyme that hydrolyses lactose into glucose and galactose. It is found in yogurt and kefir grains, but its activity is very low in kefir.[17] The bacteria in kefir are able to break down glucose to lactic acid. Both D-(−)-lactic and L-(+)-lactic acid are produced from the milk lactose, and in kefir the L+ form tends to predominate. However, the ratio of L to D depends on the source and microbial composition of the grains.[69] Applying high pressure (> 1000 MPa) appears to increase the amount of D-(−)-lactic acid produced by some kefir bacteria.[114]

4.4.4 Ethanol Content

The production of ethanol in kefir is complex; both yeasts and heterofermentative bacteria produce ethanol. The quantity of ethanol produced is dependent on the fermentation process and the type of container used (tightly capped or not). Kefir produced in small dairies in the former Soviet Union in the early 20th century contained alcohol levels between 1 and 2%. Present-day methods of production result in much lower levels of alcohol. This may be due in part to the fact that fermentations are stopped at higher pH levels than previously. The final alcohol concentration is determined for the most part by the number of yeasts present in the grains added to the milk and the time of fermentation.[115] Ethanol appears to be produced towards the end of the fermentation process, and its formation can continue even when the pH has decreased to the point where the lactic acid bacteria in the product are no longer active.[110] The ethanol concentration can be increased in the final product by increasing the temperature during fermentation.[116] Kefir produced in the laboratory from grains had a higher ethanol content (0.040–0.30%) than kefir produced commercially in Germany (0.002–0.005%).[117]

Kwak et al.[110] studied ways to control the production of ethanol in kefir made from a defined starter culture and found that a two-stage fermentation was best. They used a nonlactose fermenting yeast—*Saccharomyces cerevisia*—initially to ferment glucose added to milk and then to carry out a lactic acid bacterial fermentation using a mixture of lactobacilli, lactococcoci, leuconostoc, and propionibacteria obtained commercially. During the yeast fermentation stage, the pH remained stable; it only dropped when the lactic fermentation started. Adding 0.4 or 0.5% glucose to the starter milk resulted in ethanol production only during the yeast fermentation stage, yielding a final product with 0.07 or 0.08% ethanol. Storage experiments showed that

kefir produced with the addition of 0.4% glucose was the most stable. When 1.0% glucose was added, the production of ethanol continued in the lactic fermentation and resulted in an ethanol concentration of 0.24% in the final product.

4.4.5 Amino Acids

Kefir has the same pattern of amino acids as milk. Kefir proteins are easily digested due to the action of acid coagulation and proteolysis of milk proteins. Free amino acids found in milk are consumed during the first hours of fermentation by selective bacteria. As the fermentation slows and the kefir enters the ripening stage, the proteolytic activity of other microorganisms such as acetic acid bacteria and yeasts causes more peptides and free amino acids to be formed in a manner seen in other fermented milk products. (See Chapters 5 and 7 for more details on the hydrolysis of milk proteins.)

4.4.6 Volatile Components

Minor volatile components have been studied because of their possible contribution to kefir's unique taste. Compositional data are inconsistent, however, because of the variety of sources of the kefir analyzed. Acetaldehyde and diacetyl are two important contributors to flavor, but propionaldehyde, 2-butanone, *n*-propanol, iso-amylalcohol, acetic acid, and ethanol found in kefir may also influence the aroma.[118,119] Görner et al.[119] noted that the levels of volatiles found in kefir, particularly ethanol, changed during the course of the fermentation. Data from laboratory-produced kefir showed that maturation following fermentation lowered pH and acetaldehyde concentrations, but increased lactic acid and diacetyl levels.[100] Commercial kefir contains half as much ortic acid, twice as much pyruvic acid, nine times as much acetic acid, and about an equal amount of uric acid as does in commercial yogurt. Some kefir also contains propionic acid.[17] Dousset and Caillet[116] followed the concentrations of seven organic acids during the fermentation process and found that propionic acid was produced only in the last stages of fermentation (pH 4.33 and below) and that when the temperature during fermentation was increased from 20 to 30°C, the concentrations of citric acid, lactic acid, acetic acid, propionic acid, and isobutyric acid increased in the final product, whereas pyruvic acid levels declined.

Guzel-Seydim et al.[120] followed the production of organic acids and volatiles during the fermentation of kefir produced in the laboratory from grains obtained from Turkey. Lactate production started slowly but the concentration of lactate rapidly climbed to 6000 μg/g by the end of the 22 h fermentation. Citrate, the next most abundant organic acid, declined during fermentation from 1760 to 1440 μg/g at the end of the fermentation. Pyruvate levels increased during fermentation to a final level of 18 μg/g. Levels of orotate and urate declined over the 22 h period; hippurate was totally consumed after 15 h. Acetic acid, propionic acid, and butyric acid were not found in any samples. Diacetyl was also absent. Acetaldehyde and acetoin levels increased as the fermentation progressed. However, ethanol production did not begin until after 5 h in the fermentation. Guzel-Seydim et al.[121] also studied the changes in organic acids and volatile flavor compounds in kefir stored at 4°C for up to 21 d. Lactate was the organic acid in highest concentration (> 6000 μg/g), followed by

citrate (1500 μg/g). Lactate increased slightly during storage. Pyruvate was found at day zero, but by day seven of storage it had disappeared. The conversion of pyruvate to ethanol and CO_2 may account for this observation. This stored kefir contained no hippuric acid, acetic acid, propionic acid, or butyric acid. Acetaldehyde levels doubled (to 11 μg/g) during storage, whereas acetoin levels decreased from 25 to 16 μg/g during the storage period. This group found no diacetyl in their kefir.

Linossier and Dousset[27] showed that the yeast *Candida kefir* was capable of producing malic acid, citric acid, and pyruvic acid in milk with *Lb. kefir*, but levels of lactic acid, fumaric acid, and butyric acid were low. Citric acid was not found after 70 h of fermentation. There appeared to be a symbiotic relationship between the *C. kefir* and the *Lb. kefir*, as the growth of the bacterium was enhanced by the presence of the yeast in the media. The addition of *C. kefir* at levels as low as 0.5% of the total microbial population stimulated the growth of *Lb. kefir.*

4.4.7 The Taste of Kefir

The taste of unflavored kefir has been described as "yeasty" and the terms "prickling" and "sparkling" has been used to describe the mouth feel of kefir caused by the liberation of trapped CO_2. Complaints from long-time consumers of kefir about the taste of traditional kefir occur only when the yeast taste is very pronounced or absent.[104] However, the taste has not been scored high in sensory evaluations by North American consumers.[10,105] Kefir that scores higher than unflavored kefir in consumer acceptability tests can be produced by adding flavor—either as flavor itself or as fruit preserve. Starter cultures that contain bacteria that produce flavors can also be added to improve product acceptability.[122,123] Although flavored kefir may appeal to the North American consumer, the addition of fruit or other sources of sugars may cause unwanted fermentation by yeasts after packaging.[4]

In a sensory evaluation study carried out in Scotland, Muir et al.[124] showed that both the chemical composition and the scores given by sensory panelists differed between commercial traditional kefir made from kefir grains and modified kefir made from a defined blend of bacteria and yeasts. Traditional kefir had higher levels of lactic, acetic, and propionic acids. The sensory panelists judged the modified kefir as less acidic, with a creamier taste and had less serum separation compared to the traditional kefir. This led the researchers to conclude that modified kefir might be more acceptable to Western European consumers than traditional kefir.

Kefir produced using buffalo milk and traditional grains was reported to have chemical properties similar to kefir produced from cows' milk, and it had acceptable organoleptic properties and was deemed suitable for Egyptian tastes.[1]

4.5 NUTRITIONAL VALUE OF KEFIR

The protein, fat, and mineral content of kefir are similar to that of the milk from which it is made, and therefore, kefir has inherently a high nutritional value as a source of protein and calcium. Kefir also has a reputation as being palatable with a high digestibility, allowing for consumption of large quantities without intestinal disturbance.[125]

4.5.1 Digestibility

The fermentation process brings about denaturation of milk proteins and hydrolysis of some of the protein, resulting in smaller structures that are more susceptible to digestion by gastric and intestinal juices. In vitro experiments carried out by Alm[126] showed that kefir developed a very small curd size when exposed to simulated gastric juice even after a 3 h exposure to acidic conditions. Kefir was widely used in hospitals and sanatoria in the former Soviet Union as part of the diet for patients with gastrointestinal and metabolic diseases, hypertension, ischemic heart disease, or allergies. Evenshtein[127] reported that kefir (250 mL/d) was used successfully to stimulate gastric secretions and acid formation in patients with pulmonary tuberculosis. Kefir is particularly recommended for babies and patients with malabsorption syndromes, presumably because of the small curd size that forms in the stomach and the observation that fermented dairy products, in general, are digested without the secretion of large amounts of gastric juices.[110] Mann[2] quotes two Russian publications that highlight the nutritional value of kefir especially for infants and indicate that products based on kefir were being produced for this market.

4.5.2 Protein Nutrition

Rat studies carried out by Vass et al.[128] confirmed that kefir had a superior biological value (protein efficiency ratio—PER) than milk, which could be explained as being due to its better protein digestibility. Also, as a result of bacterial metabolism, both the total nitrogen (TN) and the nonprotein nitrogen (NPN) in kefir are higher than those in the milk it is produced from. Schmidt et al.[129] reasoned that kefir, like yogurt, had a higher protein digestibility that contributed to its higher nutritional value and capacity to regenerate liver tissue in rats that had undergone partial (70%) hepatotectomy.

During fermentation and storage, the amounts of free amino acids in kefir increase, particularly lysine, proline, cystine, isoleucine, phenylalanine, and arginine.[130–132] The bacteria and yeasts used to produce kefir cause the isomerization of the L-amino acids liberated from milk proteins to D-amino acids, particularly D-alanine, D-leucine, D-aspartic acid, and D-allo-isoleucine. D-amino acids are less common in yogurt.[133] The addition of sodium caseinate to milk results in a product that is even higher in protein content (up to 3.5%), and this has been proposed as a possible dietetic drink.[134]

4.5.3 Lactose Metabolism

Kefir contains a variety of microorganisms that have the potential to aid lactose digestion. People with lactose intolerance have an insufficient activity in their intestines of the enzyme β-galactosidase (*EC* 3.2.1.23) or lactase—the enzyme responsible for the hydrolysis of lactose into glucose and galactose. Fermented milk products offer hope to such people because some of the microorganisms used in the fermentation of these products possess lactase activity. Yogurt is often suggested as a dairy product that can be consumed by people with lactose intolerance because the lactase activity in yogurt microorganisms break down some of the milk lactose during yogurt production and storage and because lactase activity increases after

the consumption of unpasteurized yogurt due presumably to the presence of yogurt bacteria in the intestines that retain lactase activity.[135] Alm[136] studied the decrease in lactose content in several fermented milk products including kefir and found that compared to yogurt, acidophilus milk, and bifidus milk, the decrease in kefir lactose was less following fermentation and storage for up to 14 d. In their study with pigs, de Vrese et al.[137] showed that while kefir grains did have some β-galactosidase activity, kefir drink did not. The galactosidase activity in kefir made from grains is very low compared to yogurt containing live bacteria.[13] However, Hertzler and Clancy[138] reported that consumption of kefir made from a starter culture containing bacteria that do have β-galactosidase activity reduces breath hydrogen, a common measure of lactose intolerance.

The lactic acid is found in all fermented dairy products as a result of the action of homo- and heterofermentative microorganisms. Lactic acid can exist in either the L-(+) or D-(−) isomeric forms and as a 50/50 DL racemic mixture. L-(+) lactic acid is completely metabolized by the body, but D-(−)-lactic acid is used more slowly by the body, and excess D-(−)-lactic acid can cause metabolic disturbances. Alm[139] quotes World Health Organization documents that suggest that infant nutrition products containing D-(−) or DL mixture should be avoided, although it is admitted that more research needs to be done to verify this.[140] Kefir contains almost exclusively L-(+)-lactic acid; in yogurt the ratio of L-(+)/D-(−) is 58:42.[139]

4.5.4 Vitamin Content

Cows' milk is generally considered as a good source of most water-soluble vitamins, except for ascorbic acid and vitamin $B_{12.}$ Several investigators have measured the quantity of vitamins in kefir to determine whether fermentation changed levels compared to milk, but the results have often not been consistent. An early study of the vitamin B_{12} content of kefir indicated that both during the fermentation and maturation stages, the vitamin B_{12} content went down.[121] Alm[141] used commercially available grains to produce kefir that had increased folic acid content compared to the starting milk, but decreased concentrations of vitamin B_6, vitamin B_{12}, and biotin. Of the various fermented products studied by Alm, kefir had the lowest reduction (17%) in orotic acid (also referred to as vitamin B_{13}) as compared to the starting milk. Yogurt had a 47.8% reduction in orotic acid. Bossi et al.[131] reported declines in vitamin A, thiamin, riboflavin, nicotinamide, and vitamin C in laboratory-prepared kefir compared to the starting milk.

Kneifel and Mayer,[143] using ten different sources of grains obtained from Austrian households, reported increases in pyridoxine (9/10), vitamin B_{12} (5/10), and folic acid (7/10) of laboratory-prepared kefir compared to the starting cows' milk. Most kefir samples had folic acid content over 20% higher than those of the starting milk. Thiamine (8/10), riboflavin (10/10), niacin (8/10), pantothenic acid (7/10), and orotic acid or vitamin B_{13} (9/10) levels in the finished kefir were lower than in the starting milk. When different sources of milk were compared (cow, ewe, goat, and mare), ewes' milk had the largest percent increases of thiamin, pyridoxine, and folic acid, whereas goats' milk had the largest percent loses of thiamin, riboflavin, vitamin B_{12}, niacin, pantothenic acid, and orotic acid. The source of the milk may

influence the growth of particular microorganisms that, in turn, determine the final vitamin content of kefir.

Mann quotes Russian, Czech, and Polish references that describe attempts to increase the nutritional quality of kefir.[72] The total protein content of kefir can be increased by adding sodium caseinate to the starter milk. Specific bacteria can also be added to the initial mother culture to increase the levels of folic acid and vitamin B_{12}.

4.5.5 Kefir as an Infant Food

A Russian team studying on premature infants found that a mixture of kefir and Similak-type formula was well tolerated and produced adequate weight gains when fed to healthy premature infants. The blood fatty acid pattern in the infants was similar to that found in the kefir-Similak mixture that was fed.[144]

Kefir is used widely in Russian hospitals, particularly in neonatal wards, both for mothers and newborns. It is often recommended as the first food for babies after breastfeeding because of its high digestibility. Goncharova et al.[145] reported that kefir fed to premature infants did not restore their intestinal bifidobacteria nor reduce the number of disturbances of biocenosis. It appears that kefir was also used in the treatment of biliary tract diseases associated with diseases of the pancreas in children.[146] Ormisson and Soo[147] attempted to reduce the decline in buffer bases in children up to 2 yr old suffering from acute pneumonia or acute bronchitis. Kefir and sour milk both shifted the acid-base balance to acidosis, which is not desirable in sick children. It appears that some kefir products were produced specifically for children.[148]

4.5.6 Other Nutritional Uses

Kefir has been used as part of a weight reduction program in the former Soviet Union, where obese patients are given only kefir to consume every second day of treatment. Kefir has also been used in the former Soviet Union as a vehicle for increasing the essential fatty acid (EFA) intake of patients with metabolic diseases and intestinal tract disorders.[111] This EFA- enriched kefir has been prescribed to patients with atherosclerosis, ischemic heart disease, obesity, peptic ulcers, and liver and gall bladder pathologies.

A Japanese patent has been granted for the formulation of a health food that contains kefir together with enzyme inhibitors such as lipase or α–amylase.[149] This product is purported to prevent and control obesity.

4.6 PHYSIOLOGICAL EFFECTS OF KEFIR CONSUMPTION

Kefir has a long tradition of being regarded as good for health in the countries where it is a staple in the diet. However, published human-feeding trials to substantiate this view are not numerous.[7] Kefir, kefir grains, kefiran, and the bacteria found in kefir have been the subject of scientific studies to demonstrate beneficial effects on humans.

4.6.1 Kefir as a Probiotic

A probiotic is defined as a microbial preparation that contains live and/or dead cells, including their metabolites, which is intended to improve the microbial or enzymatic

balance at mucosal surfaces or to stimulate immune mechanisms.[150] Kefir contains many different bacteria and yeasts, and in a long-term feeding trial, the microbiological profile of mice was found to change viz. lactic acid bacteria counts increased in both the small intestine and large intestine, whereas *Enterobacteriaceae* and clostridia both decreased.[151] To date, however, no scientific studies have been published showing the health benefits of kefir microorganisms. A human-feeding trial comparing fecal material from subjects consuming a Hungarian–Russian type kefir made with probiotic lactic acid bacteria added to a traditional Russian kefir, showed that the total number of bacteria in fecal material and the number of certain targeted probiotic bacteria increased in both groups after four weeks of consumption (0.5 L/d).[152] This raises the possibility that kefir or one of its constituents may be a prebiotic.

The numbers of microorganims in kefir are large enough (>10^7 CFU/g) that kefir can be considered a probiotic.[153] Santos et al.[154] reported that several strains of *Lactobacillus* spp. isolated from kefir from various countries have good adhesion to Caco-2 cells, were resistant to low pH and bile acid and had antimicrobial activity against common enteropathogenic bacteria, which are popular criteria required by probiotic bacteria. In addition, during the fermentation process, microbial metabolites or degraded milk constituents may be produced that are also beneficial to human health.[155]

Studies have been published where kefir grains, kefir itself, or kefiran have been given to animals and humans to ameliorate a variety of conditions and diseases. The Russian literature contains articles describing the effects of kefir consumption, but these studies are not always readily available.

4.6.2 Antitumor Effect in Animals

The antitumor effect of kefiran was first reported by Shiomi et al.[156] They gave mice a polysaccharide isolated from kefir grains dissolved in drinking water for 7 d prior to injection with Erlich carcinoma (EC) cells and continuing for 24 d, or starting the same day as the injection of tumor cells and continuing for 23 or 24 d. A 40–59% inhibition of tumor growth was found in mice receiving 0.02% to 0.1% polysaccharide in their drinking water. The positive effect was observed for mice receiving the polysaccharide either starting before the administration of the tumor cells or at the same time as the tumor cell dose. In a second experiment, similar results (30–81% tumor growth inhibition) were found when mice were dosed with Sarcoma 180 (S180). Groups of mice were also given intraperitoneal injections of polysaccharide (0.05–2.0 mg/mouse). The polysaccharide was administered starting 7 d before or 1 d after tumor injection, and again both the growth of EC and S180 tumors were significantly reduced. Shiomi et al.[156] carried out cytotoxicity tests in which they incubated EC and S180 cells with a solution of kefir polysaccharide (1 mg/mL) and showed no effect, and this led them to conclude that the antitumor effect was host mediated.

This same group carried out another study to define the mechanism of the antitumor effect of kefir polysaccharide.[157] They showed that when mice were given polysaccharide isolated from kefir grains either by gastric intubation or dissolved in the drinking water (on days 1 and 11 of the experiment), their cell-mediated immune response was increased as measured by the delayed-type-hypersensitivity (DTH)

test. As little as a single dose of 5 mg/kg body weight was sufficient to produce a significant effect. This effect was shown to be dependent on the total dose administered and not the duration or frequency of the dose. When these same mice were then inoculated with EC cells, mice receiving as low as 10 mg/kg body weight had significantly reduced tumor weights after 14 d. This relation between increased immune response and reduced tumor growth was shown to be active in tumor-bearing mice but not in intact mice. It was suggested that kefir polysaccharide could be useful against tumor growth when administered either before or after the initiation of the tumor. However, larger doses of polysaccharide might be necessary if administered after tumor inoculation.

In a third paper, Murofushi et al.[158] investigated the antibody response of mice given kefir polysaccharide. The polysaccharide was shown to significantly enhance the antisheep red blood cell (SRBC) response, but only at a low antigen dose (5×10^6 SRBC/mouse). A treatment of 100 mg polysaccharide/kg body weight was shown to be most effective, and the data indicated that the polysaccharide was exerting its effect in early events of the antibody response. A lack of response in nu/nu, nu/+ and conventional mice to 2,4-dinitrophenyl-alanylglycylglycyl-Ficoll (DNP-Ficoll) as a TI-2 antigen or trinitrophenyl $(TNP)_{2.3}$- lipopolysaccharide (TNP- LPS) prepared as a TI-1 antigen indicated that the kefir polysaccharide was perhaps enhancing only primary T-dependent B cells and not B cells responsive to TI-1 or TI-2 antigens. Using ^{3}H-labelled polysaccharide, they were able to show that the water-soluble polysaccharide was absorbed into the body within 3 h of oral administration. Radioactivity was found in all major organs, and it was concluded that intact polysaccharide reached the spleen or thymus to activate the immune system.

The scientific literature contains a series of articles in which the antitumor properties of kefir itself are described. Furukawa et al.[159] found that mice given a subcutaneous inoculation of Lewis lung carcinoma had significantly smaller tumor weights and a 62% tumor inhibition rate 2 weeks after tumor inoculation. The mice received a gastric intubation of pasteurized kefir from day 1 to day 9 after tumor cell inoculation. Spleen weights and the number of leucocytes in tumor-bearing mice increased compared to control mice, but not in the mice receiving the kefir. This same group showed that feeding mice kefir (2 g/kg body weight) for 1 to 6 d after tumor inoculation resulted in an increase in delayed-type hypersensitivity response. However, no difference was found in the survival period of control mice versus those receiving the kefir.[160] This group's data showed that the kefir consumption significantly increased the number of leucocytes in normal mice and significantly decreased delayed-type hypersensitivity as measured by footpad swelling in mice bearing Meth-A fibrinoma. Using similar protocols and tests, Kubo et al.[161] showed that oral administration of kefir (100 or 500 mg/kg body weight for 10 d, starting 1 d after tumor inoculation) significantly reduced EC tumor weight and inhibited tumor growth by up to 54%. When mice were given kefir together with mitomycin C, the average tumor weight was even further reduced. Significant inhibition of Sarcoma 180 cell growth implanted in mice has been reported in mice eating kefir made from either cows' milk or soy milk. The positive effects of the fermented soy milk were not attributed to the genistein in the soy kefir.[162]

Furukawa et al.[163] have also looked at antimetastatic effects of two polysaccharide fractions of kefir grains. Both young (5 wk) and old (30 wk) female mice receiving the water-soluble fraction had significantly reduced pulmonary metastases of Lewis lung carcinoma. This fraction inhibited tumor growth when it was given to the young mice both before and after the tumor cell challenge. Mice given the insoluble fraction for 9 d before a challenge with B16 melanoma had an inhibition rate of 39% compared to the control mice. However, the water-soluble fraction was not protective against this highly metastatic cell line.[163]

Recent studies have looked at the antimutagenic properties of bacteria isolated from kefir in an attempt to understand their mechanism of action.[164] Starting with kefir manufactured in Mongolia, researchers isolated strains of *Steptococcus lactis* (5), *Str. cremoris* (3), *Str. faecalis* (1), *Lb. plantarum* (1), *Lb. brevis* (1), and *Leuconostoc dextranicum* (6). Using a binding assay in which the bacteria were incubated with mutagenic amino acid pyrolyzates, they showed that all the bacteria isolated from kefir had remarkable binding ability (> 98.5%) to the mutagens 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indole and 3-amino-1-methyl-5H-pyrido [4,3-b] indole but a lesser binding ability to 2-amino-6-methyldipyrido [1,2-a:3′,2′,-d] imidazole. Hosono et al.[164] concluded that these findings, along with similar results from bacteria isolated from yogurt, supported the idea that the consumption of fermented dairy products had a negative correlation with the risk for the development of colon cancer.

Miyamoto et al.[165] were able to isolate 31 strains of bacteria from kefir grains and kefir produced in western Europe, of which 3 strains of *Lactococcus lactis* ssp. *cremoris* had the strongest desmutagenic properties. These bacteria were also found to be slime-forming bacteria, and their antimutagenic properties appeared to be due to the binding affinity of AF-2 to bacterial cells. Cevikbas et al.[166] showed that intraperitoneal injection of 0.5 mL kefir/d for 20 d after the establishment of fusiform cell sarcomas significantly reduced the size of the tumors in mice and even resulted in the disappearance of tumoral necrosis in some animals.

Yoon et al.[167] used the Ames test to measure the antimutagenic properties of *Lactobacillus* spp. isolated from kefir and yogurt and were able to show that 36 out of 40 strains found in European kefir and yogurt protected *Salmonella typhimurium* TA 98 from the mutagen 2-nitrofluorene.

Kefir has been shown to have a higher antioxidant effect than vitamin E in a mouse carbon tetrachloride (CCl_4) toxicity test.[168] Mice fed kefir and exposed to CCl_4 had significantly lower malondialdehyde content in both kidney and liver tissues compared to CCl_4 exposed mice. Increased activity of glutathione peroxidase and glutathione S-transerase in the tissues of kefir-treated mice may have contributed to lowered lipid peroxide levels. To date, no human studies have been carried out to verify the antitumor effect of kefir and kefiran in humans.

4.6.3 Antibacterial, Antifungal, and Antiviral Properties of Kefir

Studies looking at the production of bacteriocins by kefir microflora found different types of bacteriocins are produced. A study on 33 sources of kefir grains from Ireland showed the presence of at least three different types of bacteriocins produced by lactococcal isolates.[169] The first bacteriocin had a narrow spectrum, inhibiting

only lactococci. A second bacteriocin inhibited strains of *Lb. casei*, *Lb. helveticus*, and *Pediococcus pentosaceus* of the strains tested. The third bacteriocin had a broad spectrum of effectiveness and inhibited all ten strains tested along with a number of other strains of *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Steptococcus thermophilus*, and *Staphylococcus aureus*. This bacteriocin was named lacticin 3147 and is produced by *Lactococcus lactis* strain DPC3147.[170] Lacticin differs from nisin in that it is plasmid encoded. Lacticin 3147 and nisin have similar inhibitory properties, and like nisin, lacticin is heat stable. Lacticin could therefore be a key factor in maintaining the integrity of the microflora of kefir grains by inhibiting the growth of foreign organisms. A recent study by Morgan et al.[171] investigated the antimicrobial potential of 38 Irish kefir grains against the pathogens *Listeria innocua* DPC1770 and *Escherichia coli* 0157:H45. It was found that 18 grains were able to fully inhibit the growth of *L. innocua*, 13 could slightly inhibit growth, and 7 had no inhibitory effect. Against *E. coli*, 34 of the grains completely inhibited growth, 3 slightly inhibited growth, and 1 source of grains had no inhibitory activity. The inhibitory effect of the kefir starters against *L. innocua* was attributed to the production of bacteriocins by the microflora. However, the inhibitory effect on *E. coli* was attributed not to a bacteriocin, but to the combined effect of acid and hydrogen peroxide.

Bossi et al.[131] tested lab-produced kefir and various combinations of bacteria found in kefir for their inhibitory effect against several intestinal pathogenic microorganisms. The kefir inhibited the growth of eight pathogens but was not effective against two strains of *E. coli* and one strain of *Staphylococcus aureus*. The growth of *E. coli* 0157:H7, *L. monocytogenes* 4b, or *Yersinia enterocolitica* 03 were not suppressed when added (at high inoculation rates) to either kefir produced from grains alone or in combination with yogurt.[172]

Kefir grains have antibacterial activity greater than kefir itself especially against Gram-positive cocci, including staphylococci, and Gram-positive bacilli. In addition, kefir was shown to have antifungal activity against a variety of fungi, yeasts, and molds. Cevikbas et al.[166] concluded that the antibacterial and antifungal activity of kefir helped to explain the wide use of kefir against infectious and neoplastic diseases. They quoted the work of Ormisson and Soo[147] as a human study that supports their conclusions. However, a translation of this later article indicates that the use of kefir in children with pneumonia and acute bronchitis as a means of improving acid-base balance was not successful.

Serot et al.[173] were able to isolate and partially characterize two antimicrobial agents from bacteria found in kefir grains. These two substances were found to be effective against Gram-bacteria found in kefir grains, effective against other Gram-positive and Gram-negative bacteria, had molecular weights of approximately 1000 Da, and were inhibited by some proteolytic enzymes. Zacconi et al.[174] showed that chicks challenged with *Salmonella kedougou* were protected by live kefir but not irradiated kefir, yogurt, or acidified milk. The treatment was most effective if the kefir was given at the same time as the *Salmonella* challenge, but it also offered protection if given 1 d or 6 d after infection.

The antimicrobial activity of fresh kefir differs from that of reconstituted lyophilized kefir. Fresh kefir had an intrinsic inhibitory power against *Staphylococcus aureus*, *Kluyveromyces lactis,* and *E. coli*, but not against *Saccharomyces cerevisiae*

or *Candida albicans*, but kefir that had been lyophilized and then reconstituted in distilled water or reconstituted powdered milk had lost this intrinsic inhibitory factor. The addition of ribitol as a cryoprotective agent appeared to be the best way of preserving the inhibitory properties of kefir after lyophilization.[175]

The antibacterial properties of kefir may be due to a combination of factors including competition for available nutrients or the production of inhibitory metabolites, such as organic acids, during fermentation. Garrote et al.[176] observed that although the bacteria in kefir have been shown to produce a bacteriocin, lactic acid and acetic acid may also contribute to the antibacterial properties of kefir. Supernatant collected from kefir produced using grains collected from Argentinian households had inhibitory effects against Gram-positive and Gram-negative bacteria, although the effect was greater against Gram-positive bacteria. Milk had no effect. Analyses of the kefir supernatants showed that they contained both lactic acid and acetic acid. When milk supplemented with lactic acid and acetic acid was incubated with *E. coli* 3, the inhibitory effect was again observed, whereas nonsupplemented milk had no effect. Milk with its pH adjusted to that of fermented kefir, or milk with added citric acid alone had no inhibitory effect against *E. coli* 3. Because the pH of the mixture determines the percentage of the acid in protonated or dissociated state, the pH of kefir supernatant (3.6–3.7) resulted in up to 1.5% of the lactic acid and 0.11% of the acetic acid being in the more powerful inhibitory undissociated form. Garrote et al. noted that previous research had shown that even these low levels were capable of inhibiting the growth of nonpathogenic *E.coli*.

Russian researchers have used a product called acipole that contains both *Lb. acidophilus* and kefir grains to manage antibiotic dysbacteriosis that is an adverse reaction to antibacterial therapy.[177] It was observed that patients suffering from pneumonia and chronic bronchitis treated with antibacterials often were susceptible to undesirable bacterial infections. Treating patients with an antibiotic and acipole lowered the frequency and severity of dysbacteriosis events.

Interferons initiate intracellular production of several kinds of antivirus proteins to indirectly induce the cells' virus resistant state. Kefir has been shown to contain a sphingomyelin that is capable of enhancing the production of interferon-β from the human osteosarcoma cell line MG-63 treated with a chemical inducer poly I: poly C. Activity was found in the range of 2 to 100 μg/mL with a maximum secretion enhancement (14 times) occurring at 25 μg/mL.[178] However, insufficient experimental protocol details were included to fully evaluate the credibility of these findings.

Besednova et al.[179] added to kefir a peptide isolated from nervous tissue of squid and found that this mixture, when fed to laboratory animals, stimulated their cellular and humoral immune systems. In mice that had originally been immune deficient, the mixture restored their immune function to normal.

Rodrigues et al.[180] used a disk diffusion method to measure the antibiotic activity of both kefir and kefiran. Both were effective against a wide range of microorganisms, but not as effective as common antibiotics. However, kefir gel applied to wounds on rats infected with *Staphyloccus aureus* did have better wound healing compared to antibiotic treated control animals. This same research group showed that both kefir and kefiran extract fed to rats had an antiinflammatory effect when the rats were subjected to cotton-induced granuloma and paw edema tests.[181]

4.6.4 Cholesterol Metabolism

The consumption of fermented milk products have long been proposed as a way of reducing serum cholesterol levels. Several human studies have been published in which yogurt was consumed. Taylor and Williams[182] summarized the results of 13 yogurt or cholesterol-feeding trials and found that yogurt consumption lowered blood cholesterol in eight trials, had no effect in four trials, and increased blood cholesterol in one trial compared to control. Several biochemical mechanisms can be proposed that would predict that consumption of fermented milk will lower blood cholesterol levels.[183] It was argued that probiotic bacteria could cause an increase in the production of short-chain fatty acids, which would in turn decrease circulatory cholesterol levels either by inhibiting hepatic cholesterol synthesis or by redistributing cholesterol from the plasma to the liver. In addition, bile acid deconjugation could be increased in the large intestine. Deconjugated bile acids then would not be absorbed, but excreted from the body. This would stimulate an increase in bile acid synthesis, taking more cholesterol out of circulation.

Vujicic et al.[184] studied the ability of kefir grains to take up cholesterol from milk during a 24 h incubation at 20°C or after incubation and a 48 h storage at 10°C. Grains were obtained from Yugoslavia, Hungary, and from the Caucasus. At the end of the fermentation, between 22 and 63% of the cholesterol in the starting milk had been assimilated; by the end of the 48 h storage period, between 41 and 84% had disappeared.

Hamsters fed a high-cholesterol (0.35%) diet that contained either lyophilized cows' milk kefir or soy milk kefir had significantly reduced serum total cholesterol, and a significantly reduced atherogenic index compared to animals receiving milk (10% skim milk or lyophilized soy milk). Part of the beneficial effect was attributed to increased excretion of neutral sterols and bile acids.[185]

Maeda et al.[186] have suggested that the consumption of kefiran may be affecting several lipid metabolism mechanisms. They show data from rats consuming high-fat diets where serum total cholesterol, LDL cholesterol, and triglycerides as well as liver cholesterol, triglycerides, and phospholipids were reduced compared to controls. Lowered blood pressure in kefiran consuming rats was attributed to lowered in vivo angiotensin l-converting enzyme (ACE) activity. Quirós et al.[187] have isolated two peptides from caprine kefir with high ACE inhibitory activity.

A randomized crossover feeding trial was carried out with 13 moderately hypercholestremic men (serum cholesterol levels 6.0–10 mmol/L). Subjects were fed 500 mL/d of kefir for 1 month or the same volume of milk and then the opposite after a 4-week washout period. By monitoring bacteria in fecal samples, it was shown that 73% of the subjects were colonized by *Leuconostoc* sp. bacteria found in kefir (see Figure 4.7). Analyses of fecal samples after the washout period indicated that once the feeding of kefir was stopped, the microbial composition of feces returned to normal. Total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and blood triglyceride levels were not changed during the period of kefir consumption.[188]

4.6.5 Other Uses

Kefir, like other probiotic products, may be most effective at influencing conditions in the gastrointestinal tract. Sukhov et al.[189] fed kefir (500 mL, 5 times/d) to

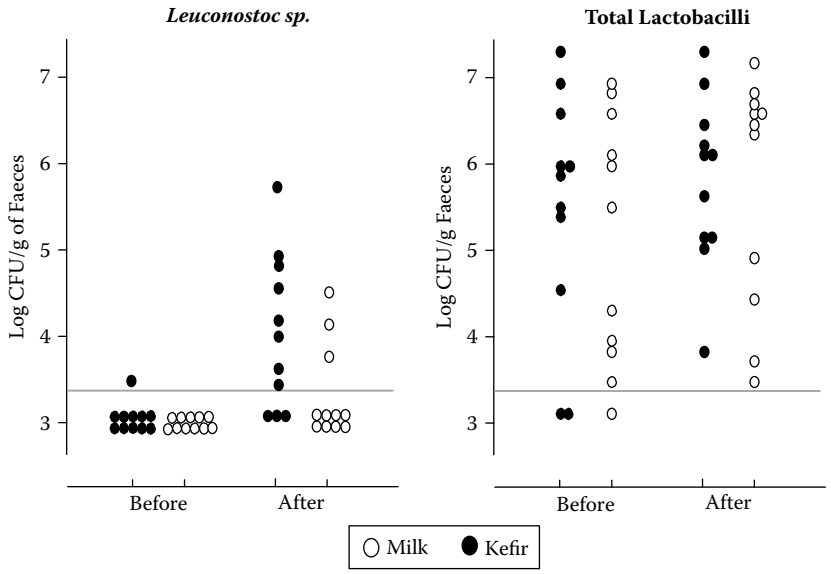

FIGURE 4.7 Colonization of subjects by bacteria during the consumption of kefir.

38 patients suffering from enteritis and found that there were no significant changes to the intestinal microflora population. Russian researchers have also used kefir to treat patients with peptic ulcers in the stomach and duodenum.[190] Japanese researchers have speculated that both kefiran and the bacteria found in kefir may be affecting the intestinal penetration of food allergens and resulting food sensitization. Mice fed kefir and then challenged with ovalbumin (OVA) had a lower plasma rise in OVA concentration, indicating something in kefir, possibly kefiran, was preventing intestinal penetration. Lowered anti-OVA IgG levels were found in mice fed kefir plus OVA, compared to mice receiving only OVA after 2 weeks of feeding.[191]

Recently, Japanese researchers have shown that rats fed kefir for 12 d prior to exposure to whole body radiation had significantly reduced apoptosis indexes in colonic crypt histological sections, 2 h after exposure to 1 Gy of radiation, compared to control animals.[192] A nonsignificant reduction in the apoptosis index continued for up to 6 h after exposure.

The abstract to a patent filed in 2000 claims that a dietary supplement consisting of milk fermented with two different sources of kefir grains (the first grains rich in bacteria and the second grains rich in yeasts) that had been filtered and then dried could be used to prevent osteoporosis.[193] It was stated that the supplement could be used in the prevention and treatment of osteoporosis and other diseases that result from calcium, magnesium, and potassium deficiencies. No data were included to support this claim.

Kefir may also stimulate the mucosal immune system. It has been recently reported that young rats (6 months old) fed kefir ad libitum and then immunized intraduodenally with cholera toxin (CT) had significantly higher (86%) serum anti-CT antibodies compared to controls.[194] This response was attributed to an enhanced in vitro antibody secretion by cultured lymphocytes isolated from the Peyer's patches

and intestinal propria. The effect of kefir was not observed in older rats (26 months old) in the same experiment. Total serum IgA titers were also not changed due to kefir consumption, but in both young and old rats, anti-CT IgG titers were lower in the kefir-fed animals. It was speculated that the immunomodulation effect of kefir may be due to bacterial wall components.

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5 Yogurt and Immunity

The Health Benefits of Fermented Milk Products That Contain Lactic Acid Bacteria

Judy Van de Water and Phornnop Naiyanetr

CONTENTS

5.1 HISTORY AND CULTURE RELATED TO FERMENTED FOOD

The history of fermented milk products is long and quite diverse culturally. Although the exact origins are difficult to ascertain, they likely date back to more than 10,000 yr ago. According to Persian tradition, Abraham owed his fecundity and longevity to the regular ingestion of yogurt. In the early 1500s, King Francis I of France was reportedly cured of a debilitating illness after eating yogurt made from goats' milk.[1] Scientific interest in the health benefits of yogurt was initiated by Élie Metchnikoff in the early 1900s. Metchnikoff proposed that the lactic acid microbes of fermentation must be antagonistic to the putrefying microbes of the gut, and once introduced into the intestine, they would prevent the breeding of the noxious microbes that required an alkaline environment. His hypothesis was stimulated by the fact that populations such as those living in the Balkans regularly ate yogurt and were noted for their longevity. He experimented on himself and reported that his health, which was generally poor, improved with regular ingestion of sour milk prepared with cultures of the Bulgarian lactic bacillus. Metchnikoff's enthusiasm about yogurt became more publicized, and doctors began recommending yogurt or sour milk as a hygienic food. Metchnikoff credited his relatively long life in part to the lactic bacilli in his diet, and hypothesized "When people have learnt how to cultivate a suitable flora in the intestines of children as soon as they are weaned from the breast, the normal life may extend to twice my 70 yr."[2] (See Chapter 1 for more details on the history of yogurt.)

Over the past several decades the consumption of fermented milk products, especially yogurt, has greatly increased. The most dramatic increase occurred during the 1980s and 1990s, which is certainly in part due to increased knowledge regarding the health benefits of yogurt and other fermented milk products.[3] Moreover, the addition of fruit and sweeteners to yogurt has made it more widely palatable. However, it is likely the increasing knowledge regarding the health benefits of fermented foods, especially live-culture yogurt, has driven the recent growth in consumption. The following sections of this chapter will discuss the fermentation process, the compositional changes of milk following fermentation, and health effects of consumption of fermented milk in both animals and humans.

5.2 YOGURT PRODUCTION

Yogurt is produced using active cultures of bacteria to ferment cream or milk. Yogurt that is produced in the United States is made with two specific live and active cultures of lactic acid bacteria (LAB)—*Lactobacillus bulgaricus (Lb. bulgaricus*) and *Streptococcus thermophilus (S. thermophilus).* These bacteria metabolize some of the milk sugar (lactose) in the milk into lactic acid. This action helps change the consistency of liquid milk into yogurt. The production of fermented milk, or yogurt, requires that the milk is first concentrated by the addition of dairy solids, evaporated, or membrane filtered. The mixture is then heated to destroy undesirable organisms, and cooled. Then, the starter cultures are added. Yogurt products may also have added ingredients such as sugar, sweeteners, fruits or vegetables, flavoring compounds, sodium chloride, coloring stabilizers, and preservatives. In the United States, *Lb. bulgaricus* and *S. thermophilus* are required by U.S. Food and Drug

Administration (FDA) standards in order for a product to be called *yogurt*. Other cultures may be added but are not required.

The fermentation process involves the inoculation of pasteurized milk that has been enriched in milk protein with concentrated cultures of bacteria, which is then incubated at 40–44°C for 4–5 h. During fermentation, lactic acid is produced from lactose by the yogurt bacteria, the population of which increases 100- to 10,000-fold to a final concentration of approximately 10^9/mL. The reduction in pH, due to the production of lactic acid, causes a destabilization of the micellar casein at a pH of 5.1 to 5.2, with complete coagulation occurring around pH 4.6. At the desired final pH, the coagulated milk is cooled quickly to 4–10°C to slow down the fermentation process.

Fermentation of milk with LAB leads to specific organoleptic characteristics (taste, aroma) of the final product. The metabolism of LAB and the interactions between the selected strains are responsible for the production of lactic acid, the coagulation of milk proteins, and the production of various compounds. Variables such as temperature, pH, the presence of oxygen, and the composition of the milk further contribute to the particular features of a specific product.[4–6] Fermented milks exhibit a wide variety of textures ranging from liquid drinks such as kefir, koumiss, and acidophilus milk to semisolid or firm products including yogurt, filmjölk, villi, dahi, and leben. (See Chapter 4 for more details on the manufacture and properties of kefir.)

Certain strains of *S. thermophilus, Lb. bulgaricus*, and other LAB, such as *Lactococcus cremoris* and some species of *Leuconostoc*, produce exocellular polysaccharides that modify the texture of a fermented milk product i.e., by increasing the viscosity or creating a "ropy" texture.[4,6–12] Lactic acid is also responsible for the slightly tart taste of the fermented milk product, whereas the other characteristic flavors and aromas are additional results of LAB metabolism. For example, acetaldehyde provides the characteristic aroma of yogurt, whereas diacetyl, produced by *Lc. diacetylactis* and *Leuconostoc cremoris,* impart a buttery taste to some fermented milks. Acetoin, acetone, lactones, and volatile acids are other important flavor components that may be present in certain fermented milks as by-products of bacterial metabolism.[8,9,12]

There is a symbiotic relationship, also known as "protocooperation," between *S. thermophilus* and *Lb. bulgaricus*, in which each species of bacteria stimulates the growth of the other. *Lb. bulgaricus* stimulates the growth of *S. thermophilus* by liberating amino acids and peptides from milk proteins; which enable *S. thermophilus* to grow faster in the early part of incubation. *S. thermophilus* in turn produces formic acid, which stimulates the growth of *Lb. bulgaricus*, resulting in a shortened fermentation time and a product with characteristics different than that of milk fermented with a single species.[4,8,9,12,13]

5.3 THE EFFECTS OF FERMENTATION ON MILK

5.3.1 Effects on Chemistry, Nutrient Content, and Activities of Enzymes

One important result of the addition of the bacteria necessary for fermentation is the resulting proteolytic activity of the yogurt bacteria. Although this activity is slight, resulting in a breakdown of only 1 to 2% of milk protein,[14] it is essential to release

small peptides and amino acids for the growth of the bacteria. *Lb. bulgaricus* is more proteolytic, but both yogurt bacteria contain peptidases that are necessary to hydrolyze large peptides into smaller peptides for transport into the cell. The principal substrate for such proteolysis is casein, but limited degradation of whey proteins may also occur.[15,16] The net effect of this proteolysis is that fermented milks have a higher content of peptides and free amino acids, especially valine, histidine, serine, and proline, than milk does.[17,18] Moreover, although the limited proteolytic action of yogurt bacteria does not significantly alter the nutritional value of milk proteins,[19] yogurt is significantly more digestible than the milk mixture from which it is made.[20] A study with rats demonstrated that feeding yogurt compared to the native milk from which it was prepared resulted in increased feed efficiency.[21] The increased digestibility of proteins in fermented milks may be related to the fine flocculation of caseins resulting from the joint action of proteolysis and acidification. (See Chapter 7 for more details on proteolysis of milk.)

With respect to the effects of fermentation on the carbohydrate fraction of milk, about 20–30% of the lactose in milk is fermented by LAB through different pathways (Figure 5.1). The bacteria used to produce yogurt are homofermentative, producing one major endproduct, lactic acid. This accounts for greater than 95% of the fermentation products found in yogurt. The final concentration of lactic acid in yogurt is 0.7–1.2% in yogurt. This lactic acid is a mixture of both the L(+) and D(–) isomers.

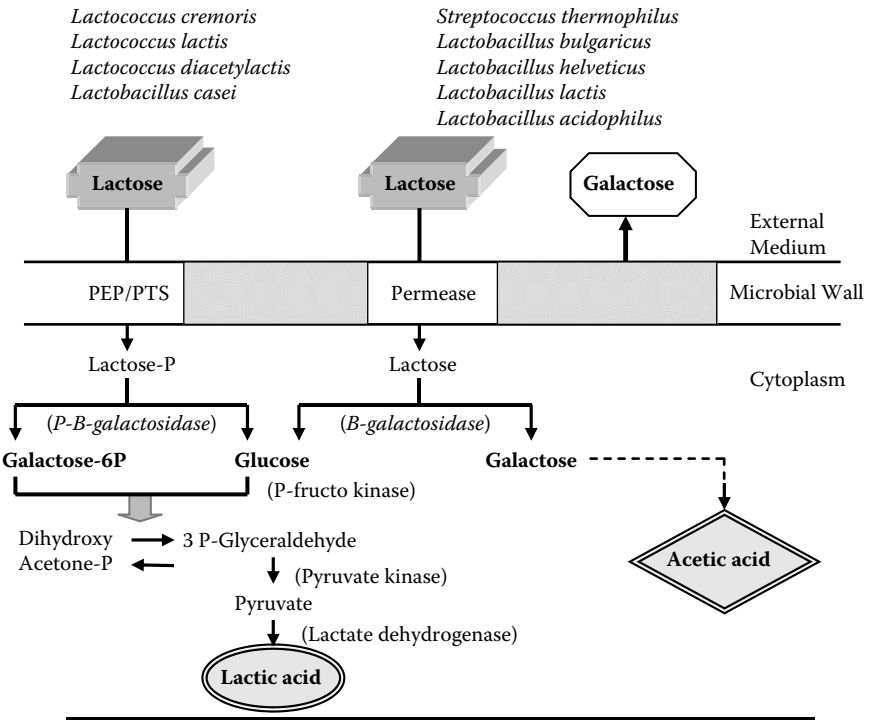

FIGURE 5.1 Major pathways of lactose metabolism by the lactic acid bacteria used for milk fermentation; PEP/PTS: phosphoenolpyruvate:sugar phosphotransferase system.

Although the quantity of each isomer present depends on the specific culture, the L(+) isomer generally represents between 50 and 70% of the total lactic acid.

It is the reduction in the lactose concentration coupled with the presence of a large number of viable bacteria containing ß-galactosidase that allows the consumption of yogurt by lactose-intolerant individuals. ß-galactosidase is protected by the bacteria from denaturation by the acid in the stomach, thus allowing delivery of the enzyme to the intestine. Once in the small intestine, bile increases the permeability of the bacterial cells, thus facilitating the entry and subsequent hydrolysis of lactose by the bacteria.[22] One study investigated the effects of acid milk and organic acids on the digestive tract in mice.[23] It was determined that lactic acid resulted in increased peristaltic movement in the duodenum, jejunum, ileum, cecum, and colon, but not the stomach or rectum. In contrast, acetic acid-stimulated movement only in the duodenum and colon. The exact role of this acid-stimulated motility on digestion remains to be clarified.

With the exception of some B vitamins, changes in the vitamin and mineral content of fermented milk products are negligible.[24] Moreover, the pasteurization of milk prior to fermentation may destroy some vitamins such as B_6, B_{12}, and folic acid, whereas the level of thermostable vitamins (niacin and pantothenic acid) remains unchanged. Some lactic acid bacterial strains produce a net increase in B vitamins, notably folates, during fermentation, whereas others result in a net loss. In general, *Lb. bulgaricus* uses folic acid, whereas *S. thermophilus* produces it.[25] However, following fermentation, the levels of some vitamins, especially B_{12} and folic acid decrease during cold storage.[26]

Yogurt, like milk, is an excellent source of calcium and phosphorous, both of which are essential for the development and maintenance of bones. In addition, yogurt also contains relatively large amounts of potassium and can also be considered as a good source of this mineral. Further, because the yogurt is usually produced from milk that has been enriched either through concentration or the addition of milk powder, it is, on a unit-to-unit basis, a richer source of these minerals than milk is. This is especially important for those individuals who are lactose intolerant and must limit their intake of dairy products.

It should be noted that postfermentation heat treatment significantly alters some properties of fermented milks. Heat treatment above 65°C appreciably reduces the level of some thermosensitive vitamins.[26] In addition, enzymatic activity, notably the activity of ß-galactosidase, is markedly reduced.[27] This reduced activity dramatically lowers the ability of lactose intolerant individuals to tolerate the same amount of lactose that otherwise could be tolerated with live-culture yogurt.

5.3.2 Health Benefits of Fermented Milk Consumption

Numerous reports and studies regarding the health benefits of yogurt and other fermented milk products have been published. Although the mechanisms behind such health claims are still being investigated, these benefits (on the immune or metabolic system) appear to be real. Many of the data collected thus far indicate that it is through the ingestion of the live LAB that these benefits are realized. The survival of bacteria administered in fermented milk products during passage through the human gut has

been investigated intensely in recent years. Well-controlled, small-scale studies on diarrhea in both adults and infants have shown that probiotics are beneficial and that they survive in sufficient numbers to affect gut microbial metabolism. Probiotics are nonpathogenic microorganisms that, when ingested, exert a positive influence on the health or physiology of the host.[28–32] They can influence intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system. Survival rates have been estimated at 20 to 40% for selected strains; the main obstacles to survival are gastric acidity and the action of bile salts. Although it is believed that the maximum probiotic effect can be achieved if the organisms adhere to intestinal mucosal cells, there is no evidence demonstrating that exogenously administered probiotics do adhere to the mucosal cells. Instead, they seem to pass into the feces without having adhered or multiplied. Thus, to obtain a continuous exogenous probiotic effect, the probiotic culture must be ingested daily. Certain exogenously administered substances enhance the action of both exogenous and endogenous probiotics. Human milk contains many substances that stimulate the growth of bifidobacteria in vitro, especially in the small intestine of infants. However, it is unlikely that these substances function in the colon. Beneficial effects may thus accrue from exogenously administered probiotics, often administered with prebiotics (nondigestible food ingredients that benefit the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon), or from endogenous bifidobacteria and lactobacilli, whose metabolic activity and growth may also be enhanced by the administration of prebiotics.[29]

Studies that have shown a sufficient level of proof to enable probiotics to be used as treatments for gastrointestinal disturbances include:

1. Increase in tolerance of yogurt compared with milk in subjects with primary or secondary lactose maldigestion[33]
2. The use of *Saccharomyces boulardii, Lactobacillus,* and *Enterococcus faecium* SF 68 to prevent or shorten the duration of antibiotic-associated diarrhea[34–37]
3. The use of *S. boulardii* to prevent further recurrence of *Clostridium difficile*-associated diarrhea[38]
4. The use of fermented milk containing *Lb. rhamnosus* GG to shorten the duration of diarrhea in infants with rotavirus enteritis (and probably also in gastroenteritis of other causes)[39,40]

Additional situations in which probiotics may be of value include mitigation of diarrhea of miscellaneous causes; prophylaxis of gastrointestinal infections, including traveler's diarrhea; and immunomodulation.[41–43] Trials in gastrointestinal diseases that involve the ecosystem, e.g., *Helicobacter pylori* infections, inflammatory bowel disease, and colon cancer are currently being performed.[44,45] However, such treatments should be thoroughly studied and carefully given to patients, because there have been some case reports of the human infections caused by these probiotic bacteria. For example, *Lb. lactis* was reported to cause a liver abscess in a regularly yogurt-consuming patient whose colonic mucosa was damaged by an ingested bone.[46] *Lb. rhamnosus* septicemia was reported in an immunocompromised patient treated with prolonged oral antibiotics for *C. difficile* diarrhea. The patient received

a short course of live yogurt; nonetheless, the link between the yogurt consumed and the septicemia was not verified.[47]

Interestingly, besides the gastrointestinal system, yogurt also enhances protective immunity against respiratory tract infections. For instance, yogurt supplementation with a balanced diet repletion in malnourished mice infected with *Streptococcus pneumoniae* improved many immunological parameters, and accelerated recovery from the infection compared to those mice repleted with a balanced diet alone.[48] The authors suggested that three factors probably contribute to these effects. First, proteins in yogurt can be easily digested and absorbed because of the predigestion of protein during casein fermentation. Second, LAB can modulate common mucosal immune systems and, therefore, enhance mucosal immunity of the respiratory tract. Third, milk components produced during fermentation also exert immunostimulatory effects. In a human study, Gluck et al. investigated the effects of a probiotic, fermented milk drink with *Lb.* GG (ATCC 53103), *Bifidobacterium* sp B420, *Lb. acidophilus* 145, and *S. thermophilus* on nasal colonization with pathogenic bacteria (*Staphylococcus aureus, S. pneumoniae,* and β-hemolytic streptococci). They demonstrated that daily consumption of probiotic yogurt for 3 weeks reduced nasal colonization with pathogenic bacteria, particularly Gram-positive bacteria, whereas volunteers who received standard yogurt showed no difference in nasal colonization.[49] Other examples of the influence of LAB on the respiratory system were shown in two different randomized, double-blind, placebo-controlled studies by Hatakka et al. and de Vrese et al.[50,51] The former group showed that there was a reduction in the number of children afflicted with respiratory tract infections in the group that received milk with *Lb.* GG, compared to those who drank milk without *Lb.* GG. De Vrese et al. studied the effects of three LAB strains (*Lb. gasseri* PA 16/8, *B. longum* SP 07/3, and *B. bifidum* MF 20/5) on the common cold. They found that daily consumption of these LAB strains for 3 months reduced the severity and duration, but not incidence, of common cold episodes in 479 healthy adults.[33] One of the most widely touted benefits of yogurt consumption is said to be the enhancement of the immune system. It has been proposed that LAB and fermented milk modulate certain parameters of both the nonspecific and specific immune responses. The link between these benefits and the immune system, however, has not been identified, and the mechanisms involved are still unknown.

5.4 FERMENTED MILK AND THE IMMUNE SYSTEM[30,52–55]

5.4.1 Overview of the Immune System

A brief overview will help to clarify the different branches of the immune system involved in the response to LAB and fermented foods. The body has a number of defense systems used against the massive invasion of foreign matter it constantly receives. The nature of this response depends on two factors:

1. The type of foreign particles (viruses, bacteria, parasites, fungi, pollens, and certain food proteins)
2. The route of entry (the skin, blood, lungs, or epithelium of the gastrointestinal tract)

The first line of defense involves physicochemical barriers such as the skin and mucus layers, e.g., in the nose and intestines, which indeed prevent the occurrence of most infectious diseases. The immune system represents the second line of defense against microorganisms, and its response involves a complex interrelation of its various components. The three principal steps in this response are

1. The recognition of the foreign molecule
2. Destruction of the foreign matter
3. Regulation of the response through multiple feedback controls

There are two major arms of the immune response—the innate or nonspecific response and the acquired or specific immune response. Generally, when one is faced with a microorganism, whether it be pathogenic or nonpathogenic, both arms of the immune system are involved in the response. The innate response consists of cells that are primarily phagocytic in nature and respond to the antigen by internalization. This in turn activates the responding cells and begins a cascade of events. Among these events, the most important are the presentation of the antigen to the acquired branch of the immune system and the activation of cells through cytokine production. Cytokines are proteins that are produced by cells and affect other cells. They bind to a receptor on the target cell and can be either stimulatory or downregulatory, depending on the cell type and the cytokine involved.

The specific or acquired immune response contains two general categories of reactivity, the cell-mediated and humoral responses. A complex interaction exists between these two systems and between the acquired response and innate immunity (Figure 5.2). Which branch of the acquired immune system is involved in a response depends on the nature of the antigen and the route of exposure. Moreover, activation requires the identification and presentation of the antigen by the innate immune system (dendritic cells, monocytes or macrophages, and sometimes B cells).

The cell-mediated immune response involves T cells that directly kill pathogen-infected cells (T-cytotoxic cells, natural killer cells) or that regulate the immune response via cytokines (T-helper cells). As mentioned earlier, these mediators are multifunctional and can affect many organ systems. Examples of cytokines include interferons (IFN), interleukins (IL), colony-stimulating factors (CSF), and tumor necrosis factor (TNF).[56] T- helper cells are divided into three subgroups, Th1, Th2, and Th3. The Th1 cells produce the more inflammatory cytokines, such as IFN-γ and IL-2, whereas the Th2 cells produce the cytokines responsible for activation of the humoral response through the production of IL-4, IL-10, and IL-13. Th3 cells produce primarily TGF-β, a cytokine that is primarily responsible for the downregulation of the immune response. There is also a feedback control system between the Th1 and Th2 cells for regulation of the response.[57]

The humoral response involves B cells that, upon stimulation by cytokines produced by helper T cells, divide and differentiate into plasma cells that secrete immunoglobulins or antibodies (Ab). Ab are capable of recognizing a specific segment of the antigen called the epitope. This can lead to neutralization of the antigen by blocking (e.g., viral attachment to cells), activation of the complement system (involved

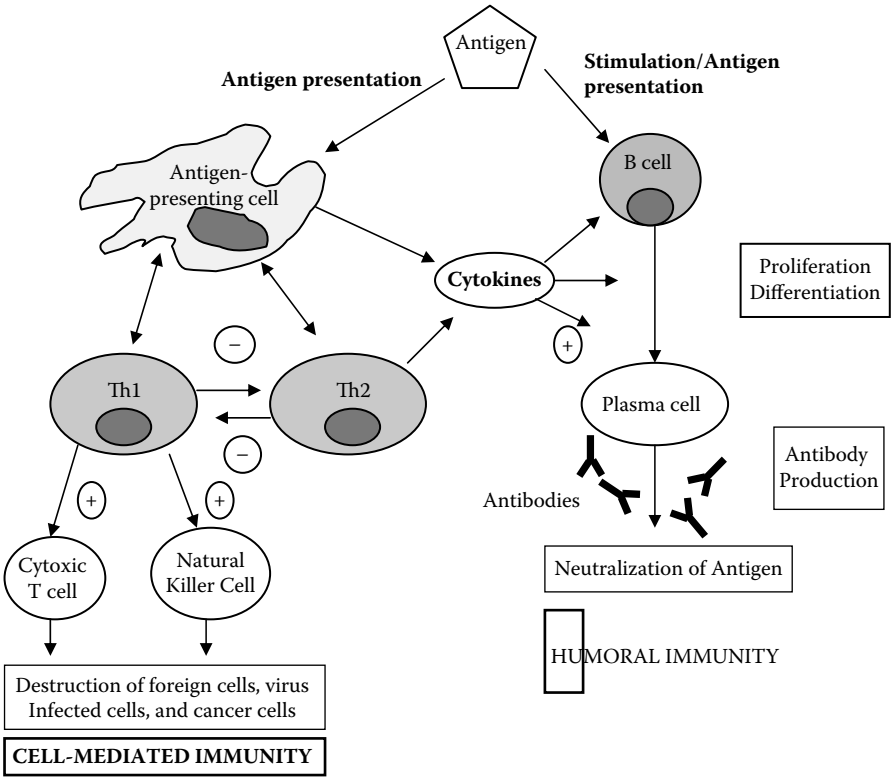

FIGURE 5.2 The acquired immune response.

primarily with bacteria), and/or clearance (through the reticuloendothelial cell and gastrointestinal systems).[56,57]

5.4.2 The Immune System of the Gastrointestinal Tract

In addition to the systemic immune response, organ-specific lymphoid tissues have unique properties not found elsewhere. The most relevant of these, in the context of this chapter on fermented foods, is the gastrointestinal (GI) tract. The GI tract contains a specialized mucosa-associated lymphoid tissue (MALT) system that also includes the lungs called the gut-associated lymphoid tissue (GALT).

The GI tract encounters a myriad of antigens from the numerous microorganisms and foreign proteins derived from various foods that enter through the mouth. A first line of defense against pathogens is provided by the barrier effect of the gastrointestinal digestive juices, the intestinal flora, and the intestinal mucus layers, each of which prevents microorganisms from entering the body. The second defense mechanism is the GALT. It is composed of organized lymphoid tissue in the ileum (Peyer's patches), as well as a large variety of immune cells (B and T lymphocytes, plasma cells, macrophages, mast cells, eosinophils, and basophils) that infiltrate the gastrointestinal mucosa. The GALT contains up to 60 billion cells and makes up 25% of the intestinal mucosa (Figure 5.3). The principal antibody synthesized by

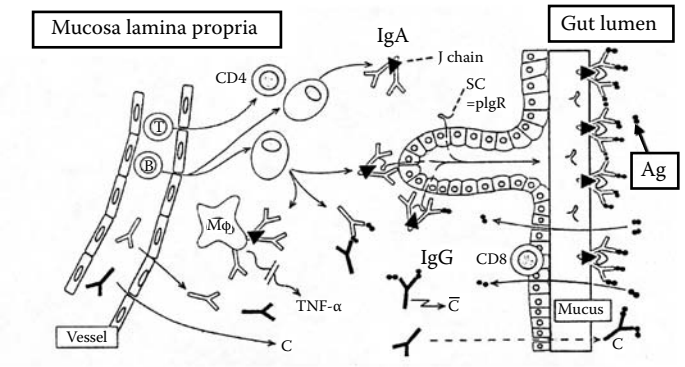

FIGURE 5.3 Gut-associated lymphoid tissue (GALT).

local plasma cells is secretory IgA (sIgA) that is found in other body secretions as well, such as in saliva, mucus, colostrum, and tears.[58] Secretory IgA is also a part of the first line of defense for the mucosal barrier, where it binds to, and blocks entry of, a variety of intestinal pathogens.

The initial establishment of the intestinal microflora is essential for the development of a fully functional and balanced immune system, including the development and maturation of IgA plasmocytes and IgA production.[59,60] Part of the intestinal IgA production is directed against commensal bacteria themselves.[61,62] Much of this IgA is reportedly induced by T-cell independent pathways that appear to be restricted to the intestinal mucosa; no specific IgA was detected in serum.[62] Interestingly, in gnotobiotic rats colonized with a combination of *Escherichia coli* and *Lb. plantarum*, a large proportion of the *E. coli*-specific IgG and IgA antibodies cross-reacted with *Lb. plantarum*. Similar cross-reactivity was observed between *Lb. acidophilus* and *E. coli*.[63] It is conceivable that some of the increased secretory Ig responses reported after supplementation with various lactobacilli during viral infections may at least partially reflect such cross-reactivity.[64–66]

5.5 EFFECTS OF LACTIC ACID BACTERIA ON THE GASTROINTESTINAL SYSTEM

Supplementation with exogenous LAB can also affect IgA production. The addition of 3 mL of yogurt containing 2×10^8 cells/mL (*Lb. delbrueckii* ssp. bulgaricus and *S. salivarius* ssp. *thermophilus*) to the diet of Balb/c mice resulted in a significant increase in IgA-secreting cells in the small intestine after 7 d. However, this increase was no longer observed after 10 d of yogurt feeding.[67] Similarly, in protein-energy deficient mice given a protein-free diet, supplementation with 3 mL of the same yogurt preparation for only two consecutive days was associated with significantly higher numbers of IgA- and IgM-secreting cells in the ileum compared to animals supplemented with unfermented milk.[68] A group supplemented with *Lb. casei* in the diet exhibited intermediate responses. Moreover, the addition of yogurt after a renutrition diet to protein-energy deficient mice exhibited an increase in IgA-secreting cell counts in the small intestine compared to those of malnourished mice given a

renutrition diet alone. Nevertheless, this effect was observed only in renourished mice fed with diluted (1:1) yogurt, but not in the group fed with undiluted yogurt. The authors concluded that yogurt supplementation to a standard renutrition diet improves the intestinal function of malnourished animals.[69] Yogurt was also most effective in improving the height of intestinal microvilli, enhancing mucus secretion, and preventing the translocation of intestinal bacteria, i.e., in improving the barrier functions of the intestine.

Swiss albino mice fed fresh yogurt for 7 d, then challenged with 20 LD_{50} of *Salmonella tyhpimurium*, exhibited significantly higher concentrations of *S. typhimurium*-specific IgA in their intestinal fluid than mice fed stored yogurt containing significantly lower numbers of live bacteria, or control mice given nonfat milk.[70] An enhanced response in antigen-specific serum IgA following vaccination with attenuated *S. typhi* Ty21a has also been reported in human volunteers consuming fermented milk containing *Lb. acidophilus* La1 (now *Lb. johnsonii* La1) and bifidobacteria (3 × 125 g/d, 10^7–10^8 colony forming units [CFU] per gram) before, during, and after vaccine administration.[71] In a similar study, volunteers were given the same vaccine and consumed *Lactobacillus* GG (ATCC 53103), *Lactococcus lactis*, or placebo for 7 d starting on the day of the first vaccine dose.[72] An increase in specific IgA was noted only in the group receiving *Lactobacillus* GG, whereas no significant differences were observed in the numbers of IgA-, IgG-, and IgM-secreting cells. The differences between this and the previously discussed study could be due to the different bacteria used, the different matrices in which they were delivered (fermented milk product versus capsules), and the different duration of LAB intake.

5.5.1 Lactic Acid Bacteria and Mucosal Health

It has repeatedly been observed that lactobacillus administration can shorten the duration of diarrhea,[45] including rotavirus-induced diarrhea in children.[64,65,73] Enzyme-linked immnospot (ELISPOT) assays of Ig-secreting cells and specific antibody-secreting cells (sASC) among circulating lymphocytes indicated that the nonspecific immune response was significantly greater in children receiving *Lactobacillus* GG than in those given a placebo.[64] In addition, although there was no difference in the number of IgA sASC during the acute phase, 90% of the lactobacillus-treated children and only 46% or the placebo-treated children had rotavirus-specific IgA sASC 3 weeks after recovery. Similarly, a higher rate of rotavirus IgA seroconversion (became positive for antibodies to rotavirus) was observed in infants given an oral rotavirus vaccine together with *Lb. casei* GG in those who received the vaccine along with a placebo.[74] It is worth noting, however, that it is currently not clear what role, if any, IgA plays in the recovery from rotavirus infection and to what extent it provides protection from future recurrence.

In children with Crohn's disease, a disease often characterized by a relative deficiency in mucosal IgA, administration of *Lactobacillus* GG (10^{10} CFU twice daily) for 10 d was associated with a significant increase in cells secreting IgA specific for β-lactoglobulin and casein.[75] Such an increase was not observed for patients with juvenile chronic arthritis or healthy controls. The numbers of specific IgG- and

IgM-secreting cells were not affected by *Lactobacillus* GG ingestion, nor was the number of nonspecific immunoglobulin-secreting cells of any isotype.

5.5.2 Lactic Acid Bacteria and Protection Against Enteric Pathogens

One of the proposed mechanisms facilitating the health benefits of fermented milk products stems from the credo that feeding a live culture LAB will inhibit the growth of pathogenic bacteria. Two of the most commonly found LAB in fermented milk products, *S. thermophilus* and *Lb. bulgaricus*, are normally isolated from green plant material and milk, respectively, and are not inhabitants of the intestinal tracts of humans and animals. These bacteria are not highly acid- and bile-resistant, with only 15% surviving the passage through the stomach and about 1% reaching the large intestine[76] where they fail to colonize.[77] However, they may still exert an effect in vivo due to intracellular enzymes, cell surface antigenic receptors, or metabolites produced during fermentation. Moreover, although yogurt bacteria and other lactic acid bacteria have been shown to inhibit pathogenic bacteria in vitro by the production of organic acids and antibiotic-like substances, this interaction has not been clearly demonstrated in vivo.[78] It has been shown in studies with mice[53,79] that feeding yogurt resulted in an alteration in the intestinal flora, stimulating the growth of lactobacilli and bifidobacteria. Changes such as these in the intestinal microflora are thought to affect the intestinal transit time and may have an impact on nutrient absorption.

It has been suggested that while the numbers of colonizing bacteria are low, they do have an impact on intestinal health. For example, in one study, the effects of chronic consumption of yogurt with (fresh) or without (heated) live bacterial cultures (*Lb. bulgaricus* and *S. thermophilus*) on plasma glucose, insulin, triacylglycerols, cholesterol, fatty acids, and short-chain fatty acids were compared in two groups of healthy men, with or without lactose malabsorption. It was determined that in men with lactose malabsorption, chronic consumption of yogurt-containing live bacterial cultures ameliorated the intolerance, as evidenced by lower breath hydrogen excretion, but increased propionate concentrations.[80]

5.5.3 Lactic Acid Bacteria, Nutrient Digestion, and Antigen Uptake

An additional vital function of LAB in the intestinal microflora is to aid in the absorption of indigestible nutrients, particularly carbohydrates (such as resistant starches, cellulose, hemicelluloses, pectins, and gums), through fermentation. Moreover, it was recently shown that colonization of adult germ-free mice with *Bacteroides thetaiotaomicron*, a major component of the intestinal microflora in mice and humans, was accompanied by marked changes in the transcription of a broad array of genes.[81] A majority of transcripts (95 of 118) increased, among them, the mRNA of many proteins involved in nutrient absorption and metabolism. Similar changes, though generally of a lesser magnitude, were seen after colonization with *Escherichia coli* or *Bifidobacterium infantis* or complete conventional mouse microflora. This suggests that colonization can play an important role in increasing the efficiency of nutrient absorption and metabolism. Interestingly, higher nutrient efficiency has been reported in animals fed a yogurt-based diet as compared to animals fed a milk-based diet, despite almost an identical nutrient composition of the diets.[82,83]

In addition to enhancing nutrient absorption, LAB also have been reported to modulate antigen uptake. In suckling rats given cows' milk in addition to maternal milk, the absorption rate of degraded horseradish peroxidase (HRP) was greater in the group receiving cows' milk with *Lb. casei* GG than in the group receiving cows' milk, which did not differ from the controls.[84] In contrast, addition of *Lb. casei* GG to the hydrolysate decreased the absorption of degraded HRP. The extent to which HRP was degraded by the mucosa was similar in controls, mice supplemented with milk plus *Lb. casei* GG, and mice receiving the hydrolysate with or without *Lb. casei* GG, but was greater in all of these groups compared to milk-supplemented animals.

When casein was first hydrolyzed with pepsin and trypsin, then additionally degraded by enzymes obtained from *Lb. casei* GG, the resulting products were found to consistently suppress Concanavalin A (ConA)- or PHA-induced proliferation in peripheral blood mononuclear cells (PBMC) from healthy volunteers.[85] In contrast, only some of the molecules resulting from casein hydrolysis with pepsin and trypsin inhibited proliferation, whereas others stimulated it. In addition, casein degraded with *Lb. casei* GG was reported to decrease the anti-CD3-induced production of IL-4 by PBMC from atopic subjects but not affecting interferon-γ (IFN−γ) secretion.[86] Intact casein stimulated the synthesis of both of these cytokines. In a similar experiment with T-cell-enriched PBMC from healthy subjects, *Lb. casei* GG degraded casein suppressed IL-2 mRNA and protein synthesis, as well as IL-4 and IFN−γ production.[87] In addition, casein degraded with *Lb. casei* GG decreased protein kinase C translocation, another marker of T-cell activation. Taken together, these results suggest that *Lb. casei* GG can digest casein, and possibly other macromolecules, into less antigenic and potentially immunomodulating peptides.

5.6 GUT-ASSOCIATED LYMPHOID TISSUE AND THE ESTABLISHMENT OF IMMUNE TOLERANCE

The gut-associated lymphoid tissue (GALT) plays an important role in the establishment of systemic hyporesponsiveness to ubiquitous antigens such as food as well as commensal bacteria, in addition to acting as an aid in mucosal defense. The nature, dose, timing, and route of entry of the antigen plays an important role in determining the nature of the T-cell responses and, thus, the outcome—sensitization or hyporesponsiveness. Cytokines produced by T cells upon antigenic stimulation are the major factors in directing the immune response.

Allergic diseases are thought to be a result of the skewing of an immune response to an allergen towards a Th2-type phenotype, i.e., an overproduction of IL-4 by $CD4^+$ helper T cells occurs along with a concomitant underproduction of IFN−γ. IL-4 promotes B cells to switch to IgE and IgG1 production, and inhibits Th1-type responses, including the production of IFN-γ. Conversely, INF-γ inhibits the proliferation of Th2 cells and the synthesis of IgG1 and IgE and induces the secretion of IgG2a and IgG3. IL-12 is a potent inducer of IFN-γ production by natural killer (NK) and Th1 cells but can also inhibit the development of a Th2-type response directly, i.e., independently of this IFN-γ induction.[57]

Interestingly, the immune responses of virtually all neonates to environmental allergens have been reported as skewed towards a Th2-type cytokine profile,

whether or not they subsequently become atopic.[88,89] In the last decades, there has been a dramatic increase in allergic diseases in developed countries, and the "hygiene hypothesis" has been formulated as a possible explanation for this development. In somewhat oversimplified terms, this hypothesis states that partial deprivation of microbial stimuli due to increased hygiene results in an imbalance between Th1- and Th2-type immune responses, favoring the development of IgE-mediated allergies.[90,91] It was originally postulated that infections with measles and other airborne viruses played a major role in providing protection from atopic diseases. It is becoming increasingly recognized, however, that the earliest and largest exposure to microbial antigens occurs during intestinal colonization by bacteria starting at birth. Thus, the intestinal microflora may provide a major stimulus in directing immune responses away from the Th2-phenotype seen in neonates and infants towards the Th1-type response that predominates in nonatopic adults.

The results from several recent studies suggest that the indigenous intestinal microflora of allergic children differs from that of nonallergic children.[92–95] In particular, a significantly lower percentage of allergic children were reported to be colonized with lactobacilli.[93] In contrast, some aerobic microorganisms, such as coliforms and *Staphylococcus aureus*, were detected more frequently in allergic than in nonallergic children. In a prospective study,[95] children who had developed atopy by the age of 2 yr were found to harbor significantly higher counts of clostridia and tended to have fewer bifidobacteria at the age of 3 weeks than children who did not develop atopic diseases. Further indications of an imbalance in the microflora of allergic children comes from observations that fecal short-chain fatty acids differed between allergic and nonallergic children at the age of 12 months.[94] Higher levels and proportions of L-caproic acid in the feces of allergic children, as compared to nonallergic children, suggested that they harbored elevated numbers of *Clostridium difficile*.

Recently, there has been a convincing evidence from animal and human studies supporting this hypothesis as well. For instance, a study using NC/Nga mice as an animal model of atopic dermatitis showed that the administration of *Lb. johnsonii* NCC533 during the weaning period in these mice prevented the development of atopic dermatitis induced by mite antigens. Skin appearance and histology were nearly normal in these mice, compared to those that were not treated with *Lb. johnsonii* NCC533.[96] A randomized double-blind placebo-controlled trial by Weston et al. has demonstrated that the supplementation with *Lb. fermentum* VRI-003 PCC in children aged 6–18 months with moderate to severe atopic dermatitis improved the severity of this disease.[97] This same research group has further investigated the mechanisms by which these LAB modulated the clinical outcome of atopic dermatitis in these children. They found that clinical improvement of the disease by LAB administration was associated with increased IFN-γ responses.[98] These findings support the hypothesis that LAB administration early in life skews the immune response toward Th1 type and, therefore, prevents or alleviates the development of atopic diseases that are caused primarily by a skewed Th2 response.

5.7 INTESTINAL MICROFLORA AND ORAL TOLERANCE

Animal studies comparing germ-free and conventional mice provide further evidence for the importance of the intestinal microflora in the development of oral tolerance. In such experiments, oral feeding of an antigen before immunization with it can induce oral tolerance with the exact outcome depending on the nature and dose of the antigen, the dosing schedule, and the age and genetic background of the animals.[99] IgG unresponsiveness to ovalbumin (OVA) lasted longer in conventional C3H mice than in germfree animals of the same strain, when both groups were fed 20 mg OVA, then immunized three times intraperitoneally (i.p.) with 10 μg OVA absorbed by alum.[100] No significant differences were observed in the duration of the IgE antibody response.

In contrast, Sudo et al.[101] reported that germfree Balb/c mice exhibited significantly higher levels of total IgE as well as OVA-specific IgE and IgG1 than conventional mice 5 and 7 weeks after OVA feeding (5 mg/d for 4 d) followed by i.p. immunization with 1 μg OVA in alum every 2 weeks. OVA-specific IgG2 levels were comparable in the two groups. Oral tolerance induction in gnotobiotic (germ-free) mice monoassociated with *B. infantis* at the neonatal stage was similar to that seen in conventional mice, although their OVA-specific IgE levels were somewhat higher. In contrast, colonization of mice with *B. infantis* at the age of 5 weeks resulted in significantly higher OVA-specific IgE, and IgG1 and IgG2a concentrations after OVA feeding before immunization, than compared to germfree animals.

In the same study,[101] OVA-stimulated IL-4 synthesis was significantly higher in splenocytes from OVA-immunized germfree animals without oral tolerance induction and was not downregulated after oral tolerance induction, unlike in conventional animals. In contrast, IFN-γ concentrations were similar in conventional and germfree animals both with and without oral tolerance induction. IL-2 and TGF-β concentrations were also higher in germfree than in conventional mice; their concentrations remained unaffected by oral tolerance induction in both groups. The authors suggested that, taken together with the data on IgG1 and IgG2a, Th1-mediated immune responses were abrogated, but Th2-mediated immune responses were unaltered by oral tolerance induction in germfree mice.

The immune response to commensal bacteria is transient and is replaced by tolerance soon after the initial colonization.[60] It has therefore been proposed that, for the intestinal microflora to play a continued role in the protection from atopic diseases, a high turnover of bacterial genera, species, and strains may be required.[91,102] Supplementation with LAB may provide such renewed stimuli for the maintenance of predominant Th1-type responses. Findings from in vitro experiments and studies in which heat-killed LAB were fed suggest that intestinal colonization is not an absolute prerequisite for such stimulation.

For the in vitro studies, splenocytes from OVA-primed mice cocultured with OVA in the presence of heat-killed *Lb. casei* strain Shirota (LcS) secreted significantly less total as well as OVA-specific IgE, whereas *Lb. johnsonii* JCM 0212 had

no effect on it.[103] LcS, but not *Lb. johnsonii*, also dose-dependently increased IFN-γ production but inhibited IL-4 and IL-5 synthesis. In addition, LcS induced IL-12 synthesis in splenic plastic-adherent cells (macrophages). Anti-IL-12 antibody abrogated the LcS-induced suppression of IgE, IL-4, and IL-5 secretion in splenocytes, whereas IFN-γ antibody was only partially effective, suggesting that IL-12 did not solely act through the induction of IFN-γ.

Balb/c mice injected i.p. with OVA on day 0 and day 4 and fed a diet containing 0.1 or 0.05% heat-killed LcS for 21 d starting on day 0 produced significantly lower levels of OVA-specific IgE and tended to have lower total serum IgE than controls not receiving LcS.[104] This was associated with an interesting switch from a Th2-type to a Th1-type cytokine pattern in splenocytes isolated from these mice and restimulated with OVA, namely, significant elevations in IFN-γ, IL-2, and IL-12 production and marked reductions in IL-4, IL-5, IL-6, and IL-10 synthesis.

DBA/2 mice fed a casein diet develop elevated casein-specific IgE in association with a Th2-like cytokine pattern. In such mice, i.p. injection of heat-killed *Lb. plantarum* L-137 inhibited casein-specific IgE production, although IgG1 levels actually increased, compared to saline injected controls.[105] There was a concomitant increase in IL-12p40 synthesis by unstimulated peritoneal macrophages from *Lb. plantarum* injected mice and an even greater increase in macrophages restimulated with *Lb. plantarum*. Secretion of IL-4 by ConA-stimulated splenocytes was diminished in *L. plantarum* injected mice, whereas IFN-γ production was not affected.

Human in vitro studies have demonstrated that three LAB strains (*Lb. gasseri* ATCC no. 19992, *Lb. johnsonii* ATCC no. 33200, and *Lb. reuteri* ATCC no. 23272) induced the activation and maturation of immature human myeloid dendritic cells, which in turn skewed CD4+ and CD8+ T cells toward a Th1 response. LAB-stimulated dendritic cells upregulated, HLA-DR, CD83, CD40, CD80, and CD86, all markers of activation. They also produced increased levels of IL-12 and IL-18 cytokines but not IL-10. CD4+ and CD8+ T cells primed by these dendritic cells secreted high levels of IFN-γ, but not IL-4 or IL-13, indicating a shift towards a Th1-type immune response.[106]

In healthy adults, it has been shown that the consumption of yogurt could decrease the serum levels of IgE, which might be advantageous to allergic individuals.[107] Additionally, Morita et al. have demonstrated that oral administration of fermented milk with *Lb. gasseri* TMC0356 for 4 weeks significantly lowered serum levels of IgE, and increased the Th1/Th2 ratio in 15 perennial allergic rhinitis patients with high serum IgE. Interestingly, serum IgE specific for Acari and Japanese cedar pollen has been significantly decreased, whereas IgE specific for house dust and fungi shows a tendency to decrease after 4 weeks, but did not reach statistical significance.[108]

Intriguingly, selective LAB strains have been successfully used as a mucosal vaccine in an animal model of allergy. That is, recombinant *Lb. plantarum* and *Lb. lactis* producing the major birch pollen allergen Bet v 1 were intranasally administered to a mouse model for birch pollen allergy. This vaccination led to the reduction in allergen-specific IgE, IL-5 production, and the upregulation of IgG2a, indicating a shift toward a Th1 response. Furthermore, it also an enhanced allergen-specific secretory IgA production in the lungs and intestines, which likely protects the mucosal system

from the allergen through a blocking mechanism. Thus, this evidence suggests that LAB can be utilized as a mucosal vaccine to prevent allergic diseases.[109]

Perdigón et al. has also proposed that LAB induce a differential mucosal immune response (specific, nonspecific, or both) in mice. Moreover, they suggest that such behavior is due to the different sites of interaction of the LAB with the gut, resulting in the induction of these different immune responses. They have demonstrated that the LAB are present in different parts of the intestine, and the pathway of internalization of the strains used to make contact with the immune system is through either the Peyer's patch M cells, follicle-associated epithelium (FAE) cells, or the epithelial cells of the small and large intestine. These findings could explain the diversity of the mucosal immune response. It is well known that M-cell interaction induces mainly a specific immune response, but when internalized through the FAE cells, the response is nonspecific or inflammatory, even though these cells can later enhance a specific immune response. The interaction with epithelial cells can lead to the enhancement of local immunity or tolerance by antigen clearance.[30,67,110]

5.8 CYTOKINES AND FERMENTED MILK

Some of the most compelling data with respect to the effects of fermented milk products on the immune system involves the production of various cytokines. Many LAB have been reported to induce the synthesis of IFN-γ in vitro, including *S. thermophilus*, *Lb. acidophilus*, *Lb. bulgaricus*, and bifidobacteria, although inconsistent results have been obtained with *Lb. bulgaricus* and bifidobacteria (see Tables 5.1 and 5.2). In contrast, most LAB studied to date have little effect on IL-4 production in vitro, except for one study reporting a downregulation of IL-4 synthesis in splenocytes from OVA-primed mice restimulated with OVA in vitro (Tables 5.1 and 5.2).[103] Studies on the effect of LAB on ex vivo cytokine production are summarized in Table 5.3.

In two separate animal-feeding trials, the effects of three forms of yogurt followed by two different unheated or heat-treated yogurts fed to B6C3F1 mice on cytokine expression in spleen, mesenteric lymph nodes (MLN), or Peyer's patches (PP) were determined.[111] All yogurts were fermented with *Lb. bulgaricus* and *S. thermophilus*; in addition, some contained *Lb. acidophilus* and/or *Bifidobacterium* sp. The effects depended on the type of yogurt fed; the presence of *Lb. acidophilus* and/or *Bifidobacterium* sp. was found not to play a role. In addition, the duration of the feeding, tissue, and cytokine examined influenced the outcome. In the first feeding trial, yogurt feeding either had no significant effect or downregulated cytokine expression in mice compared to nonfat dry milk. In particular, IL-4 mRNA was decreased in PP after 2 weeks, but not after 4 weeks, regardless of whether the yogurts contained live or heat-killed bacteria. IFN-γ mRNA was not affected in PP of any animals fed yogurt for either 2 or 4 weeks but was downregulated in MLNs by one type of live and three different heat-treated yogurts after 4 weeks of feeding. TNF-α expression was decreased after 4 weeks in spleen by all three types of yogurts; all three heat-treated yogurts also reduced TNF-α expression in MLN after both 2 and 4 weeks, but only one live-culture yogurt did so after 4 weeks. In the second feeding trial, involving two other yogurts, the results were much more variable.

TABLE 5.1
Effect of Live LAB on the Production of Various Cytokines

In vitro live bacteria Bacteria and cell type	TNF-α	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12	IFN-γ	Reference
Lb. bulgaricus E585 stimulation of PBMC	↑↑	↑↑		no effect	↑	↑	↑	no effect	145
Lb. bulgaricus stimulation of PBMC	↑	↑	no effect					↑	146
Lb. acidophilus stimulation of PBMC	↑	↑	no effect					↑	146
Lb. acidophilus stimulation of PBMC		↑			↑			↑	115
Lb. acidophilus E 507 stimulation of PBMC	↑				↑	↑ only in responders			147
Lb. acidophilus stimulation of epithelial cell/leukocyte co-cultures						no effect	↑	↑	148
Lb. casei stimulation of PBMC		↑			↑			no effect	115
Lb. rhamnosus	↑	↑		no effect	↑	↑	↑	↑	145
						no effect	↑	↑	148
Lb. sakei	↑	↑	no effect					↑	146
Bifidocacterium sp.									
Bifidobacterium			↑		↑			no effect	115
S. thermophilus stimulation of PBMC		↑			↑			↑	115
S. thermphilus stimulation of PBMC	↑	↑	no effect					↑	146

TABLE 5.2
Effect of Heat-Killed LAB on Cytokine Production

Bacteria	TNF-α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IFN-γ
Lb. acidophilus La1 (*L. johnsonii* La1) in RAW 264.7 cells	↑					↑			
Lb. bulgaricus 1489 NCK 231 in RAW 264.7 cells	↑					↑			
Lb. bulgaricus (2 strains) in RAW 264.7	↑					↑			
in PMA-stimulated EL4.IL-2 cells			↑						
L. johnsonii JCM 0212 in mouse splenocytes from OVA-primed mice				no effect	no effect			no effect	no effect
Lb. casei in x-rayed mouse splenocytes (i.e., monocytes)								↑	↑ ConA-induced IFN–γ, no effect alone
Lb. casei in mouse splenocytes from OVA-primed mice restimulated with OVA				↓	↓			↑	↑↑
Lb. casei B ATCC 11578 cell wall	↑	↑					↑		
in rat peritoneal macrophages and									
in splenocytes		↑		no effect			↑		
B. bifidum in RAW 264.7 cell	↑					↑			
in PMA-stimulated EL4.IL-2 cells			↑						
B. bifidum in RAW 264.7 cells	↑					↑			
S. thermphilus St-133 in RAW 264.7 cells	↑					↑			
S. thermphilus (4 different strains) in RAW 264.7 cells	↑					↑			
in PMA-stimulated EL4.IL-2 cells			↑						

TABLE 5.3
Effects of LAB Consumption on Ex vivo Cytokine Production

Bacteria	Treatment	Effect on cytokine production	Reference
Bifidobacterium (Bf)	B6C3F1 mice received a single dose of 109 cells	Bf decreased PMA-induced IL-6 and IFN-γ	152
Lb. acidophilus (La)	Peritoneal cells were cultured in the absence or presence of LPS or PMA	La induced IL-6 and IL-12p40, enhanced PMA-induced IFN–γ	152
Lb. bulgaricus		No effect on IL-6 or IFN–γ	
Lb. casei		Induced IL-6 and IL-12p40	
S. thermophilus		No effect on IL-6, IFN–γ, or IL-12p40	
Lb. acidophilus *Lb. rhamnosus*	Balb/c mice were gavaged with 109 CFU for 10 or 28 d, their splenocytes were cultured with ConA	↑ ↑ IFN-γ compared to milk ± IL-4	131
Lb. casei strain Shirota	Balb/c mice received 1.3×10^9 LcS orally for 7 d, their splenocytes were cultured with ConA	↑ ↑ IFN-γ ± IL-4 and IL-5[a]	144
Lb. casei strain Shirota	CIA was induced in DBA/1 mice, some received 2.5 or 5×10^8 LcS orally for 5 d, splenocytes were restimulated with denatured collagen	↓ IFN-γ compared to water-treated[a] controls	143
Lb. casei strain Shirota	Balb/c mice were i.p. injected with OVA some were fed a diet containing 0.05% (wt/wt) heat-killed LcS for 21 d, splenocytes were restimulated with OVA	↓ IL-4 , IL-5, IL-6, IL-10 ↑ IL-2, IL-12, IFN-γ	104
Lb. casei ATCC 393	Female Balb/c were immunized with Chikungunya virus and fed *L. casei* for 4 alternate days. On day 7, immunohistochemically cytokine-positive cells in the gut villi were counted	↑ TNF-α ($p < 0.05$ compared to $NAHCO_3$-treated controls); IL-1α, IL-1β, IL-2, IL-10, and IFN-γ not significantly different	66

[a] *Lb. casei* strain Shirota (LcS) appears to have opposite effects in healthy mice and mice in the early stages of collagen-induced arthritis (CIA).

Most notably, several yogurts increased IFN-γ mRNA levels in MLN; the increase being significant in the case of one heat-treated yogurt. The same yogurt, whether heat-treated or not, also increased IL-6 mRNA in MLN.

In additional mouse studies performed by Perdigón et al., the authors studied IL-4 and IFN-γ release by immunohistochemistry, due to the importance of these two cytokines in maintaining the balance between the populations Th2 and Th1, respectively. They also determined TNF-α as a measure of macrophage activity. It was determined in these studies that LAB were able to induce a diversity of responses mediated by the cytokines released, but different properties in the immunostimulation for each LAB assayed could not be determined. The authors interpreted these results as being due to the use of heterologous strains of different origins, some of them from humans, tested in a mouse model. Therefore, they then compared the behavior of *Bifidobacterium* sp. and *Lb. animalis* (isolated from mice) with the corresponding human heterologous strains. These follow-up studies demonstrated that the host specificity is important for the genus *Bifidobacterium* but not for lactobacilli. However, this specificity allows a better regulation of the dose, thus avoiding the dysregulation of the immune response.[30,110] In a more recent study, the authors also demonstrated that a homologous strain, *B. animalis*, was able to adhere and interact with mucosal epithelial cells of both small and large intestine, whereas a heterologous strain, *B. adolescentis*, was not. Nevertheless, the authors could not demonstrate that *B. animalis* modulated mucosal immunity. They proposed that bifidobacteria may not alter the immune system directly, but the health benefits may be mediated through other mechanisms, such as competitive inhibition or acid production.[112]

Several human studies also indicate that yogurt consumption may be associated with an increase in IFN-γ synthesis. Halpern et al.[79] showed an increase in IFN-γ production by T cells from subjects who consumed 450 g of live-culture yogurt per day for 4 months. This was determined by an enzyme-linked immunosorbant assay (ELISA) analysis of supernatants collected from PHA-stimulated PBMC. In a similar study by the same group of researchers, the health of a college-aged population during chronic yogurt consumption was followed. Subjects were asked to eat 200 g of plain yogurt every day for a year; one group ate heat-inactivated yogurt, and a group that ate no yogurt served as controls. The effects of year-long daily consumption of 200 g of yogurt on IFN-γ levels were measured in the two subject populations by an ELISA assay on PHA-stimulated PBMC. Although the data were quite variable, when the data were analyzed according to the subject age, there was a significant decrease in IFN-γ production in the older group of subjects.[79,113,114] Clearly, further studies involving dose kinetics would be beneficial.

In another human study, subjects eating yogurt of their own choice (i.e., with unknown numbers and types of live or inactive bacteria) for 2 weeks showed a significant increase in 2-5A synthetase activity.[115] This enzyme is reportedly a specific marker for the production of interferons. Significant upregulation of 2-5 A synthetase activity was also reported in volunteers who consumed 100 g yogurt containing 10^9 *Lb. bulgaricus*/g and 10^9 *S. thermophilus*/g compared to volunteers consuming milk.[116] Enzyme activity was further increased after volunteers ate 250 g yogurt/d for 15 d.

In a double-blinded crossover design experiment by Wheeler et al., the effect of consuming live-culture yogurt (450 g/d) with or without *Lb. acidophilus* was studied in adult patients with moderate asthma. After two 1-month crossover test periods, no significant changes were noted in peripheral cell counts, IgE, IL-2, or IL-4, when the two diets were compared to each other. In addition, Con A-stimulated lymphocytes from patients who consumed yogurt containing *Lb. acidophilus* produced borderline elevated interferon gamma levels ($p = 0.054$). No differences were noted in the mean daily peak flows or changes in spirometric values, and the quality of life indices were unchanged. The authors concluded that the live-culture yogurt generated trends towards an increase in IFN-γ and decrease in eosinophilia.[117]

5.9 LACTIC ACID BACTERIA AND IMMUNE CELL FUNCTION

Highly variable results have been reported from both in vitro and ex vivo experiments assessing the effects of various LAB on lymphocyte proliferation. Whether LAB enhance or inhibit proliferation appears to depend not only on the specific bacterial strain investigated, and the type and concentration of the mitogen used to induce proliferation, but also on the activation state of the immune system of the experimental animals or humans.

In vitro, incubation of human PBMC with live *Lb. johnsonii* or *Lb. sakei* for 5 d resulted in a strong proliferative response.[118] The effect obtained with *Lb. johnsonii* was significantly greater than that of *Lb. sakei* and was of a magnitude similar to that observed with PHA (10 μg/mL). Heat-killed bacteria produced similar results. Homogenates of *Lb. rhamnosus GG*, *B. lactis*, *Lb. acidophilus*, *Lb. delbrueckii* ssp. bulgaricus, and *S. thermophilus* all suppressed the proliferation of human PBMC induced with a very high concentration (125 μg/mL) of PHA.[119] This effect was significantly reduced, but not completely eliminated, by heat inactivation. Cytoplasmic extracts, whether unheated or heated, significantly inhibited PHA-induced proliferation, whereas cell wall extracts had no effect.

In mice fed yogurt for 10 months, splenocyte proliferation in response to ConA and PHA was significantly increased compared to milk-fed controls.[82] In contrast, after a shorter feeding period (4 weeks), the ConA-, PHA-, and LPS-induced proliferation of splenic and intestinal lymphocytes from DBA/2J mice fed yogurt or dried milk powder as 50% of their energy for 4 weeks, did not differ significantly.[83] When, however, such mice were challenged with an LD_{50} dose of *Salmonella typhimurium*, proliferation of the splenocytes after ConA stimulation was significantly higher in the yogurt-fed than in the milk-fed group, whereas the response to PHA or LPS was similar in the two groups. The mitogenic response of intestinal lymphocytes in response to ConA and LPS was also significantly higher in yogurt-fed than in milk-fed mice. The proliferative response to PHA, though greater in yogurt-fed animals, was not significantly different from that in milk-fed animals. Similarly, the proliferative response to ConA and LPS was significantly increased in splenocytes from *S. typhimurium*-challenged Balb/c mice fed a diet supplemented with 30 g of yogurt (8×10^8 LAB/g) for 12 d, compared to powdered milk-supplemented controls.[120] A slight, statistically nonsignificant, increase in proliferation was also observed in mice fed heat-treated yogurt. In another study by the same group, supplementing the

diet of unchallenged Balb/c mice with 20% heat-treated yogurt resulted in similar proliferative responses of PP to PHA as did yogurt containing live LAB (*S. thermophilus* and *Lb. bulgaricus*) after 14 or 21 d.[53] Only yogurt containing live LAB, however, increased the LPS-induced proliferation of PP after 7, 14, and 21 d. These enhancing effects of yogurt consumption on the mitogenic response were transient and were no longer observed after 28 d of supplementation with yogurt containing live or heat-killed LAB.

Basal and LPS-stimulated proliferation was increased in splenic lymphocytes from mice fed 10^9 viable *Lb. acidophilus* per kg body weight for 7 d compared to saline-treated controls.[121] In contrast, the same number of bacteria from the strains *Lb. casei*, *Lb. gasseri*, and *Lb. rhamnosus* inhibited basal proliferation and proliferation stimulated with supraoptimal concentrations of LPS or ConA. In a similar study by the same authors, however, oral supplementation with *Lb. rhamnosus* for 7 d did not affect basal proliferation, whereas administration for 14 d enhanced it.[122] Unlike in the previous study, an increase in proliferation stimulated with the optimal concentrations of ConA or LPS after 7 d was also observed.

Intraperitoneal administration of *Lb. casei* to MRL/lpr mice (a model for systemic lupus erythematosus) was also reported to significantly inhibit the ConA-, LPS-, or pokeweed mitogen-induced proliferation of splenocytes compared to saline-injected controls.[123] In mesenteric lymph node cells, however, *Lb. casei* injection did not significantly affect the proliferative response to these mitogens.

5.9.1 Innate Immune Responses

The innate immune system plays an important role as a first line of defense against invading pathogens. It also provides signals that activate the adaptive arm of the immune system. Several studies have reported that the immunomodulatory effects of LAB are mainly on the innate immune response. Vinderola et al. found that *Lb. casei* CRL 431 interacted with epithelial cells through toll-like receptor 2 (TLR2), a pattern recognition receptor, and this interaction induced IL-6 secretion.[124] Subsequently, by using mice as an experimental model, they demonstrated that *Lb. casei* CRL 431 administration increased the expression of TLR2 and CD206, a mannose receptor, on dendritic cells and macrophages from Peyer's patches and lamina propria of the small intestine. There was also an increase in IgA+ cells and IL-6-producing cells in the lamina propria of the small intestine. However, the resident T-cell population and IL-5-producing T cells remained unchanged. These findings indicated that *Lb. casei* CRL 431 predominantly modulates the innate immune response.[125] The mechanisms by which LAB influences innate immunity were further elucidated by an in vivo study by Kim et al.[126] Splenocytes from mice orally administered with *Lb. casei* ATCC27139 exhibited an upregulation of innate cytokines, including TNF-α, IL-12, IL-18, and IFN-γ, as well as pattern recognition receptors, such as TLR2 and nucleotide-binding oligomerization domain (Nod). Moreover, this study suggested that signaling pathways mediated by NF-κB and p38 MAP kinase are crucial in innate immune modulation by *Lb. casei* ATCC27139.

5.9.2 Phagocytic Activity of Macrophages and Granulocytes

Peritoneal macrophages from mice supplemented with milk fermented with *Lb. acidophilus*, *Lb. casei*, or both (both isolated from human feces) for 8 d exhibited significantly increased phagocytic activity compared to the peritoneal macrophages from nonsupplemented mice.[127] Colloidal carbon clearance as a measure of in vivo phagocytic activity was similarly enhanced. In a similar study by the same group of researchers,[128] oral administration of *Lb. casei* enhanced in vitro and in vivo phagocytosis, whereas *Lb. bulgaricus* had a little effect on in vitro phagocytosis, but nonetheless significantly increased the in vivo colloidal carbon clearance rate. Macrophage activation has also been observed in tumor-bearing mice fed yogurt mixed into their diets.[129,130] In mice immunized with cholera toxin or tetanus vaccine, supplementation with 10^9 CFU of *Lb. acidophilus* (HN017), *Lb. rhamnosus* (HN001), or *B. lactis* for 10 or 28 days was associated with a significant increase in phagocytic activity of both PBL and peritoneal macrophages, when compared with controls given skim milk without LAB.[131]

In a study where human volunteers consumed milk for 3 weeks, followed by fermented milk providing either 7×10^{10} CFU *Lb. acidophilus* La1 or 1×10^{10} CFU *B. bifidum* strain Bb 12 for another 3 weeks, there was a highly significant increase in leukocyte phagocytic activity in both groups, with granulocytes showing a greater increase than monocytes (measured as uptake of opsonized *E. coli*).[132] A significant elevation in phagocytic activity could still be detected 6 weeks after the cessation of fermented milk intake, even though fecal counts of bifidobacteria and lactobacilli, respectively, had returned to baseline levels by day 12 after the end of bacterial supplementation. Another study by the same group, using a similar protocol, compared the effects of supplementation with 150 mL/d of a milk fermented with *S. thermophilus* to those seen after the consumption of the same product supplemented with *Lb. johnsonii* La1, either fresh or stored for 21 to 28 d, to yield a 10-fold lower bacterial count.[133] Despite the somewhat lower daily dose compared to the previous study (1.5×10^9 CFU *Lb. johnsonii*/day), a significant increase in leukocyte phagocytic activity and respiratory burst were observed in the group receiving fresh fermented milk with *Lb. johnsonii* La1. Similar trends were seen in the group consuming stored fermented milk with *Lb. johnsonii* La1, but this did not reach statistical significance for either parameters. The authors concluded that the minimal effective dose for modulating granulocyte or monocyte activities was 10^9 CFU/d. It should be noted, however, that storage of fermented milk products results in changes other than the decrease in viable bacteria due to the accumulation of metabolites generated by these bacteria. Hence, whether the lack of a significant effect in the group receiving stored fermented milk with *Lb. johnsonii* La1 was due to the lower cell count or to other factors (or a combination of the two) is not clear. In order to establish a true dose response, it will be necessary to test products in which differences in the number of viable bacteria are obtained by the appropriate changes in the manufacture, rather than through prolonged storage.

In addition, another research group has more recently demonstrated that the lack of fermented food consumption led to a reduction in the phagocytic activity of granulocytes. Furthermore, supplementation with either conventional yogurt containing

Lb. delbrueckii sp. bulgaris or probiotic yogurt containing *Lb. gasseri* CECT5714 and *Lb. coryniformis* CECT5711 reversed this effect.[134]

In healthy subjects, consumption of milk supplemented with 2.6×10^8 CFU *Lactobacillus* GG resulted in a significant upregulation of the expression of phagocytosis receptors (CR1, CR3, FcγRI, and IgαR) on neutrophils, whereas the increases on monocytes were not significant, compared to the period prior to milk consumption without *Lactobacillus* GG.[135] Milk alone did not affect receptor expression. In contrast, in milk-hypersensitive subjects, there was a considerably higher expression of all of these receptors on monocytes and neutrophils during milk consumption. When, however, *Lactobacillus* GG was added to the milk, receptor expression did not significantly differ from that seen before milk challenge, suggesting that *Lactobacillus* GG downregulated the milk-induced phagocyte activation.

The cytokines TNF-α and IL-1β are cytokines predominantly produced by activated macrophages. As summarized in Tables 5.1 and 5.2, numerous in vitro studies have reported an upregulation of the synthesis of these cytokines when a variety of cell types, including those used for the production of yogurt or commonly added to it, were incubated with live or heat-killed LAB. This further suggests that numerous LAB have a stimulatory effect on macrophage activities.

Interestingly, growth phase, heat treatment, and interactions between the two were found to influence the ability of three different lactobacilli to induce TNF-α human peripheral blood monocytes.[136] Live bacteria from the logarithmic growth phase stimulated considerably higher amounts of TNF-α secretion than heat-killed cells. In contrast, heat-killed bacteria harvested at the stationary phase induced 4- to 5-fold higher levels of TNF-α than live cells did.

5.9.3 Natural Killer Cell Activity

Natural killer (NK) cells are thought to play an important role in inhibiting carcinogenesis. IL-12 is a strong inducer of NK cells, and—as discussed earlier and summarized in Tables 5.1 and 5.2—a variety of LAB including LcS[103,104] have been shown to stimulate IL-12 production. The enhancement of NK activity and a significant reduction in tumor incidence have been reported after LcS supplementation in 3-methylcholanthrene-induced carcinogenesis in C3H/HeN mice.[137,138] Such inhibition was not observed in NK cell deficient beige mice,[138] suggesting that a major mechanism by which LcS inhibits carcinogenesis in this model is through the enhancement of NK cytotoxicity.

In mice, oral administration of *Lb. rhamnosus* resulted in a significant increase in NK cell activity, whereas the increase after *Lb. acidophilus* or *B. lactis* supplementation was not significant.[131] Daily intake for 3 weeks of *B. lactis* in low-fat milk by human volunteers, however, was associated with significantly higher NK cytotoxicity compared to consumption of low-fat milk alone.[139] In addition, Olivares et al. demonstrated that, in a randomized, double-blind, placebo-controlled human clinical trial, consumption of yogurt containing two new probiotic strains, *Lb. gasseri* CECT5714, and *Lb. coryniformis* CECT5711, increased the proportion of NK cells, whereas the percentage of NK cells in control subjects receiving standard yogurt decreased. Interestingly, the increased percentage of NK cells in the former group

occurred only in subjects who initially had a low or average proportion of NK cells but not in subjects with high numbers of NK cells.[107] These findings emphasize the fact that the immunomodulatory effects of probiotics depend on not only the strains of the bacteria but also the immune status of the hosts.

In addition, under the stress of academic examinations, which has been shown to suppresses the immune system, students who consumed yogurt plus *Lb. casei* DN-114001 were able to maintain the numbers of CD56+ cells (NK cells and a subpopulation of cytotoxic T cells), whereas students who received semiskimmed milk had decreased CD56+ cell counts.[140] Students consuming yogurt also showed significant increases in lymphocyte counts compared to those receiving milk. Moreover, this study demonstrated that yogurt consumption tended to prevent an increase in serum cortisol level under stress; however, there was no statistical significance between these two groups ($p = 0.062$).

Perdigón et al. studied the mechanisms by which yogurt was able to inhibit the growth of a chemically induced intestinal tumor. They demonstrated that yogurt can downregulate the inflammatory response induced by the carcinogen by:

1. The increase of IgA+ producing cells and CD4+ T cells
2. The diminution of T cells, CD8+, and cytotoxic activity
3. The increase of cellular apoptosis of infiltrating immune cells
4. Apoptosis, which was favored by an increase in the levels of TNF release induced by yogurt
5. Induction of IL10 release, which plays an important role in the mechanisms of downregulation

The authors concluded that the mechanisms involved in the immunostimulation by LAB or yogurt are multiple. These findings indicate that it will be very difficult to establish a reliable test for selection of LAB with immunostimulatory capacity. However, the knowledge of the mechanisms controlling the response to an individual strain or yogurt will allow a better choice for therapeutic purposes.[130]

5.9.4 Immunostimulating versus Immunosuppressive Effects

TNF−α and IL-1β are proinflammatory cytokines, and stimulation of their production suggests that LAB may have proinflammatory activities. Such activities would be undesirable in situations already characterized by inflammation, such as allergy, asthma, and several autoimmune diseases. LAB therapy has been examined in several of these conditions, and to date, no exacerbation of inflammation has been observed. Instead, in children with atopic eczema and cows' milk allergy, consumption of extensively hydrolyzed whey formula supplemented with *Lactobacillus* GG, but not the same formula alone, was associated with a significant decrease in fecal TNF−α, although TNF−α release by ConA-stimulated PBMC was similar before and after treatment.[141] This may be a strain-dependent effect; it may, however, also reflect the potential of LAB to have differential effects in health and disease.

In contrast to the immunostimulatory effects of LAB through TNF−α and IL-1β production, certain strains of LAB have been shown to induce IL-10-producing

regulatory T cells, and thus exert an immunosuppressive effect. It has been demonstrated that *Lb. reuteri* and *Lb. casei,* but not *Lb. plantarum,* primed monocyte-derived dendritic cells through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN). These LAB-primed dendritic cells induced the development of IL-10-producing regulatory T cells, which inhibited the proliferation of bystander T cells.[142] This could be a benefit in those diseases of the gut that are proinflammatory in nature such as Crohn's disease.

Indeed, it has repeatedly been reported that various LAB differentially affect the immune responses of healthy animals or volunteers, and those whose immune system is activated due to pathogenic challenge or autoimmune disease. For example, *Lactobacillus* GG downregulated phagocyte receptor expression induced by milk challenge in milk-hypersensitive subjects but stimulated receptor expression in healthy subjects.[135] Administration of LcS was associated with increased ConA-stimulated IFN−γ production by splenocytes from healthy Balb/c mice, but had the opposite effect in DBA/1 mice in the early stages of collagen-induced arthritis (see Table 5.3).[143,144] Mitogen-induced proliferation of splenic lymphocytes from DBA/2J was not enhanced after yogurt feeding.[83] When, however, such mice were challenged with an LD_{50} dose of *Salmonella typhimurium*, the proliferative response to ConA was significantly higher in the yogurt than in the milk group. Nonetheless, because of the species and strain dependence of the immunomodulation by LAB, thorough assessment of LAB in vitro and in animal models will be crucial before they can be used to modulate diseases characterized by immune deviation.

5.10 CONCLUSIONS

An aura has surrounded the association of fermented milk products, especially yogurt, and health for more than a century. The pivotal finding within this association has been the direct effect of yogurt upon the immune system. Although this area has been studied in both humans and mice for some time, a significant amount of research is still required to address the fundamental basis for the mechanisms of yogurt's biological consequences. It is perhaps the complexity of the immune system, coupled to an equally complex microbial physiology in vivo, that limits rapid progress within this area. Despite these limitations, the yogurt-immunity connection remains as an exciting and attractive area of research for a variety of disciplines. Within the foreseeable future, it is envisioned that these disciplines will collectively develop a core of paradigms regarding the science of yogurt and immunity that allows rigorous examination and verification with respect to human health. Moreover, through these efforts, new methodology, procedures, microbial strains, and research findings will consolidate the variation in studies extant today, and drive the development of improved fermented milk products.

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6 Health Properties of Milk Fermented with *Lactobacillus casei* strain Shirota (LcS)

Kouji Miyazaki and Takeshi Matsuzaki

CONTENTS

6.1 HISTORY AND BACKGROUND

Throughout history, humans have made use of lactic acid bacteria (LAB), which are distributed widely in nature. LAB have traditionally been employed to produce fermented milk products, including yogurt, leiben, dahi, kefir, and koumiss. Currently, they are also used to produce many processed foods, such as fermented meat products, brewed products, Japanese pickles, and bread, as well as silage.[1,2]

LAB were first discovered by Pasteur in 1857. In 1878, Lister reported the isolation of LAB from rancid milk. Subsequently, these bacteria were also isolated from the intestinal tract. *Bifidobacterium* species were discovered by Tissier[3] in 1889, and *Lactobacillus acidophilus* was discovered by Moro[4] in 1990. Soon afterwards, attempts were made to classify these new species of LAB. Although there was some confusion for a while, Orla-Jensen[5] laid the foundation for an overall classification of LAB in 1919.

The genus *Lactobacillus* comprises Gram-positive, nonsporing, noncatalase-producing facultative anaerobic rods that commonly produce lactic acid as their major metabolite. *Lactobacilli* are generally isolated from fermented milk and from the intestinal tract of humans and other animals.[6] Major *Lactobacillus* species isolated from the human intestinal tract include *Lb. gasseri, Lb. crispatus, Lb. johnsonii, Lb. salivarius, Lb. reuteri, Lb. casei, Lb. ruminis, Lb.itulinus, Lb. lantarum,* and *Lb. revis.*[7] *Lb. acidophilus* is rarely isolated from the human intestinal tract.

Lb. casei strain Shirota (LcS) was isolated and cultured stably in 1930 by Minoru Shirota (1899–1982) at the Microbiological Laboratory of the Faculty of Medicine at Kyoto University, Kyoto, Japan. This organism is resistant to gastric acid and bile acids, so that live bacteria can reach the lower intestine after oral administration. Dr. Shirota developed "Yakult," which is a dairy product manufactured using LcS. Yakult was first produced in 1935 and is based on his hypothesis that daily oral intake

of LAB promotes intestinal health and prevents diseases, thereby prolonging the life-span of humans. Yakult that contains LcS has been sold for over 70 yr in Japan. The products contain more than 15 billion living LcS per bottle in Japan, and about 25 million bottles per day were consumed in 26 countries and territories in 2006. Subsequent studies of LcS have focused on the taxonomy, genetics, and physiology of *Lactobacillus* species and other LAB as well as the nutritional value of fermented milk products, their beneficial effects on fecal properties, and their preventive action against gastrointestinal infection. Additional recent studies have included the investigation of the beneficial effects of fermented milk products as well as the effects of cellular constituents and metabolic products of LAB used to manufacture these products on host homeostasis, e.g., prevention of carcinogenesis, beneficial effects on lipid metabolism and blood pressure, and immunomodulatory effects. The results obtained from these studies have shown that LcS and dairy products manufactured using this *Lactobacillus* strain have a range of biological activities.

Recently, several strains of LAB have attracted the attention of investigators as probiotics that are defined as "viable bacteria that exhibit beneficial effects for health based on an improvement in the balance of intestinal bacterial flora" or "live microorganisms which when administered in adequate amounts confer a health benefit on the host."[8,9] Also, synbiotics have been recognized as potent new agents by clinicians, and describe a combination of probiotics (e.g., LAB) and prebiotics, which are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon.[10] LcS has been considered one of the most widely investigated and typical probiotic strain.

The present chapter summarizes various findings on the biological activities of LcS that can impact favorably on human health. This review presents information from studies performed both within and outside the Yakult Institute[11] (Yakult Central Institute for Microbiological Research, Tokyo, Japan).

6.2 GENERAL PROPERTIES OF LcS

6.2.1 Morphology and Structure of LcS

LcS is an average-sized bacillus. Each cell is about 1 to 2.5 μm long and has a diameter of about 0.5 μm as observed under the electron microscope (Figure 6.1). In general, the size of bacterial cells changes slightly during culture. In the case of LcS, cells longer than 4 μm sometimes appear at the late stage of culture, when cell division ceases. LcS is normally roundish and has a smooth surface without any of the flagella or cilia possessed by *Escherichia coli* (Figure 6.2).

Each LcS organism is surrounded by a cell wall about 20 to 30 nm thick that is composed of peptidoglycan (murein), teichoic acid, and proteins, as is the case for other Gram-positive bacteria. After fixation by the Ryter-Kellenberger method[12] the standard fixation method for bacteria, LcS appears to have a polysaccharidelike coat outside the cell wall. This coating may not be an intrinsic structure like a capsule, but instead may be a layer of metabolic products adhering to the cell wall, as no such

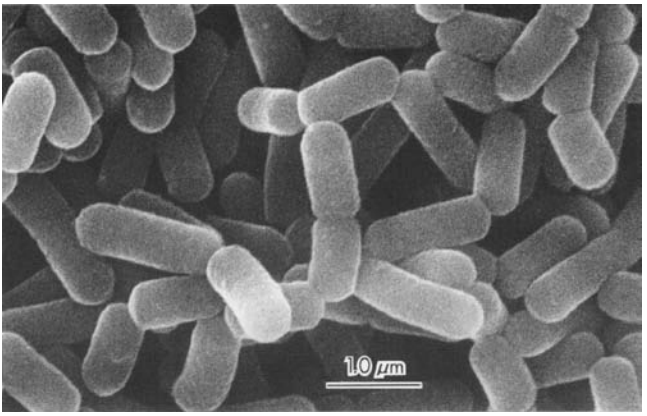

FIGURE 6.1 Scanning electron micrograph of *L. casei* Shirota (LcS), showing bacilli about 0.5 mm in diameter and about 1.5 mm in length (× 17,000). (From Lactobacillus casei *strain Shirota—Intestinal Flora and Human Health*, Yakult Central Institute for Microbiological Research, Tokyo, 1990, p. 23. With permission.)

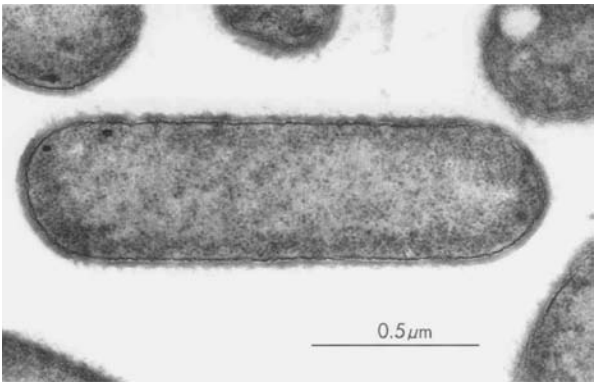

FIGURE 6.2 Electron micrograph of an ultrathin section of LcS, showing a relatively smooth cell surface (× 66,000). (From Lactobacillus casei *strain Shirota—Intestinal Flora and Human Health*, Yakult Central Institute for Microbiological Research, Tokyo, 1990, p. 24. With permission.)

coat is observed after double fixation with glutaraldehyde and osmium tetroxide or after rapid freezing and substitution fixation. LcS does not have a regular array layer composed of fine protein granules.[13]

Chemical analysis shows that LcS is surrounded by a cell wall composed of peptidoglycans to which polysaccharides are bound as accessory polymers, whereas lipoteichoic acid and other polymers extend toward the cell wall from the cell membrane as in other Gram-positive bacteria.[14] Some other Gram-positive bacteria have a capsule as the outermost structure or have M protein or S layers on the cell wall.[15] However, no such structures have been detected in LcS. Thus, the major constituents of the surface of LcS appear to be lipoteichoic acid, a peptidoglycan layer, and polysaccharides attached to the cell wall (Figure 6.3).

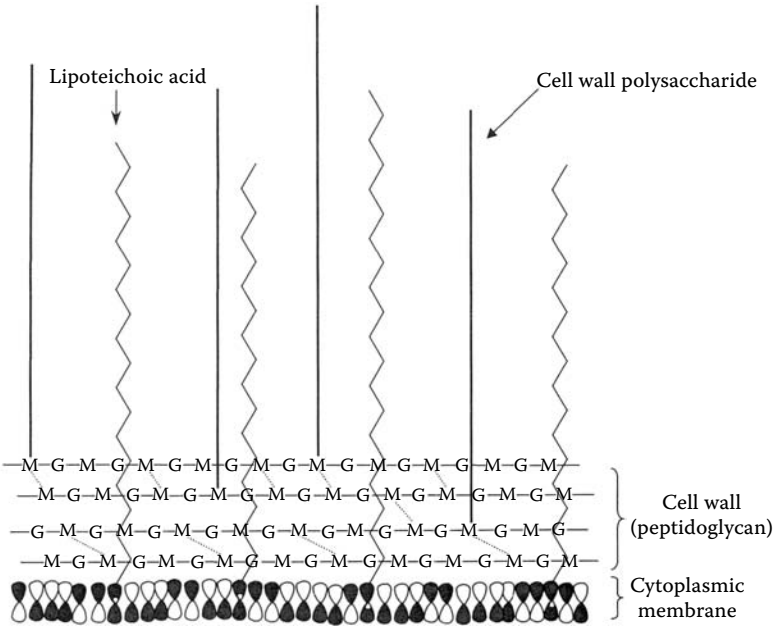

FIGURE 6.3 Diagram of the cell surface structures of Gram-positive bacteria. M: *N*-acetylmuramic acid, G: *N*-acetylglucosamine, -----: Interpeptide bridge. (From Lactobacillus casei *strain Shirota—Intestinal Flora and Human Health*, Yakult Central Institute for Microbiological Research, Tokyo, 1990, p. 31. With permission.)

6.2.2 Energy Metabolism of LcS

In LcS, the phosphoenolpyruvic acid-dependent phosphotransferase system (PTS) may facilitate cellular uptake of lactose. When grown in a medium with glucose or lactose as the major carbohydrate source, LcS produces lactic acid as the predominant product of fermentation. Thus, this strain belongs to the homofermentative group of LAB. Homofermentative LAB produce 2 moles of lactic acid and 2 moles of ATP from 1 mole of glucose. The efficiency of adenosine triphosphate (ATP) formation in the process of homolactic acid fermentation is twice that of heterolactic acid fermentation. Therefore, the efficiency with which LcS obtains energy is higher than that of heterofermentative lactic acid bacteria.[16,17]

LAB are generally classified as homofermentative or heterofermentative. Homofermentative bacteria are further classified into obligate homofermentatives (which are always homofermentative under any culture conditions) and facultative homofermentatives (which can switch to the heterofermentative pattern with a change of carbohydrate source or culture conditions). Because LcS also produces trace amounts of acetic acid and ethanol, this strain is a facultative homofermentative in the strict sense.[18,19]

6.2.3 Nutritional Requirements of LcS

The nutritional requirements of LcS are very complicated, as are those of many other LAB. To achieve the maximum growth of LcS in a synthetic culture medium,

TABLE 6.1
Nutritional Requirements of LcS

Nutrients eliminated from the basal synthetic medium	Viability	Nutrients eliminated from the basal synthetic medium	Viability
Amino acids		***Vitamins***	
Alanine	+	Thiamine	+
Arginine	–	Riboflavin	–
Aspartic acid	–	Pyridoxal	–
Cysteine	±	Biotin	+
Glutamic acid	–	Pantothenic acid	–
Glycine	+	Nicotinic acid	–
Histidine	+	Folic acid	±
Isoleucine	–	*p*-Aminobenzoic acid	+
Leucine	–		
Lysine	–	***Purines and pyrimidines***	
Methionine	–	Adenine	±
Phenylalanine	±	Cytosine	+
Serine	+	Guanine	+
Proline	–	Thymine	+
Threonine	–	Uracil	±
Tryptophan	–	Xanthine	±
Tyrosin	–		
Valine	–		

Note: After incubation at 37°C for 16 h in Rogosa medium, bacteria were harvested and washed twice with saline. Then the organisms were incubated into liquid basal synthetic medium containing all but the specified nutrient and were incubated at 37°C for 3 d. During incubation, the turbidity of each culture was examined at 24-h intervals and graded as + (good growth), ± (slight growth), or – (no growth).

Source: Morishita, T., Fukuda, T., Shirota, T., and Yura, T., *J. Bacteriol*, 120, 1078–1084, 1974.

as many as twelve amino acids and four vitamins are required.[20] Several additional amino acids, vitamins, and nucleic acid bases have also been found to promote the growth of this strain. LcS is auxotrophic for valine, glutamic acid, nicotinic acid, and pantothenic acid, which are required for the growth of almost all species of LAB. Various minerals are also required by LcS; the concentration of manganese required is particularly high (see Table 6.1).

6.2.4 Genome Analysis of LcS

The genome analysis of bacteria has progressed rapidly since the entire genome of *Haemophilus influenzae* were sequenced in 1995. The whole genome sequence has already been reported for the following LAB: *Lactococcus lactis* ssp. *lactis, Lb. plantarum, Lb. johnsonii, Streptococcus thermophilus,* and *Bifidobacterium longum*. The accumulation of genome data makes it possible to characterize and compare with the sequenced strains in LAB at the genome level.

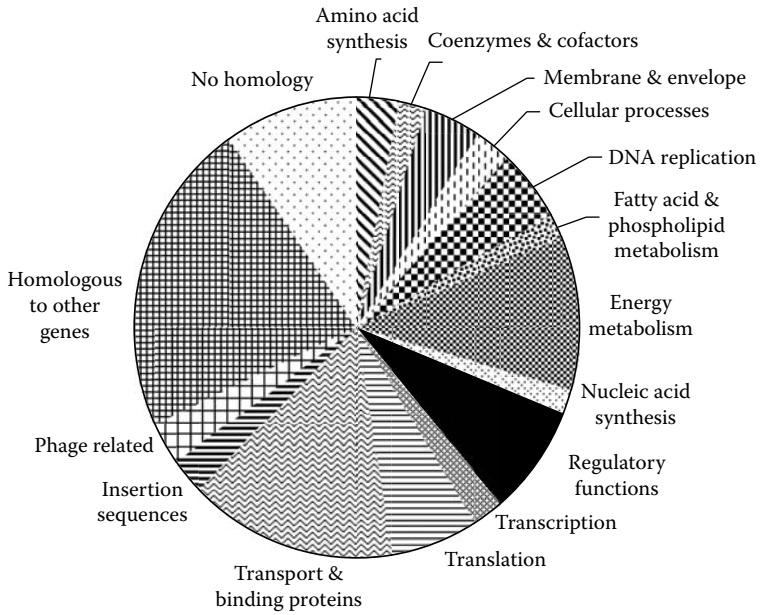

FIGURE 6.4 Classification of genes of LcS.

Sato et al.[21] have recently published the entire genome sequence of LcS, constructing a random library of DNA using the shot-gun library method, assembling with contig sequences, and filling the gaps between different contigs followed by sequencing data from primer walking. A chromosome of LcS was constructed with 3,035,753 bp of nucleotides, and estimated 2760 genes, 5 copies of rRNA operon, 58 copies of the tRNA gene and 70 copies of insertion sequence. Figure 6.4 shows the classified genes of LcS. LcS had some deficiencies in genes associated with TCA cycles and amino acid synthesis. Also, there were no major homologous regions between LcS and *Lb. plantarum* WCFS1 on the genome sequence level.

These findings indicate that LcS has a lower efficiency than general aerobic bacteria in the production of ATP and complex nutritional requirements. The gene analysis clearly demonstrates that LcS adapts well to the environment of the human intestine, which has little oxygen and is rich in nutrients.

6.3 FERMENTATION PROCESS OF LcS

6.3.1 Cultivation of LcS

LcS has been shown to have various biological effects, which will be described in the following sections. It should be noted that the cell components and biological activities may change with different culture conditions. Therefore, it is important to study the growth characteristics of this strain in detail, and to optimize the culture conditions for mass production of the cells with the desired biological activity. The most commonly used media for cultivating LAB are MRS medium[22] and Rogosa

medium.[23] For experimental use, LcS is cultured for 48 h at 37°C in Rogosa's medium. After cultivation, cells are collected by centrifugation, washed with sterile distilled water, and lyophilized.

For industrial use, corn-stepped liquor CSL medium, which is composed of glucose and corn steep liquor, is more popular because it is less expensive and ensures favorable bacterial growth.

6.3.2 Basic Growth Aspects of LcS

LcS is a homofermentative organism that derives energy from glucose by the following metabolic reaction:

$$C_6H_{12}O_6 + 2ADP + 2Pi \rightarrow 2C_3H_6O_3 + 2ATP + 2H_2O$$

The metabolic product, lactic acid, accumulates in the medium, and the pH of the medium is decreased. When the lactic acid concentration in the medium increases to 16 g/L, the pH is decreased to nearly 4, and cell growth ceases due to the high acidity (static culture). The cell concentration when growth ceases is 5×10^9/mL (82.2 g-cell/L). When the pH of the medium is constantly adjusted to about 7.0 with an alkaline agent such as ammonia or sodium hydroxide (constant pH culture), the cells continue to grow until the cell concentration plateaus at 1 to 2×10^{10}/mL (5.0 g-cell/L).

6.3.3 Optimization of Culture Temperature and pH

The parameters that most significantly affect the growth of LAB are pH and temperature. As the pH continually changes in static culture, it is difficult to optimize the conditions for growth.

To determine the optimum culture conditions in constant pH culture, batch culture was performed with the two major variables, pH and temperature, changed within the range of 5.0 to 8.0 and 25 to 45°C, respectively. The relationships of these variables with the growth time (T h), final cell concentration (X g-cell/L), cell yield of glucose (Yx g-cell/g-glucose), and cell productivity (Px g-cell/h/L) were determined. X and Yx remained relatively constant independent of both variables, at 4.8 to 5.0 g-cell/L and 0.11 to 0.12 g-cell/g-glucose, respectively. In contrast, T and Px were significantly affected by both temperature and pH. The optimum pH and temperature were found to be 6.5 and 35°C, respectively, for maximum mass production of LcS.[24]

6.3.4 Effect of Temperature on the Survival of LcS in Dairy Products

Strains of LAB used to manufacture fermented milk or sour milk beverages usually have a high proliferative activity, show high LAB production in a specific medium, and create no metabolites that adversely affect the flavor of the product. On the other hand, the strains used are also required to create little or no lactic acid in the final product during storage. During transportation of the final product to the point of sale, the ambient temperature must be controlled strictly to suppress lactic acid production by the bacterial strain and thus maintain product quality.

Figure 6.5 shows the results of a study on the effects of storage temperature on the viability of LcS in a fermented milk product together with affects of acidity and pH of the product. The viable cell count was determined by culture on agar plates containing bromocresol purple. This culture medium is officially sanctioned by the Ministry of Health and Welfare in Japan and available commercially.[25,26]

With a rise of storage temperature, activation of bacterial metabolism results in an increase in acidity and a decrease in pH, causing a decrease in the number of live

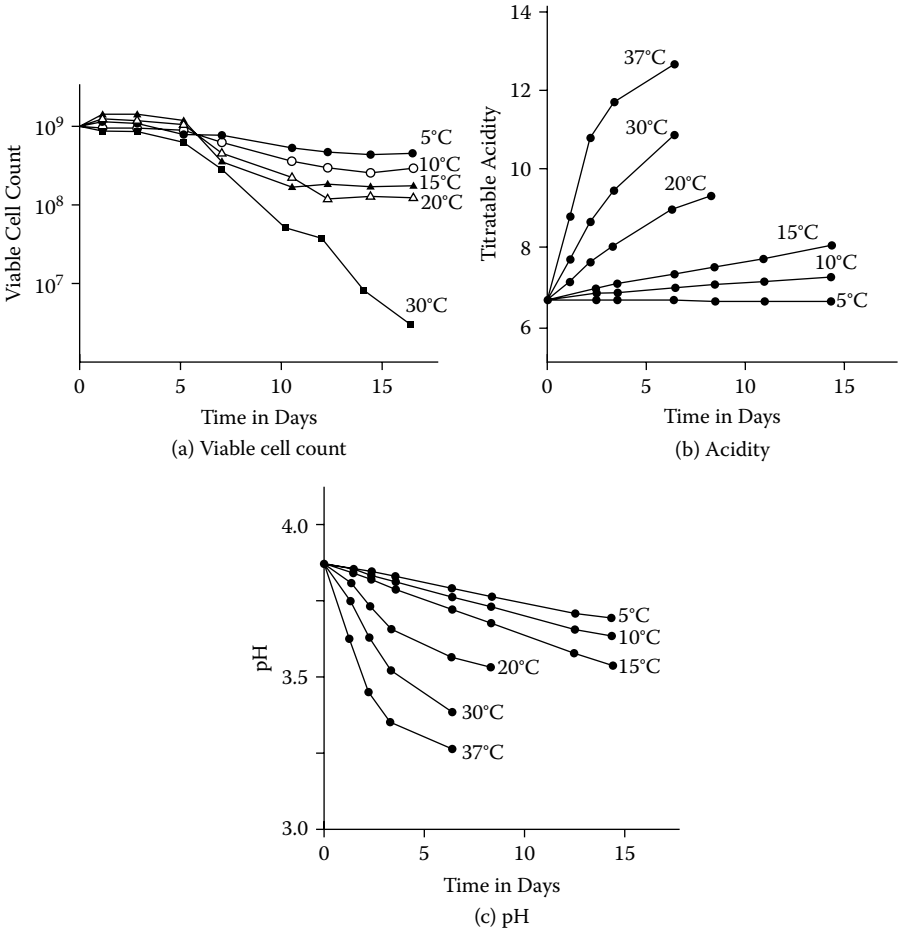

FIGURE 6.5 Effect of storage temperature on the viable cell count (a), titratable acidity (b), and pH (c) of LcS fermented milk (Yakult). Sample batches were serially diluted tenfold with sterile saline to obtain bacterial growth of 30 to 300 colonies per plate. A 1-mL aliquot of the dilution obtained in this way was mixed well with 15 mL of heated medium in a petri dish. After solidification, the plates were inverted and cultured at 35 to 37°C for 72 h. At the end of incubation, the yellow colonies on each plate were counted. Titratable acidity: volume of 0.1 *N* NaOH required to neutralize a 10-mL sample. (From Lactobacillus casei *strain Shirota—Intestinal Flora and Human Health*, Yakult Central Institute for Microbiological Research, Tokyo, 1990, p. 91. With permission.)

bacterial cells. At a storage temperature above 15°C, these changes become marked. Such changes may impair the balance of metabolites that determines the product's flavor and may reduce product quality with respect to taste, aroma, and flavor.

It was concluded that fermented milk and sour milk beverages should be stored at 10°C or lower to maintain their quality for a specified period after manufacture. In general in Japan, the expiry date of such dairy products is set at 14 d after manufacture, provided that the products are stored at 10°C or lower. During storage at 10°C or lower, the viable cell count in the product shows only a slight decrease in number, which is not associated with any noticeable changes in taste or flavor.

6.4 MODIFICATION OF GASTROINTESTINAL FUNCTION BY LcS

Digestion and absorption of nutrients are the major functions of the stomach and small intestine. It has long been considered that fermented milk and yogurt prepared using lactobacilli promote lactose digestion and absorption, and may be of nutritional benefit for individuals with low lactase activity.[27]

Promotion of lactose absorption by fermented milk and yogurt has been explained by prolongation of the gastric emptying time and small intestinal transit time, as well as by hydrolysis of lactose via the lactase activity in these products. The former effect may be attributed to the physicochemical properties and fat content of the fermented milk. Gastric emptying functions independently for liquids and solids, with liquids being transported more rapidly. Transit of liquid gastric contents is controlled by mechanical stimulation through changes of the intragastric pressure or chemical stimulation caused by lipids, carbohydrates, or acids, which induce contraction of the pyloric sphincter via mechanoreceptors of chemoreceptors present in the duodenal mucosa.[28]

Studies on the function of the stomach and small intestine and gastrointestinal kinetics of absorption were carried out in rats using a fermented milk drink containing LcS; L-lactic acid, glucose, sucrose, and lactose concentrations were measured. Following intragastric administration of the fermented drink to rats, the amounts of these ingredients that remained unabsorbed in each segment of the small intestine were determined. L-lactic acid was not absorbed in the stomach, reached the ileum in 15 min after administration, and was absorbed in the small intestine without passage through the ileocecal valve. Glucose and sucrose were absorbed in the jejunum, while some lactose remained undigested and passed through the ileocecal valve. In an experiment involving intestinal perfusion of anesthetized rats, L-lactic acid showed a more rapid absorption in the ileum than in the jejunum or colon, which suggests that there is a specific mechanism for L-lactic acid absorption in the ileum. The gastric emptying time, and the small intestinal transit time after oral administration of the fermented milk prepared using LcS, were compared with these same parameters in rats after administration of a solution with the same carbohydrate composition and after administration of unfermented milk. The gastric emptying time was longest after administration of the fermented milk, followed by unfermented milk, and then the carbohydrate solution, whereas the small intestinal transit time was similar in all three cases (Figure 6.6).

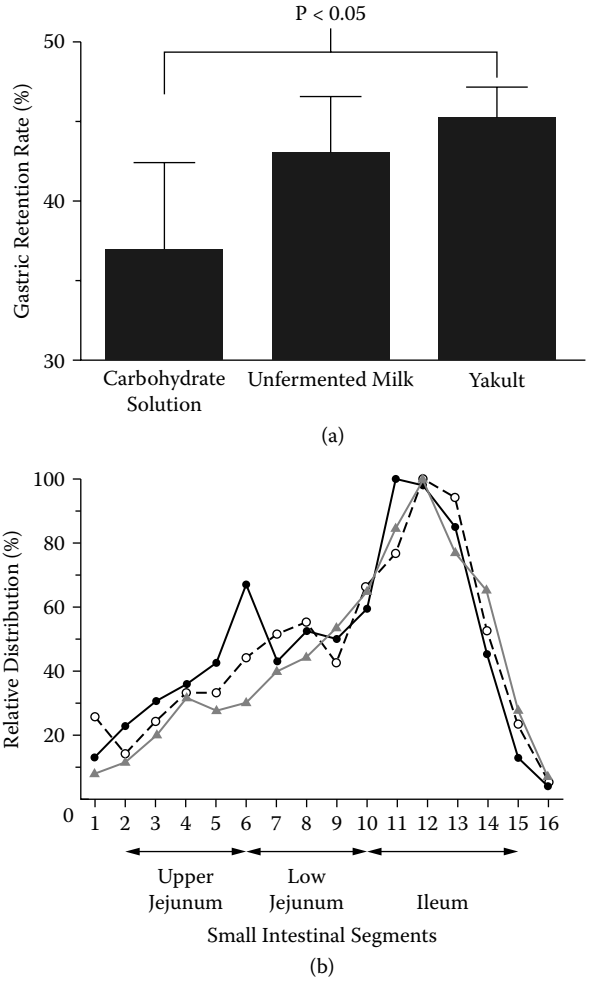

FIGURE 6.6 Gastric retention rate (a) and distribution of phenol red (a marker of unabsorbed substances) in 16 small intestinal segments of equal length (b) after 30 min administration of 2 mL of Yakult (●), a solution with the same carbohydrate composition (○), or unfermented milk (▲) in normal rats fasted for 24 h. Data on the distribution of phenol red are expressed as a percentage of the maximum value. (From Ohashi, Y., and Umesaki, Y., *Dig. Absorp.* 20, 119–123, 1997. With permission.)

In normal and gnotobiotic rats, the gastric emptying time was longer after oral administration of the fermented milk prepared using LcS than after administration of a control carbohydrate solution.[29] This suggests that lactic acid or other fermentation products of LcS act as chemical stimuli in the upper gastrointestinal tract. LcS may help increase carbohydrate absorption in individuals who have impaired absorption in the small intestine. Administration of LcS has also been reported to improve colonic function by increasing the frequency of bowel movements and relieving abdominal symptoms associated with constipation (unpublished data). Although the

exact mechanism of these effects is not clear, LcS may improve colonic motility by establishing a normal intestinal flora.[30]

6.5 EFFECTS OF LcS ON EXPERIMENTAL ANIMALS

6.5.1 Antitumor Activity of LcS

In 1981, Yokokura et al.[31] screened 26 strains of 14 species of LAB for *in vivo* antitumor activity against sarcoma 180, a transplantable mouse tumor, and found that some strains had potent antitumor activity. Among them, LcS had an especially high potency. The antitumor effect of LcS administered at various times and via various routes of administration was assessed using transplantable mouse tumors (Table 6.2). Intravenous or intraperitoneal administration of LcS caused a dose-dependent inhibition of the growth of subcutaneously implanted sarcoma 180 in ICR mice as well as Meth A fibrosarcoma and another methylcholanthrene-induced tumor (MCA K-1) in syngeneic BALB/c mice.[32] LcS also exerted a potent antitumor effect on Lewis lung carcinoma and B16 melanoma, as well as on highly metastatic variants of B16 melanoma (B16-BL6 and B16-F10), in syngeneic C57BL/6 mice. Intrapleural administration of LcS has been shown to markedly improve the survival of mice with carcinomatous pleurisy in humans. Mice inoculated intrapleurally with Meth A fibrosarcoma cells eventually die of massive pleural effusion associated with tumor cell proliferation. The pattern of proliferation of tumor cells in the pleural cavity is quite similar to that of carcinomatous pleurisy in humans. Therefore, this system may provide an appropriate animal model of carcinomatous pleurisy. When administered intrapleurally, LcS showed a significantly greater survival benefit than other bacterial preparations—OK-432, *Corynebacterium parvum*, and bacillus Calmette-Guerin (BCG) —and has been established as a useful therapy for malignant pleural effusion.[33] Also, it has been reported that oral administration of LcS effectively inhibited methylcholanthrene-induced carcinogenesis in mice.[34]

LcS has been reported to augment the antitumor effect of a combination chemotherapy with agents such as doxorubicin, mitomycin-C, cyclophosphamide, bleomycin, and 5-fluorouracil. (See Table 6.1) Significant synergism between LcS administered via various routes (intrapleural, intraperitoneal, intravenous, and subcutaneous), and these cytotoxic drugs have been observed in mice with various transplantable tumors, including mouse Meth A fibrosarcoma, L1210 leukemia, and Lewis lung carcinoma.[35]

6.5.2 Augmentation of Host Immune Cells by LcS

The initial response induced by administration of LcS in a host is an activation of neutrophils, macrophages, or natural killer (NK) cells. In particular, LcS has a strong potential to augment host NK activity as well as the activation of macrophages.[33,36] Even in mice inoculated with Meth A fibrosarcoma cells, administration of LcS induced a high level of NK activity in splenocytes. As shown in Table 6.3, lymphocytes in pleural exudate exhibited a strong NK activity from 3 d after administration onward, and still remained active at 7 d.[33] Also, it was reported that oral administration

TABLE 6.2
Antitumor Effect of LcS Administered via Various Routes

Route of administration	Dose (mg/kg) × dose number	Transplantable tumor	Inoculation site	Antitumor effect
Intrapleural (i.pl.)	4 × 5	Meth A	i.pl.	T/C 250
Intraperitoneal	5 × 5	Meth A	i.p.	T/C > 157 (1/10)
(I.p.)	10 × 3	L1210	i.p.	T/C 138
	2 × 5	C57AT1	i.p.	T/C 134
	2 × 5	Sarcoma 180	i.p.	T/C > 209 (1/9)
	10 × 5	Meth A	s.c.	I.R. 87.6
	10 × 5	MCA K-1	s.c.	I.R. 61.3
Intravenous (i.v.)	10 × 5	Meth A	s.c.	I.R. 88.2
	2 × 5	MCA K-1	s.c.	I.R. 60.4
	10 × 5	Sarcoma 180	s.c.	I.R. 75.9
	10 × 4	3LL	s.c.	T/C > 132 (2/7)
	10 × 5	B16	s.c.	T/C > 150 (1/10)
	10 × 5	B16-F10	i.v.	T/C 134
	10 × 10	AH 130 (Rats)	i.v.	T/C 181
	10 × 10	AH 66 (Rats)	i.v.	T/C 159
	10 × 10	AH 7974 (Rats)	i.v.	T/C 139
	10 × 10	AH 41C (Rats)	i.v.	T/C > 178 (2/6)
Subcutaneous	30 × 7	Meth A	s.c.	I.R. 72.9
(s.c.)				
Intratumoral (i.t.)	4 × 5	Meth A	s.c.	I.R. 88.2
	4 × 5	Meth A	s.c.	T/C > 152 (2/10)
	4 × 5	K234	s.c.	T/C > 162 (2/10)
	10 × 4	B16-BL6	s.c.	I.R. 81.9
	10 × 4	B16-BL6	s.c.	T/C 156
	10 × 5	B16-F10	s.c.	T/C 142
	4 × 4	Line-10 (Guinea pigs)	Intradermal	(5/6)

Note: T/C: Survival benefit (%) = mean survival time (days) of treated animals/mean survival time (days) of control animals × 100; I.R.: Inhibition rate (%) = (1 – mean tumor weight in treated animals/mean tumor weight in control animals) × 100. Animals used were BALB/c mice (Meth A fibrosarcoma, MCA K-1 sarcoma, K 234 sarcoma), DBA/2 mice (L1210 leukemia), C57BL/6 mice (C57AT1 virus-induced tumor, B16 [B16-F10 and B16-BL6] melanoma, Lewis lung carcinoma), ICR mice (Sarcoma 180), Donryu rats (AH 130, AH 66, AH 7974, and AH 41C ascitic hepatoma), and strain-2 guinea pigs (Line-10 hepatoma). Allogeneic tumors are underlined. Numbers in parentheses represent the percentage of animals surviving for 40 d showing complete tumor regression.

Source: Yokohura, T., et al. *Intestinal Flora and Carcinogenesis*, Mitsuoka, T., Ed., Japan Scientific Societies Press, Tokyo, 1981, pp. 125–137. With permission.

of LcS significantly enhanced splenic NK activity in a murine carcinogenesis model[37] (see Figure 6.7). Furthermore, oral administration of LcS significantly delayed the onset of carcinogenesis in mice treated with 3-methlycholanthrene and recovered the decreased T-cell responses such as concanavalin A-induced T cell proliferation and interleukin-2 production[38] (see Figure 6.8). Important antitumor mechanisms induced by LcS include enhancement of such nonspecific antitumor activity and induction of

TABLE 6.3
NK Activity of Pleural Exudate Cells Harvested after Intrapleural Administration of LcS

Days after administration of LcS[a]	Cytotoxicity (%) against YAC-1 lymphoma[b]	
	100:1[c]	50:1
Untreated control	1.8 ± 1.3	1.7 ± 1.8
1	2.6 ± 1.8	1.3 ± 0.6
3	52.4 ± 3.0	47.0 ± 7.7
5	47.0 ± 9.2	34.6 ± 5.0
7	57.0 ± 7.0	50.6 ± 6.9

[a] Pleural exudate cells were obtained after intrapleural administration of LcS (100 μg) to BALB/c mice.
[b] Cytotoxicity was determined by the ^{51}Cr-release method.
[c] Effector/target ratio.
Source: Matsuzaki, T., Yokokura, T., and Mutai., M., *Cancer Immunol. Immunother.*, 26, 209–214, 1988. With permission.

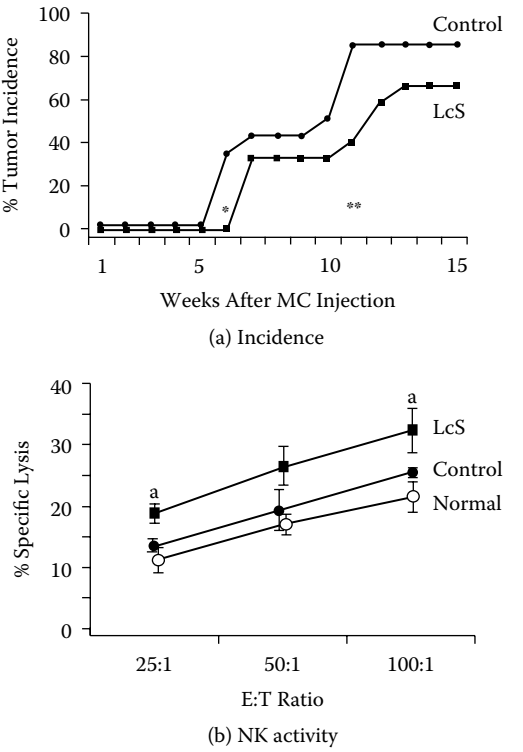

FIGURE 6.7 (a) Inhibition of 3-methylcholanthrene (MC)-induced carcinogenesis and (b) enhancement of natural killer activity by LcS. * $P < 0.05$ at 6 weeks, ** $P < 0.05$ up to 11 weeks in the cumulative incidence; E:T, effector cell to target cell; (a) $P < 0.05$ vs. control. (From Takagi, A., Matsuzaki, T., Sato, M., Nomoto, K., Morotomi, M., and Yokokura, T., *Carcinogenesis*, 22, 599–605, 2001. With permission.)

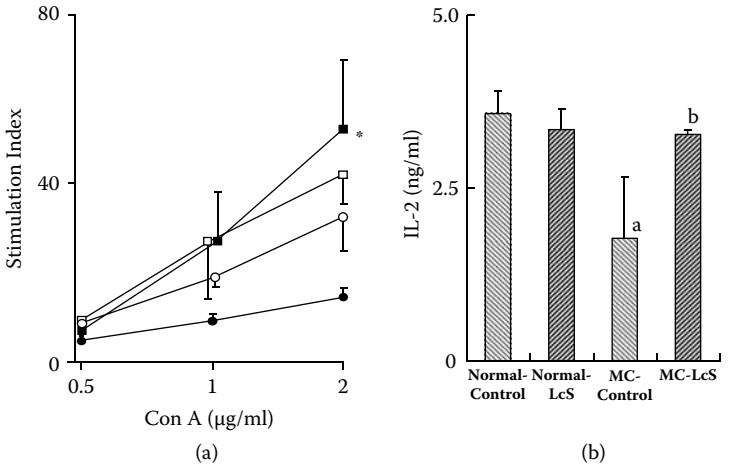

FIGURE 6.8 Improvement of methylchorantherene (MC)-induced suppression of proliferative responses of spleen cells to concanavalin (Con) A by LcS. The spleen cells obtained from the mice at week 16 after MC treatment were cultured with Con A, and the splenic cell proliferation (a) and the release of interleukin (IL)-2 (b) were measured. (a) $P < 0.01$ vs. normal control; □, non(MC)-treated; ■, MC-treated; ○, non(MC)-treated; ●, MC-treated. (From Takagi, A., Matsuzaki, T., Sato, M., Nomoto, K., Morotomi, M., and Yokokura, T., *Med. Microbiol. Immunol.*, 188, 11–116, 1999. With permission.)

cell-mediated immunity through subsequent activation of T cells. LcS was shown to be potent in inducing cell-mediated immunity.[30] In BALB/c mice, tumor-specific immunity was induced by intraperitoneal or subcutaneous inoculation of a mixture of LcS and tumor cells.[39] Induction of tumor-specific antitumor immunity was observed in some strain-2 guinea pigs that showed complete tumor regression after intratumoral administration of LcS.[40] Splenic and peritoneal T cells obtained from these animals exhibited cytostatic activity against tumor cells in a neutralization test. In C57BL/6 mice, priming with LcS augmented the antitumor and antimetastatic effects of LcS. This may have resulted from enhancement of tumor-specific effector cells by various cytokines produced by LcS-specific helper T cells.[41]

6.5.3 Protection Against Bacterial Infection by LcS

LcS has been reported to protect a host against infection by the pathogens *Listeria monocytogenes*, *Mycobacterium fortunum*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, murine cytomegalovirus, and herpes simplex virus. Prior intraperitoneal administration of LcS increased the resistance of mice to infection induced by intraperitoneal inoculation of a lethal amount of *P. aeruginosa* compared with untreated mice. The protective effect of LcS against *P. aeruginosa* infection was also noted in mice with severe neutropenia following whole-body X-ray irradiation, whereas no such effect was observed in mice treated with carrageenan, which causes a specific inactivation of macrophages.[42] These findings suggested that macrophages can act as effector cells in protection against infection.

Listeria monocytogenes, a Gram-positive bacillus forming short rods, is an intracellular parasite that is widely distributed in nature. *L. monocytogenes* has been isolated from numerous species of mammals, birds, fish, crustanceans, and insects. Because of its widespread occurrence, *L. monocytogenes* has many opportunities to enter food production and processing environments. Recently, one outbreak of gastroenteritis with fever but without progression to invasive disease was linked to the consumption of milk highly contaminated by *L. monocytogenes*, adding *Listeria* to the causes of food poisoning.[43] However, the pathogenesis of *L. monocytogenes* in human infection is unclear. The protective effect of LcS against *L. monocytogenes* infection was assessed in mice[44] (see Table 6.4). Administration of LcS caused sustained activation of macrophages. Mice inoculated intravenously with a lethal dose of *L. monocytogenes* were protected if they had received LcS for 3 weeks prior to the lethal dose. Some mice died even though they had received *Corynebacterium parvum* 1 week prior to the lethal dose. In this experimental system, the number of viable bacteria in the liver was markedly reduced in mice pretreated with LcS compared with untreated mice and mice pretreated with *C. parvum*. In mice treated with LcS, there was increased production of bactericidal lysosomal enzymes (e.g., β-glucuronidase) and bactericidal oxygen radicals (superoxide radical; $\bullet O_2^-$) by peritoneal macrophages and Kupffer cells in the liver, and this change was more sustained than that caused by *C. parvum*.

Shiga toxin-producing *Escherichia coli* O157:H7 (STEC) is characterized by the production of two kinds of Shiga toxins (Stxs), Stx1 and Stx2, which cause hemorrhagic colitis. Another characteristic of STEC is the ability to cause infections with only about 100 organisms. Ogawa et al.[45] reported a protective effect of LcS against STEC in an infant rabbit infection model. The LcS group was fed sterilized artificial milk supplemented with LcS (from 6×10^8 to 1.4×10^9 CFU/rabbit), and the control group was fed only sterilized artificial milk. Feeding was carried out

TABLE 6.4
Protective Effect Against *Listeria monocytogenes* Infection of Pretreatment with LcS or *Corynebacterium parvum* in Mice

	Survival rate (%)[a]	
Treatment[b]	LcS	*C. parvum*
–7	9/9 (100)[c]	9/9 (100)
–14	9/9 (100)	0/8 (0)
–21	9/9 (100)	0/9 (0)
–28	1/8 (12.5)	0/10 (0)

[a] After inoculation of 5×10^4 CFU of *L. monocytogenes* into the tail vein, survival was monitored for 14 d. The survival rate of untreated control mice was 0.0% (0/15 mice).

[b] Each mouse received an intravenous dose of 1 mg of LcS (10^9 cells) or *C. parvum* at 7, 14, 21, or 28 d before inoculation with *L. monocytogenes*.

[c] Number surviving/number treated (% survival).

Source: Nomoto, K., Miake, S., Hashimoto, S., Yokokura, T., Mutai, M., Yoshikai, Y., and Nomoto, K., *J. Clin. Lab. Immunol*., 17, 91–97, 1985.

twice a day from 1 to 10 d of age after birth. At 3 d of age, STEC (1×10^3 CFU/rabbit) was inoculated orally through a catheter tube. Daily oral administration of LcS dramatically decreased the severity of diarrhea, lowered the STEC colonization level in the gastrointestinal tract 100-fold, and reduced both Stx1 and Stx2 concentrations in the intestines on day 7 after infection (see Figure 6.9). Also, administration of LcS increased levels of IgA against Stx1, Stx2, and formalin-killed STEC cells in the colon approximately two-, four- and threefold, respectively, compared with those of the control by day 7 after infection. However, there was no significant difference in pH or the concentration of undissociated lactic acid in the gastrointestinal content between the groups. The results suggest that administration of LcS enhances the local immune response to STEC cells and Stxs, and leads to elimination of STEC and thus a decrease in the concentration of Stxs in the intestines. In conclusion, preventive administration of LcS may lead to enhanced resistance to acute STEC infection.

6.5.4 Protection Against Viral Infection by LcS

It has been reported that the oral administration of LcS suppresses the upper respiratory infection of influenza virus (IFV) in aged and infant mice.[46,47] The intake of a LcS diet (containing 0.05% heat-killed LcS) by aged mice for 4 months before the infection of IFV significantly augmented the NK activity of splenocytes and lung cells and the production of IFN-gamma and TNF-alpha in nasal lymphocytes, and significantly reduced the titer of IFV in the nasal washings after the infection of IFV (see Figure 6.10). Furthermore, the oral administration of LcS (about 10^8 CFU/mice) to infant mice for 3 weeks before the infection of IFV significantly augmented the pulmonary NK cell activity and the IL-12 production by mediastinal lymph node cells, and diminished the titer of IFV in the nasal washings. The survival rate of infant mice infected with IFV was significantly higher in the LcS group than in the control group (administered saline).

These results suggest that the oral administration of LcS has the potential to enhance not only systemic cellular immunity but also local cellular immunity in the respiratory tract, and to protect against influenza virus infection.

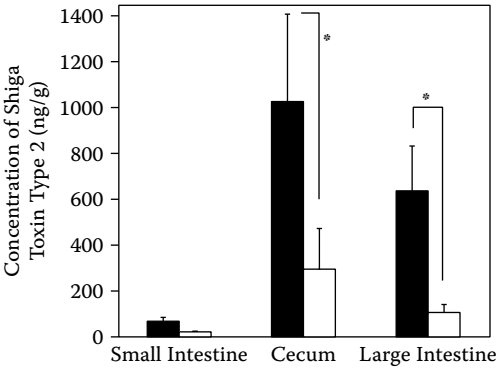

FIGURE 6.9 Inhibition of Shiga toxin type 2 production by LcS in a Shiga toxin-producing *Escherichia coli* (STEC) infection model.

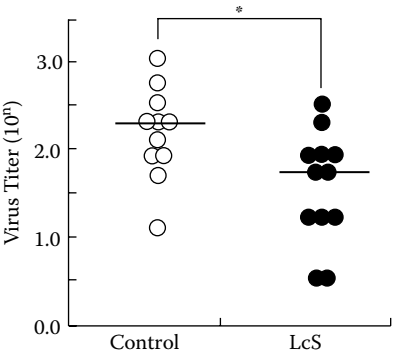

FIGURE 6.10 Effect of oral administration of LcS on viral titers in nasal washings.

6.5.5 Antihypertensive Effect of Oral Administration of LcS

The antihypertensive effect of LcS was assessed in spontaneously hypertensive rats (SHR), an animal model commonly used for antihypertensive drugs.[48] A single oral administration of LcS (100mg/kg) had no effect on blood pressure in normotensive Wistar-Kyoto rats (WKY), whereas the same dose significantly decreased the blood pressure of SHR. At this dose, LcS did not affect the heart rate of WKY or SHR. In addition, long-term administration of LcS at 100 or 1000 mg/kg/d suppressed the development of systolic blood pressure (SBP) in SHR (Figure 6.11).

A water-soluble fraction of LcS was obtained with high yield by autolysis at 55°C and pH 7.0 for 2 h followed by heating at 100°C for 10 min. This soluble fraction was lyophilized and termed LEx, and the antihypertensive effect of LEx was studied. This soluble fraction was composed of sugar (about 20% by the phenol-sulfuric acid method), protein (about 45% by Lowry's method), nucleic acid (about 10%), and ash (about 10%). A single oral administration of LEx at a dose of 10mg/kg lowered the SBP in SHR. Moreover, long-term oral administration of 1mg/kg suppressed the development of SBP.[49]

6.5.6 Inhibitory Effect on Immunoglobulin (Ig) E Production

Shida et al.[50] have reported that some probiotics exert a regulatory effect on the immune response, resulting in the inhibition of immunoglobulin E (IgE) production by murine spleen cells in vitro. Also, it has been demonstrated that LcS has a preventive effect on IgE production in BALB/c mice in vivo.[51] The mice were immunized by intraperitoneal injection of ovalbumin (OVA) and $Al(OH)_3$ on d 0 and 14. Seven days after final immunization, blood was collected from all mice and assayed for OVA-specific serum IgE, while spleen cells were prepared for assays of OVA-specific IgE production and OVA-induced cytokine production. The level of OVA-specific IgE in serum in each group is shown in Figure 6.12. In the group fed a diet containing 0.05% (w/w) LcS, inhibition of OVA-specific IgE production was evident at 3 weeks of feeding of the LcS-containing diet, compared with the response of the control group. In the mice fed LcS, the level of production of Th1-associated cytokines, such as IFN-γ

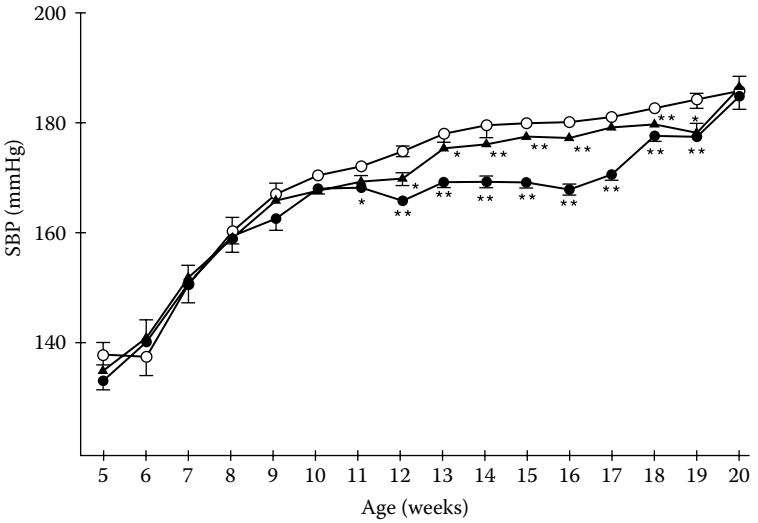

FIGURE 6.11 Effect of long-term oral administration of LcS on SBP in SHR. LcS was dispersed in distilled water (DW) at appropriate concentrations that resulted in oral doses of 100 (▲) and 1000 (●) mg/kg/day delivered in a volume of 0.5 mL/100 g body weight; (○) control rats. Administration was performed daily for 11 weeks from 5 to 16 weeks of age. ** $P < 0.01$ vs. control. (From Furushiro, M., Sawada, S., Hirai, K., Motoike, M., Sansawa, H., Kobayashi, S., Watanuki, M., and Yokokura, T., *Agric. Biol. Chem*., 54, 2193–2198, 1990. With permission.)

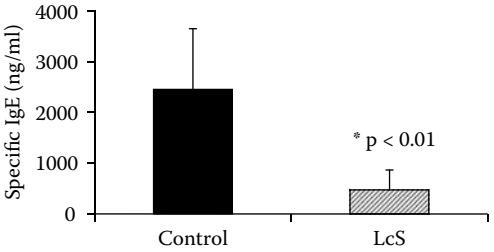

FIGURE 6.12 Effect of LcS on ovalbumin (OVA)-specific IgE production.

and IL-2, by spleen cells was higher than that in the control group (see Figure 6.13a). In contrast, the level of production of Th2-associated cytokines, such as IL-4, IL-5, and IL-6, by spleen cells from the mice fed LcS was lower than that of the control group (see Figure 6.13b). In addition, the level of production of IL-12, which augments IFN-γ production, by spleen cells from the mice fed LcS was also higher than that of the control group (see Figure 6.13c). Furthermore, it has been reported that the intraperitoneal injection of LcS (200 μg, three times/week) not only induces an increase in IL-12 in the serum and lowers serum levels of OVA-specific IgE and IgG1, but also diminishes systemic anaphylaxis in a food allergy model with ovalbumin-specific T cell receptor transgenic mice fed a diet containing OVA for 4 weeks.[52]

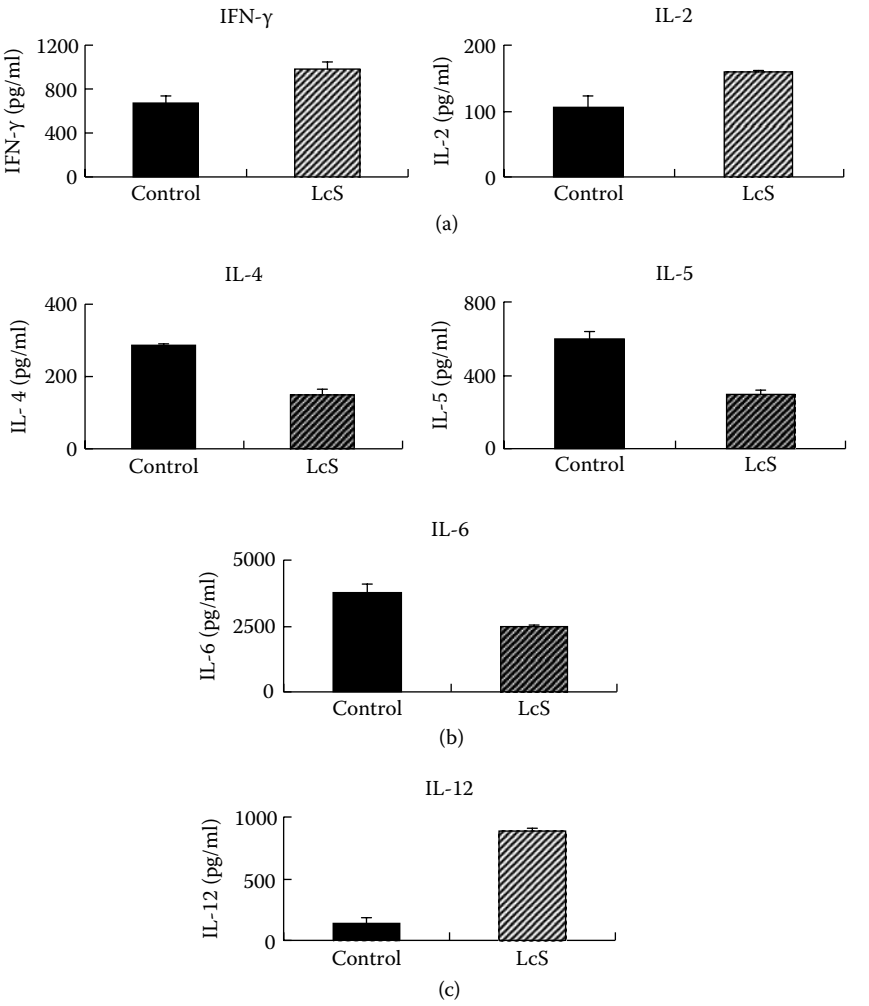

FIGURE 6.13 Production of interferon (IFN)-γ, interleukin (IL)-2 (a), IL-4, IL-5, IL-6 (b) and IL-12 (c) by spleen cells in vitro. BALC/c mice were intraperitoneally injected on day 0 and 14 with 20 μg of OVA and 2 mg of $AL(OH)_3$ in a total volume of 0.2 mL. The mice were then fed a diet containing (wt/wt) 0.05% LcS for 21 d. Spleen cells were collected on day 21 and were co-cultured with OVA (final concentration, 100 μg/mL) for 24 h. The amounts of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-12 in the supernatant were measured by ELISA. (From Matsuzaki, T., Yamazaki, R., Hashimoto, S., and Yokokura, T., *J. Dairy Sci.,* 81, 48–53, 1988. With permission.)

These results indicate that LcS induces a Th1 response rather than a Th2 response. Therefore, it may be concluded that functional augmentation of Th1 cells and inhibition of Th2 cells by LcS (or LcS components) are probably critical in the inhibition of IgE production and the suppression of allergy onset in mice.

6.5.7 Impact of LcS on Autoimmune Diseases

It has been reported that oral administration of LcS effectively inhibits the onset of diabetes in an insulin-dependent diabetes mellitus model, the nonobese diabetic (NOD) mouse.[53] In this study, 4-week old female NOD mice were fed a diet of either standard laboratory chow or the same chow containing 0.05% (by weight) LcS, and the onset of diabetes was thereafter recorded. The incidence of diabetes was significantly higher in the control group (10/12) than that the LcS-treated group (3/12) (P <0.01). Pathological analysis in the LcS-treated group revealed strong inhibition of the disappearance of insulin-secreting β cells in Langerhans islets caused by the autoimmune disease (see Figure 6.14).

It was also shown that oral administration of LcS significantly prolonged the life span of MRL/lpr mice that develop autoimmue disease resembling human systemic lupus erythematosus. In addition, LcS accelerated macrophage recruitment and prevented the expansion of $B220^+$ T cells without affecting the functions of T cells in MRL/lpr mice.[54]

Kato et al.[55] reported the effect of the oral administration of LcS on the development or type II collagen (CII)-induced arthritis (CIA) in DBA/1 mice. It was shown that the LcS-treatment significantly reduced the incidence and the development of CIA and the levels of antibody to CII in serum compared with the control group. The CII-specific IgG2a and IgG2b antibodies in serum were also downregulated in the LcS-treated group. Also, LcS inhibited the delayed-type hypersensitivity response to CII in DBA/1 mice immunized with CII and complete Freund's adjuvant, and suppressed the CII-specific secretion of interferon-γ from splenocytes. Taken together, this suggests that LcS has the potential to ameliorate or prevent autoimmune diseases through modification of the humoral and cellular immune response in the host.

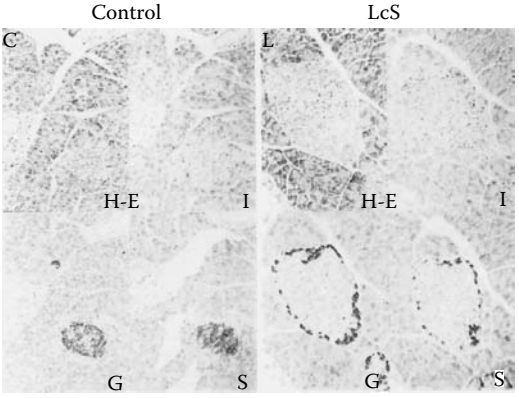

FIGURE 6.14 Photomicrographs of islets of Langerhans from NOD mice. Hematoxylin (H-E) staining and immunoperoxidase staining (I: insulin. G: glucagon, S: somatostatin) (X 120). (From Matsuzaki, T., Nagata, Y., Kado, S., Uchida, K., Kato, I., Hashimoto, S., and Yokokura, T., *APMIS*, 105, 643–649. With permission.)

6.5.8 Preventive Effect on Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD), ulcerative colitis, and Crohn's disease are chronic inflammatory diseases of the intestines that are increasing in prevalence in advanced countries. Although the pathogenesis of IBD is unclear, it is widely accepted that aggressive cell-mediated immune responses to certain commensal enteric bacteria play key roles in IBD.

Matsumoto et al.[56] demonstrated that the intake of a diet containing 0.05% of heat-killed LcS for 8 weeks significantly reduced the mortality rate, the colitis score, and improved colon length in a murine model of chronic colitis induced with dextran sodium sulphate. A significant improvement in histological scores was also observed on the intake of a diet with heat-killed LcS for 10 weeks in SAMP1/Yit mice, a murine model of Crohn's disease (see Figure 6.15). Furthermore, a heat-killed LcS significantly inhibited the production of IL-6 after LPS-stimulation in RAW264.7 cells (a cell line of macrophages), large intestinal lamina propria mononuclear cells isolated from mice with chronic colitis, and peripheral blood mononuclear cells derived from patients with ulcerative colitis in vitro.

It is suggested that the intake of probiotics like LcS has the potential to prevent IBD via the regulation of aggressive cell-mediated immune responses to certain commensal bacteria in the intestines.

These findings in several animal models reveal that the immuno-modulating activities of LcS may play important roles in the prevention or suppression of tumors, infections, allergies, autoimmune diseases, IBD, and others.

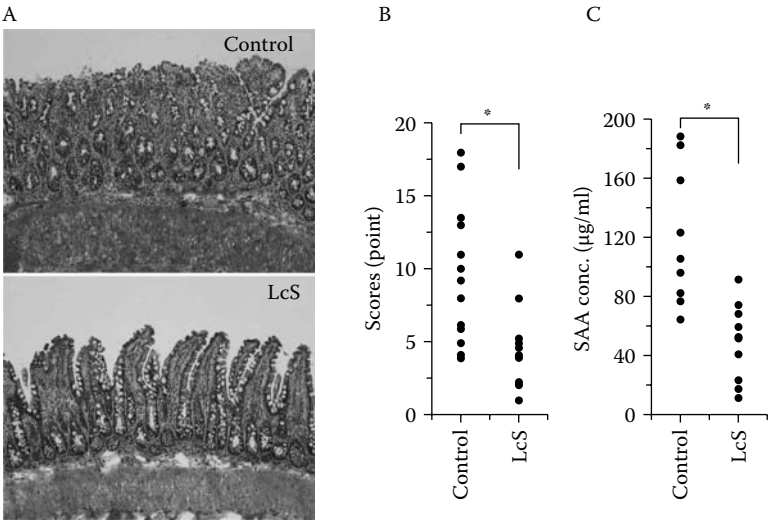

FIGURE 6.15 Improved effect of LcS on a murine model of Crohn's disease.

6.6 EFFECTS OF LcS IN HUMAN TRIALS

6.6.1 Survival of LcS in the Gastrointestinal Tract and Modification of Intestinal Flora

6.6.1.1 Survival of LcS in Infants

Aritaki and Ishikawa[57] studied the effect of a daily intake or 30 mL of a fermented milk containing LcS (1×10^7 to 3×10^8 cells/mL, given as three doses of 10 mL each) on the intestinal flora of 10 healthy formula-fed infants (aged 2 to 5 months). The intestinal flora were assessed by classifying 50 to 200 colonies grown on a smear of an appropriate dilution of feces by Gram staining and morphological observation. The organisms were classified as lactobacilli, bifidobacteria, Gram-positive bacilli, Gram-negative bacilli, or cocci, and the percentage of colonies in each group was calculated. The mean percentage of *Lactobacillus* species increased from 4.5% before drinking fermented milk to 20.9% after 3 d and to 53.8% after 7 d. After cessation of the drinking of the fermented milk, the percentage of *Lactobacillus* species remained high, being 52.5% and 32.2% at 3 and 6 d afterwards, respectively. The percentage of Gram-negative bacilli decreased from 43.1% before administration to 29.4 and 14.7% after 3 and 7 d of administration, respectively. The percentage of this bacterial group remained lower after cessation of administration. Yamagishi et al.[58] also studied the effect of a fermented milk containing LcS on intestinal flora in 12 infants (aged 4 to 19 months) hospitalized in a nursing home. Each infant was given 65 mL of the drink daily for 60 consecutive days, and lactobacilli were determined. The results were similar to those reported by Aritaki and Ishikawa.[57] After administration of fermented milk, the number of lactobacilli in the feces and the number of infants excreting lactobacilli both increased.

6.6.1.2 Survival of LcS in Children

Shirota et al.[59] studied the fecal recovery of LcS during the long-term intake of a fermented milk containing LcS and the effect of habitual intake of this fermented milk on the intestinal flora in 30 children aged 2 to 6 yr (Figure 6.16). The children were divided into two equal groups following randomization with respect to age, sex, and living environment. One group drank 50 mL of a fermented milk containing live LcS (1×10^8 to 2×10^8 cells/mL) together with 180 mL of milk every morning. The other group drank 50 mL of a fermented milk sterilized at 80°C for 30 min together with 180 mL of milk daily for the same period. The fecal recovery of LcS increased rapidly after the start of daily intake of fermented milk containing live LcS. The number of bacteria recovered per gram of feces was in the range 10^6 to 10^8 (mean: 2.0×10^8) after 1 week of administration and 6×10^7 to 1×10^9 (mean: 2.0×10^8) after 2 weeks. Constant fecal recovery of the bacteria continued thereafter. Following cessation of the drinking of the fermented milk containing live LcS, the fecal recovery of LcS did not decrease rapidly and remained at 10^6 to 10^7 cells/g in 4 of the 15 children after 2 weeks. After 3 weeks, however, no LcS cells were detected

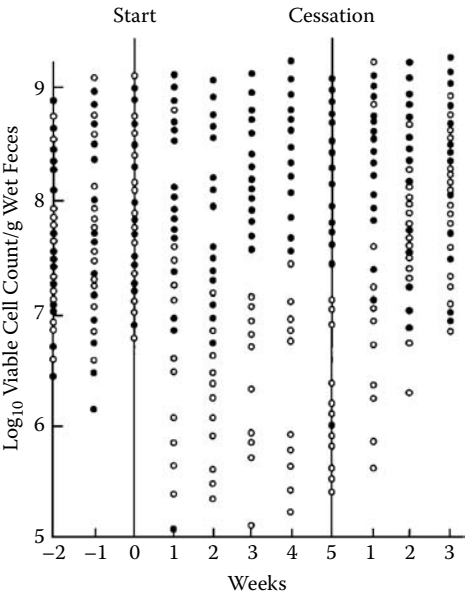

FIGURE 6.16 Changes of fecal LcS and lactobacilli in healthy infants who drank the fermented milk containing viable LcS and the sterilized fermented milk. ○, Yakult group; ●, sterilized Yakult group. (From Shiroa, M., Aso, K., and Iwabuchi, A., *Jpn. J. Bacteriol.*, 21, 274–283, 1966. With permission.)

in the feces of any subject. In both groups receiving either live or killed bacteria 10^6 to 10^9 (mean: 10^8) cells of Enterobacteriaceae were detected per gram of feces at baseline of detection limit. In the children drinking fermented milk containing LcS, the number of Enterobacteriaceae decreased to 10^4 to 10^7 (mean: 10^6) cells per gram after 2 to 3 weeks. However, the number of bacteria rapidly returned to the baseline values after the cessation of the intake of live LcS. In the control group given sterilized fermented milk, fecal Enterobacteriaceae remained unchanged. The number of *Enterococcus* in the feces decreased daily in a similar manner after the start of intake of fermented milk with LcS; values gradually returned to baseline values after cessation of LcS intake. Other bacterial species, such as anaerobes (e.g., bifidobacteria), staphylococci, and yeasts were not affected by fermented milk with live LcS. Hanada et al.[60] also assessed the effect of a daily intake of a fermented milk (30 mL/d, containing 1×10^7 to 2×10^8 cells/mL of LcS) for 10 weeks on fecal lactobacilli, Enterobacteriaceae, group A enterococci (e.g., *Enterococcus* sp.), and group B enterococci (e.g., *Staphylococcus* sp.) in a controlled study involving 20 children aged 4 to 7 yr. In children drinking the fermented milk with LcS, LcS was detected in the feces after 1 to 2 weeks. Fecal recovery transiently decreased around the fifth week and increased again from the sixth week. During the period that fermented milk containing LcS was ingested, Enterobacteriaceae, *Enterococcus* ssp., and *Staphylococcus* ssp. decreased.

6.6.1.3 Survival of LcS in Adults

Tanaka et al.[61] orally administered live LcS (1×10^{10} cells) together with 200 mL of milk to 5 healthy adults (aged 25 to 32 yr) daily for 5 weeks. They found that the feces of these subjects contained 10^7 to 10^8 cells of LcS/g. Lactobacilli increased in all five subjects, while bifidobacteria increased in four of them (see Figure 6.17). Tanaka and Ohwaki[62] also performed a double-blind controlled study to confirm this finding. Twenty adult men consumed either 80 mL of a fermented milk (containing 7.5×10^8 cells of LcS/mL) or nonfermented milk three times daily for 4 consecutive weeks. In the fermented milk group, *Lactobacillus* spp. significantly increased, and LcS was the predominant strain of this bacterial group in the fecal flora. Bifidobacteria also increased, while Enterobacteriaceae decreased.

Spanhaak et al.[63] performed a similar study to assess the effect of a fermented milk containing LcS in European individuals. There were two 2-week untreated control periods before and after the treatment period. Ten subjects drank 100 mL of a fermented milk (containing 10^9 cell of LcS/mL) three times daily for 4 weeks, while 10 control subjects drank unfermented control milk in the same manner. During the two control periods, all subjects received 100 mL of sterilized milk three times daily. None of the fermented milk recipients complained of any adverse effects during the

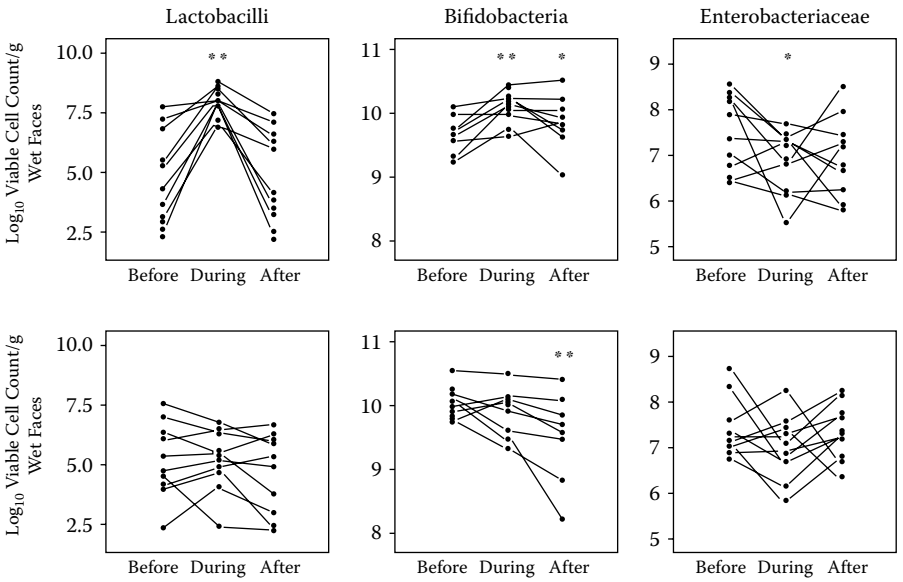

FIGURE 6.17 Changes of fecal microflora in healthy adults who drank the fermented milk containing viable LcS (top) and unfermented milk (bottom). (From Tanaka, R., Tohyama, K., Morotomi, M., Takayama, H., Nanno, M., Kuroshima, T., and Mutai, M., Effect on the fecal flora and urinary metabolites of the administration of *Lactobacillus casei* and *Bifidobacterium breve*, in *Proceeding of I RIKEN Symposium on Intestinal Flora: Intestinal Flora and Cancer*, Mitsuoka, T., Ed., Japan Scientific Societies Press, Tokyo, 1981, pp. 79–103. With permission.)

treatment period. More than 10^7 cells of LcS were recovered per gram of feces, and bifidobacteria also increased.

6.6.2 Modification of Bowel Movements by LcS

LAB have long been used and have an established efficacy for treatment of defecation disorders (e.g., diarrhea and constipation) and various associated abdominal symptoms.[64] Kawamura et al.[65] evaluated the efficacy of *Lactobacillus* powder (containing 1×10^{10} viable cells of LcS/g) in 30 patients with a long history of defecation disorders (diarrhea and constipation) or unidentified abdominal complaints, including abdominal pain and bloating. The patients were given 1 g of the *Lactobacillus* preparation orally once daily for 5 to 82 d (mean: 14.4 d). The number of bowel movements, the properties of the feces, and the severity of abdominal pain, bloating, anorexia, and nausea were measured during the treatment period. The patients were rated as markedly improved when all complaints improved, moderately improved when more than half of the complaints improved, and slightly improved when fewer than half of the complaints improved. A rating of unchanged was assigned when none of the complaints improved. Fifteen patients (50%) showed some response, with the rating being markedly improved for eight patients, moderately improved for three patients, and slightly improved for four patients. A response was obtained in 10 of the 15 patients without organic disease (e.g., those with irritable bowel syndrome or habitual constipation) and in 2 of the 5 patients with organic disease (chronic colitis, Crohn's disease, and diverticular disease of the colon). When stratified by symptoms, the highest response rate (45%; 9/20) was obtained for bloating, followed by abnormal bowel movements (9/27), abnormal fecal properties (10/29), abdominal pain (6/22), and anorexia (3/8).

Recently, a double-blind, placebo-controlled, randomized study was conducted over a 4-week period in 70 patients with symptoms of chronic constipation in Europe. All patients drank a fermented milk product containing more than 6.5×10^9 viable LcS/65 mL/bottle/d or a sensorially identical placebo. The consumption of LcS for 2 weeks resulted in a significant improvement in the self-reported severity of constipation and stool consistency. At the end of the 4 weeks, the occurrence of moderate and severe constipation, and the occurrence of hard stools were significantly less in the LcS group (see Table 6.5.)[66] One week after the intervention period, 89% of the LcS group reported a positive effect on constipation, significantly more than the placebo group (56%).

Another randomized, placebo-controlled, cross-over study was performed in 40 healthy volunteers who drunk fermented milk containing more than 4.0×10^{10} viable LcS/80 mL/bottle/d or a placebo drink for 2 weeks. The defecation frequency significantly increased in the LcS group compared with that before the intake. The number of *Bifidobacteria* and their percentage in the total number of fecal bacteria were significantly higher in the LcS group than placebo group. In 21 volunteers with a stronger tendency to have constipation, and with a defecation frequency below 4 times/week, the frequency of defecation was higher in the LcS group than the placebo group.[67]

These results indicate a beneficial effect of LcS on gastrointestinal symptoms of patients with defecation disorders (diarrhea and constipation) and individuals in a

TABLE 6.5
Improvement of Constipation by Drinking of a Fermented Milk Product Containing LcS in a Randomized Placebo Controlled Trial

		End of intervention	
	Baseline (n = 70)	**Treatment (n = 35)**	**Placebo (n = 35)**
Occurrence of moderate and severe constipation (%)	96	34	83***
Occurrence of hard stools (%)	94	29	82***

Note: Seventy patients with symptoms of chronic constipation drank either the fermented milk product containing LcS (treatment) or the placebo milk (placebo) daily for 4 weeks. Severity of constipation and stool consistency were assessed by a questionnaire before and at the end of the intervention. Data represent the median. *** $P < 0.001$ between treatment and placebo.

Source: Koebnick, C., Wagner, I., Leitzmann, P., Stern, U., and Zunft, H.J., *Can. J. Gastroenterol.,* 17, 655–659, 2003.

suboptimal state of health with a stronger tendency to have constipation. The administration of probiotic LcS is also recommended as an adjunctive therapy in cases of chronic constipation.

6.6.3 Suppression of Intestinal Putrefaction by LcS in Healthy Adults

The nature of the bacterial metabolites formed in the intestine depends on the characteristics of the bacterial flora. In the intestine, proteins are degraded by bacterial fermentation into potentially toxic metabolites such as amines, NH_3, phenol compounds, indole compounds, and thiols that cause intestinal putrefaction. Amines, phenols, and indoles have been implicated in the pathogenesis of bladder and bowel cancers. NH_3 has been shown to be a pathogen of hepatic dysfunction.

To evaluate the effect of LcS on intestinal putrefaction in humans, seven healthy adult men were given 10^{10} CFU of LcS daily together with 200 mL of milk for 5 weeks (Figure 6.18).[68] Throughout the experimental period, the urine excreted after rising (morning urine specimen) was collected and assayed to determine indoxyl sulfate, *p*-cresol, and phenol excretion. The mean urinary concentrations of indoxyl sulfate and *p*-cresol during the treatment period were significantly lower than those before the administration when only milk was fed to the subjects ($P < 0.05$).

Five subjects were enrolled for a second similar study and also received 10^{10} CFU of LcS daily for 5 weeks. In this study, only the urinary concentration of phenol decreased significantly ($P < 0.05$), whereas the urinary concentrations of indoxyl sulfate and *p*-cresol showed a tendency toward reduction.

The data obtained from 12 subjects in the two studies were combined and analyzed together. As shown in Figure 6.18, the mean urinary concentrations of indoxyl sulfate ($P < 0.01$), *p*-cresol ($P < 0.05$), and total phenol ($P < 0.05$) during treatment with LcS were significantly lower than the levels in the pretreatment period when only milk was fed to the subjects. Moreover, on withdrawal of LcS, the urinary

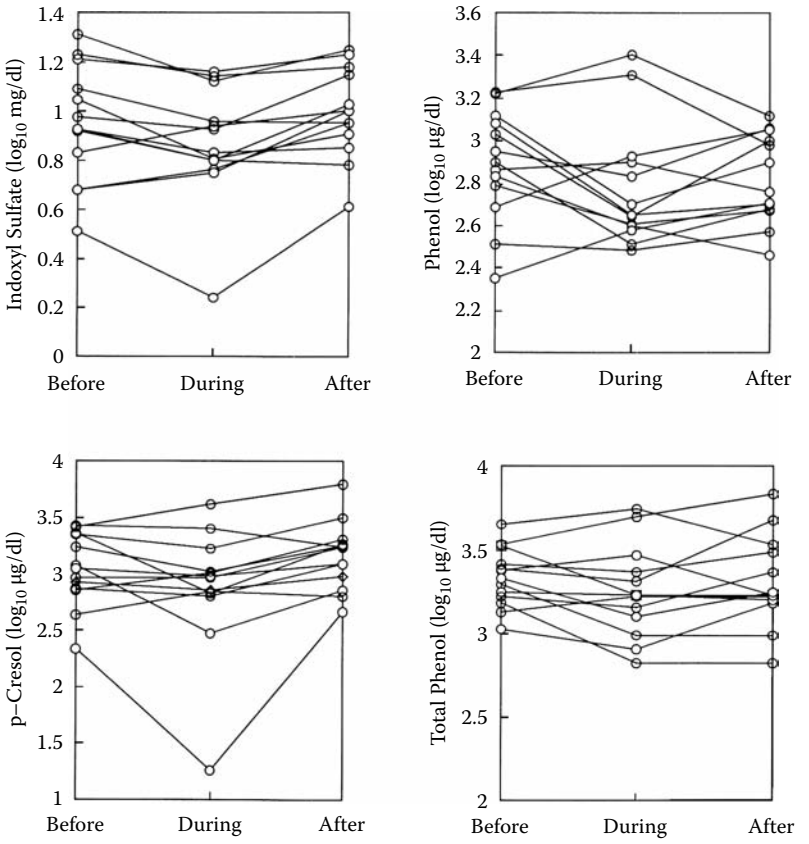

FIGURE 6.18 Suppression of urinary excretion of indoxyl sulfate and phenols in morning urine specimens from healthy adults receiving treatment with LcS. (From Tohyama, K., Kobayashi, S., Kan, T., Yazawa, K., Tershima, T., and Mutai, M., *Microbiol. Immunol.*, 25, 101–112, 1981. With permission.)

concentrations of indoxyl sulfate and *p*-cresol returned to their respective pretreatment levels ($P < 0.05$).

Recently, a randomized, placebo-controlled, cross-over study was performed with ten healthy volunteers consuming the stable isotope-labeled biomarkers lactose-[^{15}N]ureide and [$^{2}H_4$]tyrosine, at the start and end of a 2-week intake period, to assess the generation and accumulation of toxic fermentation metabolites (NH_3 and cresol). The intake of a fermented milk product containing more than 6.5×10^9 viable LcS/65 mL/bottle for 2 weeks twice daily significantly suppressed the urinary excretion of^{15} N, a biomaker of NH_3, and p-[$^{2}H_4$]cresol as compared with a placebo.[69]

These results suggest that LcS suppresses the generation and accumulation of potentially toxic fermentation metabolites by putrefactive bacteria in the human intestine. It is assumed that increased numbers of lactic acid producing bacteria, and production of lactic acid on the intake of LcS, suppress the growth of putrefactive bacteria and improve the intestinal flora.

6.6.4 Antitumor Effects in Humans

6.6.4.1 Antitumor Activity of Heat-Killed Cells of LcS

As the impact and the safety of LcS has been confirmed in several experimental tumor models, the clinical efficacy of subcutaneous administration of heat-killed cells of LcS (LC9018) combined with radiotherapy has been assessed. In a late Phase II study and a Phase III study involving patients with cervical cancer, LC9018 combined with radiotherapy caused significantly greater tumor regression and was of greater survival benefit than radiotherapy alone.[70]

Because intrapleural administration of LC9018 significantly suppressed pleural effusion and inhibited tumor growth in mice with experimental carcinomatous pleurisy, its clinical use for the treatment of carcinomatous pleurisy was assessed in 47 patients with malignant pleural effusion secondary to primary lung cancer. When combined with chemotherapy agents, LC9018 administered at doses of 0.5, 0.2, and 0.1 mg achieved a response rate of 90.0%, 83.3%, and 67.7%, respectively. Intrapleural administration of LC9018 was found to be effective as an antitumor agent for controlling malignant pleural effusion, while improving the quality of life (QOL) of cancer patients.[71,72]

6.6.4.2 Preventive Effect of LcS on the Recurrence of Bladder Cancer

Aso et al.[73,74] conducted two clinical studies to assess the preventive effect of a LcS preparation (BLP which contained viable LcS at 10^{10} cells/g, Yakult Honsya, Tokyo) on the recurrence of bladder cancer after transurethral resection. The first study was a randomized controlled study designed to determine the preventive effect of BLP on the recurrence of superficial bladder cancer after transurethral resection. Patients assigned to the BLP group received 1 g of the preparation three times daily for 1 yr, or until tumor recurrence. Following surgery, all patients underwent cystoscopy and cytological examination of the bladder every 3 months to detect cancer recurrence. The time to the first recurrence was compared between patients treated with BLP and those not treated with BLP (control group). The 50% recurrence-free interval was 352 days in the BLP group compared with 208 days in the control group. This represented a 2.4-fold higher risk of tumor recurrence in the control group than in the BLP group.[73]

Subsequently, a double-blind, placebo-controlled study was conducted to obtain more objective evidence of the efficacy of BLP against superficial bladder cancer (Figure 6.19). In this study, the 50% recurrence-free interval was 688 days in the BLP group compared with 543 days in the placebo group. Based on these results, Aso et al.[74] concluded that BLP significantly prolonged the time to recurrence of bladder cancer after transurethral resection. Furthermore, the conclusion was followed by a case-control study that showed a reduction in the risk of developing bladder cancer with the habitual intake of dairy products containing LcS in 180 cases and 445 population-based controls matched by gender and age.[75]

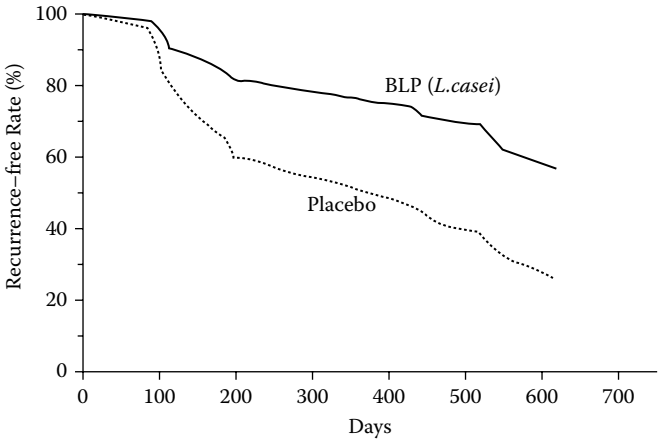

FIGURE 6.19 Corrected cumulative recurrence-free rates for patients treated with BLP containing LcS and placebo recipients in a double-blind placebo-controlled study. (From Aso, Y., Akaza, H., and the BLP Study Group, *Urol. Int.*, 49, 125–129, 1992. With permission.)

6.6.4.3 Preventive Effect of LcS on the Recurrence of Colorectal Cancer

Ishikawa et al.[76] conducted a randomized, controlled study to examine whether dietary fiber and a LcS preparation (BLP which contained living LcS at 10^{10} cells/g) prevented the occurrence of colorectal tumors. Subjects who had had at least two colorectal tumors removed but were presently free of tumors were randomly assigned to four groups, with 95, 96, 96, and 93 assigned to the wheat bran, LcS (3 g/d), both, and no treatment groups, respectively. In all subjects, the fat intake was restricted to 18 to 22% of total energy intake. The presence or absence of new colorectal tumor(s) was diagnosed by colonoscopy after 2 and 4 yr. The multivariate adjusted odds ratio (OR) for the occurrence of tumors was 1.31 (95% confidence interval [CI] 0.87 to 1.98) in the wheat bran group and 0.76 (95% CI 0.50 to 1.15) in the LcS group compared to the control group after 2 yr. For the occurrence of tumors with a grade of moderate or severe atypia, the adjusted OR was 0.80 (95% CI 0.52 to 1.22) after 2 yr in the group administered LcS and 0.65 (95% CI 0.43 to 0.98) after 4 yr, showing a significant decrease after 4 yr (Figure 6.20). These results suggest that LcS prevents atypic colorectal tumors.

6.6.4.4 Augmentation of Host Immune Parameters

Sawamura et al.[77] determined the natural killer (NK) activity and the response to phytohemagglutinin of lymphocytes obtained from the peripheral blood and regional lymph nodes before and after tumor resection in patients with Dukes A colon cancer who were treated with BLP. Flow cytometric analysis of lymphocyte subsets showed an increase of helper T cells and NK cells and a decrease of suppressor T cells in the peripheral blood of patients with BLP (Figure 6.21). Among the lymphocytes in the regional lymph nodes obtained from the treated patients, suppressor T cells were slightly decreased and suppressor/inducer T cells were decreased. Because treatment with BLP increased helper T cells and NK cells, and

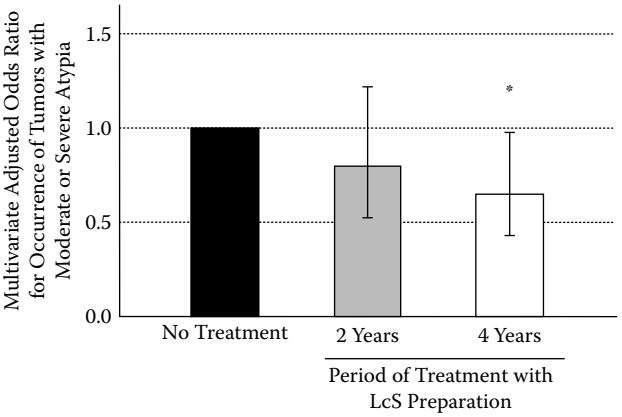

FIGURE 6.20 Prevention of atypia of colorectal tumors by treatment with LcS preparation in a randomized trial.

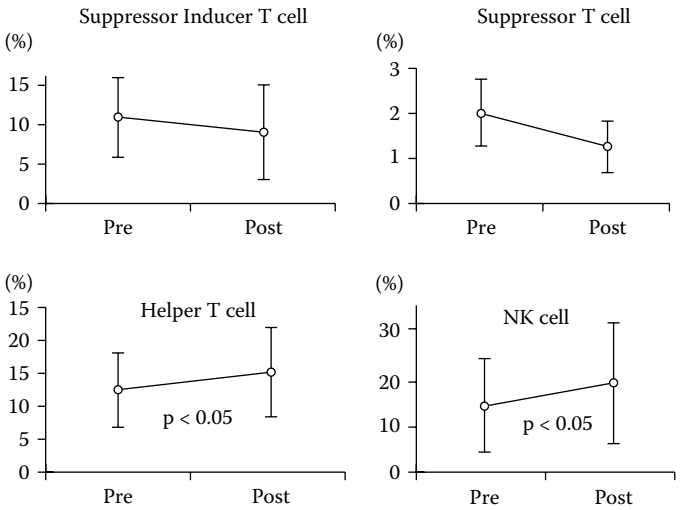

FIGURE 6.21 Effect of oral administration of BLP containing LcS on peripheral blood lymphocyte subsets in patients with colon cancer. From Aso, Y., Akaza, H., Tsukamoto, T., Imai, K., Naito, S., and the BLP Study Group, *Eur. Urol.,* 27, 104–109, 1995. With permission.)

also decreased suppressor T cells in the regional lymph nodes as well as in the peripheral blood, BLP seems to have a systemic immunopotentiating effect.

Recently, it was reported that the intake of a fermented milk product containing more than 4×10^{10} viable LcS/80 mL/bottle/day for 3 weeks augmented host NK cells in the peripheral blood of healthy volunteers (Figure 6.22)[78] and habitual smokers[79] in a pilot study and a double-blind, placebo-controlled, randomized study, respectively. The effects were particularly prominent in low-NK individuals. Moreover, the intake of the fermented milk containing living LcS twice a day for 4 weeks not only augmented host NK cells but also improved spasticity and urinary symptoms in patients

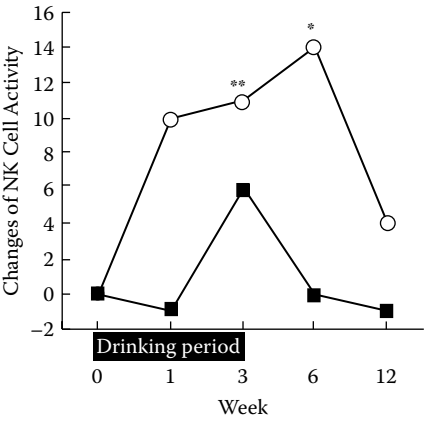

FIGURE 6.22 Changes of NK cell activity during the study period.

with human T-cell lymphotropic virus type-1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis in an uncontrolled preliminary trial.[80]

These findings suggest that it is possible to prevent disorders such as cancer, autoimmune diseases, virus-associated diseases, etc., by taking some kind of dairy product. It was demonstrated that medium and high level of NK activity of peripheral blood lymphocytes are associated with a reduced cancer risk, whereas low activity is associated with increased cancer risk, suggesting a role for the natural immunological host defense mechanisms against cancer.[81]

6.6.5 Clinical Application

6.6.5.1 Pediatric Patients

In the pediatric field, there are patients with abnormal intestines that can't absorb nutrients well and develop intestinal immunity. Most such patients are stressed by surgical operations, the administration of antibiotics, and restricted lactation after birth. They frequently develop severe diarrhea, enteritis, and/or sepsis, and have an unbalanced intestinal flora with low numbers of bifidobacteria and high numbers of harmful microorganisms (pseudomonad, MRSA [methicillin resistant *Staphylococcus aureu*s] and *Candida*).

Candy et al.[82] reported a case study in which 10 mL of a fermented milk product containing more than 6.5×10^9 viable LcS was administered to a patient with short bowels at 12 months of age. The administration of LcS resulted in abundant lactobacilli in stools after 3 d, a significantly increased urinary concentration of sodium ion, and a decrease in stool frequency from 12 to 4/d. After 2 yr, when the administration was continued, the subject consumed a variety of normal foods supplemented with an elemental diet. Although the sodium ion supplement was reduced, his development was normal.

Kanamori et al.[83–86] used LcS and galactooligosaccharides as a synbiotic therapy in pediatric patients. The patients were administered an LcS preparation (BLP, which contained more than 1.0×10^9 living LcS/g, 3 g/d), a *B. breve* preparation (BBG-01

which contained more than 1.0×10^9 living *B. breve* strain Yakult/g, Yakult Honsya, Tokyo; 3 g/d), and galactooligosaccharides (Oligomate HP; Yakult Honsya, 3 g/d).

The synbiotic therapy for more than 1 yr improved the intestinal bacterial flora and increased short-chain fatty acids in feces in 7 malnourished short bowel patients with refractory enterocolitis. All pediatric patients but one, exhibited an accelerated body weight gain.[86] Figure 6.23 shows a clinical case of a 3-yr, 4-month-old girl with short bowel syndrome who received the synbiotic therapy for about 2 yr. The synbiotic therapy not only accelerated body weight gain dramatically, but also suppressed episodes of high fever attack and metabolic acidosis, increased the number of bifidobacteria and lactobacilli, and decreased the number of harmful bacteria (*Candida* and *E. coli*) and the ratio of total facultative anaerobic bacteria to total bacteria in the intestinal bacterial flora.[83]

A critically ill 9-month-old girl with laryngotracho esophageal cleft (type IV) was treated by synbiotic therapy. Abundant amounts of synbiotic bacteria were detected in her feces, suggesting that these administered bacteria affected intestinal function in situ. Bowel movements resumed soon after the commencement of synbiotic therapy, and considerable amounts of short chain fatty acids were detected in the feces. Growth of the patient was satisfactory under this treatment.[84]

Synbiotic therapy was designed for a 3-month-old patient with fulminant MRSA enterocolitis who was successfully treated with vancomycin and synbiotics. Vancomycin eradicated MRSA colonizing the intestinal lumen first, and the administration of synbiotics effectively helped to reestablish the anaerobic bacteria as dominant in the intestinal bacterial flora and reinforced colonization resistance.[85]

These findings suggest that LcS has the effect of improving intestinal flora and functions, and increasing body weight in pediatric patients in the presence or absence of other probiotics (*B. breve* strain Yakult) and prebiotics (galactooligosaccharides). Synbiotics may be a potent modulator to treat several pediatric diseases.

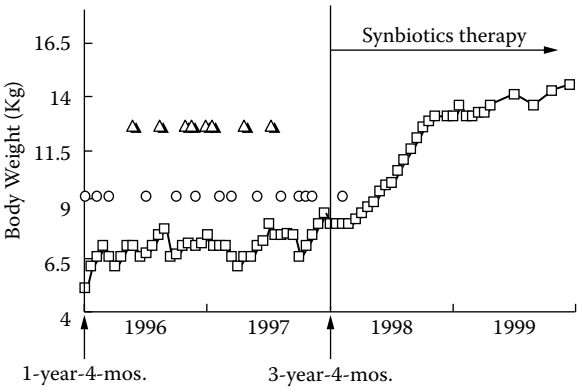

FIGURE 6.23 The clinical course of the patient as defined by changes in body weight gain (□) and episodes of repetitive high fever attacks (○) and severe metabolic acidosis (△) before and after the start of the synbiotic therapy.

6.6.5.2 Surgical Patients

Prevention of infectious complications after surgery for digestive organs is a major clinical task. However, the use of antibiotics must be restricted to suppress the generation of MRSA. To address these issues, synbiotics have recently been introduced. Kanazawa et al.[87] and Sugawara et al.[88] conducted two randomized controlled trials to assess the effect of synbiotics on surgical outcome in biliary cancer patients undergoing hepatectomy.

In the first trial, patients with biliary cancer were randomly allocated to two groups before hepatectomy. From postoperative day 1 to day 14, one group received postoperative enternal feeding that included synbiotics, which were the LcS and *B. breve* preparation (Yakult BL Seichoyaku, which contained 1.0×10^8 living LcS/g and 1.0×10^8 living *B. breve* strain Yakult/g, Yakult Honsya, Tokyo; 3 g/d) and galactooligosaccharides (Oligomate HP, Yakult Honsya, 12 g/d). The other group received enternal feeding only. In the synbiotic group (21 patients), the intestinal flora were dramatically improved with the increase of *Bifidobacterium* and *Lactobacillus* and the decrease of harmful bacteria (enterobacteriaceae, *Pseudomonas,* and *Candida*) (Figure 6.24), and the intestinal organic acid concentration recovered to the normal level after surgery, but did not change in the control group (23 patients). Moreover, the synbiotic therapy significantly reduced the incidence of infectious complications from 52 to 19%.[87]

In a second trial, patients with biliary cancer were randomly divided into two groups. All patients were administered with synbiotics according to the same protocol as in the first trial from postoperative day 1 to day 21. Group A did not receive synbiotics during the same preoperative period. From preoperative day 7 to day 21, group B received a LcS fermented milk product (containing viable LcS more than 4.0×10^{10}/80 mL/bottle/d), a *Bifidobacterium* fermented milk product (containing viable *B. breve* strain Yakult more than 1.0×10^{10}/100 mL/bottle/d), and galactooligosaccharides (5 g × 3/d). Preoperatively in group B (41 patients with pre- and postoperative

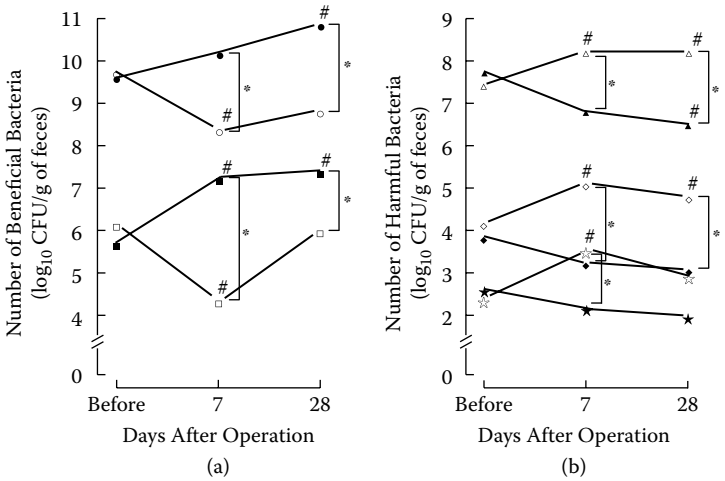

FIGURE 6.24 Improvement of disrupted fecal bacterial flora by synbiotics (LcS, *Bifidobacterium breve* and galactooligosaccharides) after hepatectomy.

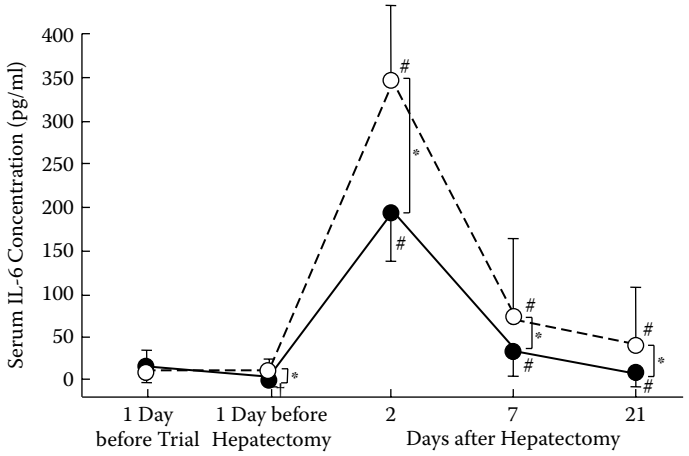

FIGURE 6.25 Changes of serum IL-6 before and after hepatectomy.

synbiotics), NK activity in the blood was augmented, whereas the serum interleukin-6 (IL-6) level (Figure 6.25) decreased significantly. The postoperative serum IL-6 level (Figure 6.25), C-reactive protein level, and number of leucocytes were significantly lower in group B than group A (40 patients with postoperative synbiotics). Finally, the incidence of infectious complications, postoperative hospital stay, and cumulative length of antibiotic therapy were significantly lower in group B than in group A (Table 6.6). All patients tolerated surgery in both trials (mortality 0%).[88]

These results indicate that the pre- and/or postoperative administration of synbiotics, LcS, *B. breve* strain Yakult, and galactooligosaccharides, can reduce postoperative infections and benefit surgical patients. The beneficial effects presumably involve the correction of abnormalities, such as intestinal imbalances, a lowered natural host defence, and augmented inflammation, which are caused by surgical and other stresses.

The clinical application of probiotics (LcS, *B. breve* strain Yakult) with or without prebiotics (galactooligosaccharides) supports the goal that the health-related quality of life in patients is enhanced by the improvement and/or augmentation of physiological functions, natural host defense, and bacterial flora in the intestines.

6.7 SAFETY OF LcS

LcS exhibited no acute toxicity, such as death, soft stools/diarrhea, weight loss or abnormal autopsy findings, in rats given a single oral administration at the highest dose (2 g/kg, equivalent to about 10^{12} live cells/kg), and no chronic toxicity, which was evaluated based on body weight, organ weight, general condition, food/water consumption, ophthalmologic findings, urinalysis, hematological analysis, biochemical analysis, and histopathological examination The rats were given repeated oral administration for 1 or 6 months at the maximum technically feasible dose (1 g live cells/kg, corresponded to about 10^{11} live cells/kg). LcS also showed no mutagenicity in the Ames test using *Salmonella typhimurium* and *E. coli* as test strains with a

TABLE 6.6
Postoperative Infectious Complications, Hospital Stay, and Mortality of Patients with Postoperative Synbiotics or Pre- and Postoperative Synbiotics in Biliary Cancer Surgery

	Group A (postoperative synbiotics, *n* = 40)	Group B (pre- and postoperative synbiotics, *n* = 41)	*p*
Patients with any infectious complications (*n*)	12 (30.0%)	5 (12.1%)	0.0491
Bacteremia	5 (12.5%)	1 (2.4%)	
Intra-abdominal abscess	7 (17.5%)	4 (9.7%)	
Wound infection	6 (15.0%)	2 (4.8%)	
Pneumonia	3 (7.5%)	1 (2.4%)	
Postoperative hospital stay (days)	44.0 ± 13.8 (median, 38)	34.9 ± 14.9 (median, 30)	0.0447
Cumulative length of antibiotic therapy (days)	44.0 ± 13.8 (median, 38)	44.0 ± 13.8 (median, 38)	0.0355
Mortality	0	0	

Note: LcS, *B. breve* strain Yakult and galactooligosaccharides were orally administered as synbiotics.
Source: Sugawara, G., Nagino, M., Arai, T., Nishio, H., Ebata, T., Takagi, K., Ueyama, J., Suzuki, T., Takagi, K., Asahara, T., Nomoto, K., Tanaka, R and Nimura, Y., *Ann. Surg*. 244, 706–714, 2006.

crude extract obtained by ultrasonic disruption of 6.3×10^9 live cells at the highest dose and in the micronucleus test using peripheral blood of mice administered as a single oral dose of 1.5×10^{12} live cells/kg.[11]

Most strains of LAB are considered commensal microorganisms with no pathogenic potential, because of the long-term, widespread use of LAB in fermented foods and dairy products. Therefore, members of *Lactobacillus,* particularly those inhabiting the human intestines, are generally recognized as safe. However, in rare cases, *Lactobacillus* bacteremia, caused by a few strains of lactobacilli, can develop.[89] Infective endocarditis (IE) is the most common infection associated with lactobacilli.

Asahara et al.[90] evaluated several *Lactobacillus* strains for pathogenicity in a rabbit infective endocarditis model involving an injection through a catheter into the left ventricle of the heart. Two bacteremia-associated strains of lactobacilli and the *Lb. rhamnosus* type strain colonized with moderate infectivity and developed the vegetation but the probiotic LcS did not (Table 6.7).

In a pilot study using 28 critically ill children admitted to a pediatric intensive care unit, the safety of LcS used as a probiotic was assessed by bacteriologic surveillance in surface swabs and endotracheal aspirates (colonization) as well as blood, urine, and sterile body fluid cultures (invasive infection/bacteremia). LcS was cultured from the feces of five of the six study subjects whose bowels were opened during their stay in the care unit. There was no evidence of either colonization or bacteremia with LcS in bacteriologic cultures obtained from the study subjects. The preparation was well tolerated with no apparent side effects.[91]

TABLE 6.7
Induction of Infective Endocarditis by Several *Lactobacillus* Strains in a Rabbit Model

Test strain	Inoculum (CFU/rabbit)	Vegetation		
		Wt (mg)[a]	Incidence of infection[b]	Log_{10} CFU/g of vegetation[ac]
Lb. casei strain Shirota	1.3×10^9	15 ± 9	0/5	< 1.0
	6.7×10^7	11 ± 8	0/5	< 1.0
Lb. casei PHLS A357/84[d]	4.1×10^8	76 ± 37	5/5	7.7 ± 1.0
Lb. rhamnosus PHLS A103/70[d]	6.0×10^8	126 ± 53	5/5	8.6 ± 1.2
Lb. rhamnosus ATCC 7469[T]	3.0×10^9	No tested	6/6	8.3 ± 0.5
	6.0×10^7	No tested	4/5	8.7 ± 1.5
Lb. acidophilus ATCC 4356[T]	1.5×10^9	9 ± 7	0/4	< 1.0
Lb. gasseri DSM 20243[T]	2.4×10^9	18 ± 13	0/3	< 1.0

Note: Three to six rabbits per group were injected with the test strains through a catheter to the heart and were killed for bacteriological and other examinations on the 14th day after the injection.

[a] Values are the mean ± standard deviation.

[b] Number of infected rabbits/total number of rabbits.

[c] CFU of the test strain in vegetation homogenates was counted on agar plates after appropriate dilutions, plating, and incubation.

[d] Isolates from clinical endocarditis.

Source: Asahara, T., Takahashi, M., Nomoto, K., Takayama, H., Onoue, M., Morotomi, M., Tanaka, R., Yokokura, T., and Yamashita, N., *Clin. Diagn. Lab. Immunol.,* 10, 169–173, 2003.

There have been no side effects from the drinking of fermented milk containing viable LcS for over 70 yr, or in trials with healthy volunteers and infants, children, adults, and the elderly using fermented milk or LcS preparations with or without *B. breve* strain Yakult and galactooligosaccharides. These findings suggest that LcS is very safe in not only healthy subjects, but also patients with a variety of diseases and conditions.

6.8 CONCLUSIONS

Various biological activities of LcS were discussed based on data obtained from animal models and human trials (Table 6.8). Also, many data proved the safety of LcS in healthy subjects and patients suffering from various diseases. Lactobacilli including LcS have traditionally been used for food processing and have been ingested orally as live cells in yogurt and other fermented foods. Therefore, the modulating effect on intestinal function of LAB and other metabolites has been thought to be their primary benefit for human health. In recent years, many researchers and clinicians have recognized the advantage of probiotics. Probiotics with or without prebiotics have been applied to not only the maintenance of human health but also support in therapy for patients. With recent technological advances, this field will show even more progress in the future.

TABLE 6.8
Positive Effect of LcS in Several Models

Disease/condition	Treatment/dose	Effect of LcS	Ref.
		Animal	
Bowel movement	p.o. LcS 109/kg	Normalization	29, 30
Tumors	i.p., i.v./100–250 μg	Inhibition	31–33
Immune modulation	i.p., i.v., i.pl., s.c., p.o./100–250 μg	Regulation (augmentation)	34, 35, 37–39
Metastases	i.v., i.l./100–250 μg	Inhibition	36, 40, 41
Viral infection	p.o./0.05%-containing diet (0.05% LcS)	Inhibition	42, 43
Bacterial infection	i.p., s.c./100–250 μg, p.o./ LcS 108-109	Inhibition	44, 46, 47
Immunoglobulin E	p.o./0.05% LcS	Inhibition of IgE and modulation of cytokine production	51
Anaphylaxis	i.p./200 μg	Inhibition of anaphylaxis and modulation of cytokine production	52
Hypertension	p.o./10 mg/kg	Inhibition of increased blood pressure	49
Diabetes	p.o./0.05% LcS	Inhibition of onset	53
Autoimmune	p.o./0.05% LcS	Inhibition of onset	54
Arthritis	p.o./100–200 μg	Inhibition of onset	55
Inflammatory bowel diseases	p.o./0.05% LcS	Inhibition of onset	56
		Human	
Normalization of intestinal flora	p.o./LcS 107-1010, p.o./ LcS 109 (synbiotics)	Normalization	57–62, 85
Constipation	p.o./LcS 109-1010	Decreases constipation	65–67
Intestinal Putrefaction	p.o./LcS 1010	Inhibition	68–69
Uterine/lung cancer	s.c., i.d., i.pl.	Prolongation of survival	70–72
Bladder cancer	p.o./LcS 1010	Prevention of recurrence and risk reduction	73–75
Colon cancer	p.o./LcS 1010	Prevention of recurrence	76
Immune modulation	p.o./LcS 1010	Augmentation of NK activity	77–79
HTLV-1-associated myelopathy	p.o./LcS 1010	Improvement of spasticity and urinary symptoms	80
Short bowel disease	p.o./LcS 109, p.o. /LcS 109 (synbiotics)	Improvement of intestinal function	82, 83, 86
Laryngotracho-esophageal cleft	p.o./LcS 109 (synbiotics)	Improvement of intestinal function	84
Bacterial infection	p.o./LcS 108-1010 (synbiotics)	Inhibition	87, 88

Note: i.v., intraveneous injection; i.p., intraperitoneal injection; i.l., intralesional injection; i.pl., intrapleural injection; s.c., subcutaneous injection; i.d., intradermal injection; p.o., per oral administration; synbiotics, administration with LcS, *B. breve* strain Yakult and galactooligosaccharides.

6.9 ACKNOWLEDGMENTS

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7 Biologically Active Peptides Released in Fermented Milk

Role and Functions

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CONTENTS

7.1 INTRODUCTION

Recent years have seen a growing interest in the health enhancement effects of lactic acid bacteria (LAB).[1] More evidence is accumulating that probiotics—foods that contain live bacteria—might constitute a valuable therapeutic and preventive tool against a number of diseases in humans and in animals. Probiotics confer on their host multiple beneficial effects including prevention and treatment of diarrhea, induction of protective immunity against pathogens and tumors, prevention of allergies, controlling inflammatory diseases, modulating gastrointestinal functions, and alleviation of lactose intolerance and hypertension.[2] Numerous health and functional attributes of fermented dairy products are ascribed to the microorganisms that induce physical and chemical modifications of milk components. The mechanism responsible for health benefits of LAB is multifactorial, and probably relates to the complex interaction between milk components, LAB and their constituents, and the intestinal mucosa.

Recent studies have reported the particular role of the metabolic products derived from milk fermentation by probiotics. An important metabolic activity that occurs during milk fermentation is proteolysis. Proteolysis ensures LAB growth in the medium, and consequently, might influence the potential release of physiologically active peptides. These peptides are encrypted in the milk protein sequences in a latent state, and might be released during food processing or after degradation by digestive enzymes. They have been shown to possess opiate, antithrombotic, antihypertensive, immunomodulating, antibacterial, antigastric, and mineral carrier properties. Hence, milk-related bioactive peptides, and other derived metabolites from fermentation, might play an important role in health enhancement and reducing the incidence of many diseases. Physiologically active peptides might particularly contribute to the phenomenon of probiotics due to their hormone-like activities. Many of these sequences can be found in the milk of a large number of mammalian species. Some multifunctional bioactive sequences present in overlapping sequence, in the β-caseins for example, are endowed with multiple physiological activities (morphinomimetic and immunomodulating). Bioactive sequences are also found in plant and animal proteins as immunomodulating peptides in soybean[3,4] and rice albumin.[5]

Several peptides derived from milk proteins that have effects on behavioral, neurological, physiological, and vasoregulatory responses have been identified. Table 7.1 lists the major peptides identified in cows' milk and their physiological activities.[6,7]

This chapter will review the occurrence of biologically active peptides in fermented milk and their functional and potential physiological activities, specifically opioid, antihypertensive, and immunomodulating activities.

7.2 RELEASE OF BIOLOGICALLY ACTIVE PEPTIDES PRODUCED DURING FERMENTATION

The amino acids present in milk have limited nutritional support for LAB growth; therefore, LAB rely on a complex proteolytic system to ensure optimal growth in

TABLE 7.1
Bioactive Peptides Derived from Milk Proteins

Name	Protein precursor	Physiological activity
Casomorphins	α-, β-Casein	Opioid agonist
α-Lactorphin	α-Lactalbumin	Opioid agonist
β-Lactorphin	β-Lactoglobulin	Opioid agonist
Lactoferroxins	Lactoferrin	Opioid antagonist
Casoxins	κ-Casein	Opioid antagonist
Casokinins	α-, β-Casein	ACE-inhibitory
Lactokinins	β-Lactalbumin, β-Lactoglobulin, serum albumin	ACE-inhibitory
Antihypertensive peptides	β-Casein	Antihypertensive
Immunopeptides	α-, β-Casein	Immunomodulatory
Lactoferricin	Lactoferrin	Antimicrobial
Casocidin	α_{S2}-Casein	Antimicrobial
Isracidin	α_{S1}-Casein	Antimicrobial
Casoplatelins	κ-Casein	Antithrombotic
Phosphopeptides	α-, β-Casein	Mineral binding

Source: Adapted from Meisel, H. and Bockelmann, W., *Antonie Van Leewenhoek*, 76, 207–215, 1999; Clare, D.A. and Swaisgood, H.E., *J. Dairy Sci.,* 83, 1187–1195, 2000. With permission.

milk. The proteolytic system is composed of proteinases, peptidases, and transport systems. These LAB cells possess:

1. Proteinases located in the microbial cell envelop that permit the degradation of caseins into oligopeptides
2. Peptide transport systems that allow the internalization of the released oligopeptides
3. Intracellular peptidases that hydrolyze the oligopeptides into peptides or into amino acids to be used by the cell[8–11]

The first step in milk protein breakdown involves proteinases that are responsible for the release of peptides from caseins. Then, the peptides released are hydrolyzed by peptidases to release amino acids and small peptides that are taken up in the cell by transport systems.

7.2.1 Proteinases

Most of the proteinases are located in the cell envelop. Lactococcal proteinases, that have been extensively studied (for a review, see Law and Haandrikman[12]), are very large proteins with a molecular weight of 140,000 kDa and pH optima of 5.5 to 6.5. They are classified as serine proteinase inhibitors, and have a conserved active site triad consisting of aspartic acid, histidine, and serine. Proteinases can be classified

as PI, when β-casein is the principal protein degraded by the enzyme, or PIII, when a different specificity toward the β-casein is shown in addition to the αs_1-casein hydrolysis.[13] When purified, these enzymes can strongly degrade caseins, and release a large number of oligopeptides. Approximately 20% of the oligopeptides released are small enough to be taken up by the transport system of *Lactoccus lactis*.[14]

Proteinase genes were found to be encoded on a plasmid. Molecular cloning of proteinase genes in *Lactococcus* and its transfer into a Prt^- *L. lactis* ssp. *lactis* strain resulted in its conversion to Prt^+ phenotype.[15] Proteinase genes prtP and prtM encoded respectively a 200 kDa protein and a 32 kDa lipoprotein. The latter is implicated in the conversion of the inactive proteinase to its active form during and after translocation across the membrane.[16,17] The prtP gene codes for a preproteinase with a signal peptide at the N-terminus, and a membrane anchoring sequence at the C-terminus. The C-terminus of the protein consists of membrane spanning domains with a hydrophobic α-helix. Many basic amino acids constitute the C-terminal region of the helix, and it is believed that their role is related to the interaction with charged phosphate groups of the phospholipid bilayer acting then as a stop-transfer signal.[12,18] *Lactobacillus helveticus* and *Lb. delbrueckii* ssp. *bulgaricus* were shown to possess stronger enzymatic activity than lactoccocci. The activity of a cell wall from *Lb. helveticus* L89 was studied by Martín-Hernández et al.[19] Foucaud and Juillard[20] demonstrated that a high peptide content is found after a 24 h fermentation of milk. At the end of the growth period, proteinase activity still occurs at low pH, but peptides cease to be taken up by the cell because the transport system does not operate at low pH.

Proteinase activity, which differs markedly among species of LAB, may also have a major impact on the peptide pattern. Each probiotic enzyme possesses a unique enzymogram that differs from one species to another, as well as from one strain to another within the same species, contributing to the release of a different pool of potentially bioactive peptides. Thus, proteinases, by their specificities and activities, play a primary role in the generation of the peptide pool in fermented milk and potentially the release of bioactive peptides.

7.2.2 Peptidases

Lactic acid bacteria possess a large spectrum of proteolytic enzymes including endopeptidases, aminopeptidases, tripeptidases, and dipeptidases. The biochemical characterization of these peptidases reflects their intracellular location. An endopeptidase PepO was located by Tan et al.[21] The enzyme is an intracellular metalloproteinase, capable of hydrolyzing oligopeptides such as bradikinin. A specific endopeptidase pepX degrades oligopeptides by releasing dipeptides containing proline.[22] Aminopeptidases act on oligopeptides released by a proteinase by cleaving a single N-terminal amino acid residue. Aminopeptidases designed PepN and PepC were found to possess a broad specificity toward peptide bonds. Others, such as the PepA that releases the amino-terminal glutamate residue, are less specific.

Dipeptides and tripeptides can also be cleaved by specific peptidases. Some of these enzymes have a broad specificity, cleaving all dipeptides and tripeptides except those containing proline. The presence of proline-specific peptidases is necessary for

optimal growth of LAB because of their ability to degrade proline-rich oligopeptides from caseins. Proline amminopeptidases PIP and PepI specific for dipeptides and tripeptides with N-terminal proline residue were isolated from two strains of LAB.[23,24]

7.2.3 Peptide Transport

The oligopeptides and the small peptides are taken up by the cell by a dependent oligopeptide transport system (Opp), the dipeptide and tripeptide transport system (DtpT), and DtpP. The Opp system comprises two ATP-binding proteins—OppD and OppF; two integral membrane proteins—OppB and OppC; and a substrate binding protein—OppA.[25] Only a limited number of peptides resulting from the proteinase action are transported by the ologopeptide transport system. Foucaud and Juillard[20] noted that < 25% of casein-derived peptides were used to sustain LAB growth. Most of these oligopeptides do not promote growth of LAB because they are not transported into the cell. In addition, the uptake of small peptides via Opp is limited by the size of the peptides. The transport system of LAB enables them to internalize oligopeptides up to 18 amino acids in length, although only peptides smaller than 11 amino acid residues were found to be transported.[10] Thus, the nontransported peptides left in the medium constitute an important pool for potential biologically active peptides.

7.2.4 Biopeptides from Fermented Milk and Cheese

Cell wall-bound proteinases of LAB, as well as enzymes from the endogenous microflora of milk enzymes, including digestive enzymes, have the capacity to release bioactive peptides from within the sequences of milk proteins. However, microbial proteolysis is highly specific, leading to the release of very potent bioactive peptides. Microbial proteolysis has also an impact on the hydrolytic pattern of milk protein before the action of digestive enzymes. It was shown from high pressure liquid chromatography (HPLC) analysis of the gastrointestinal digesta of human subjects, that the release of peptides is greater after ingestion of yogurt than with nonfermented milk.[26] The peptidic profile of milk proteins is significantly different after microbial fermentation (Figure 7.1) suggesting that microbial proteolysis can be a potential source of bioactive peptides.[27] The degradation of milk proteins produced by proteolysis with *Lb. helveticus* was confirmed by size-exclusion HPLC. The fermentation of milk by this LAB increased the amount of smaller weight protein-derived compounds from the degradation of milk proteins. This is shown by the appearance of peaks caused by the proteolytic system of the bacteria after milk fermentation (Figure 7.2) that were not present before fermentation (Figure 7.1a). In addition to the release of biologically active peptides after fermentation, microbial proteolysis may also expose the inner protein bonds, favoring the action of digestive enzymes and the release of potentially active peptides. α_{s1}-Casein and β-casein are major proteins found in large quantities in milk; they are very susceptible to proteolysis. With 199 and 209 amino acid residues in their sequences, respectively, these proteins have the capacities to release more than 20,000 different peptides.[28]

It has been shown that milk fermented by *Lb. helveticus* R389, a bacterium that has strong protease and peptidase activities compared to other lactic acid bacteria,[28]

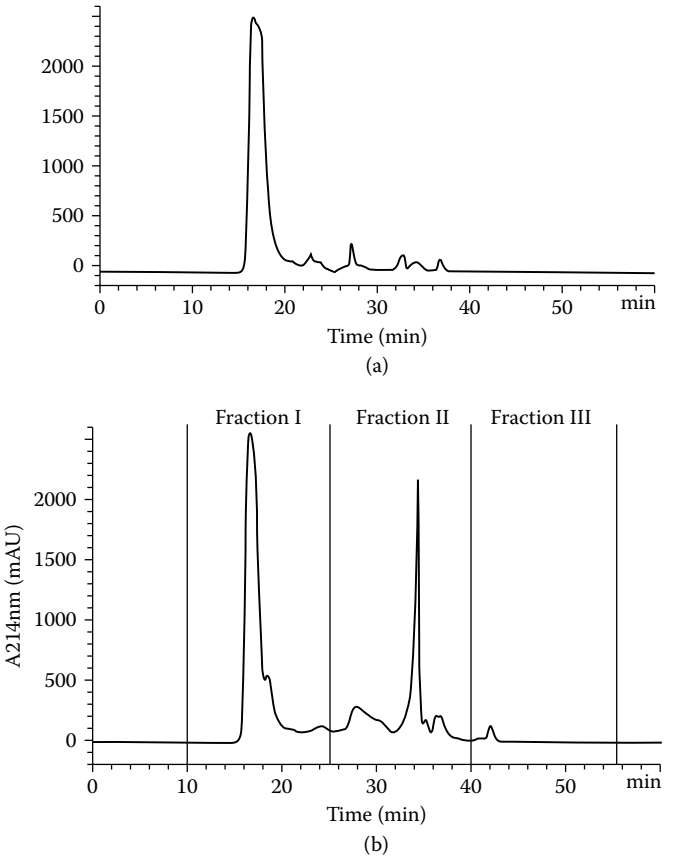

FIGURE 7.1 Size-exclusion HPLC profile of unfermented milk (a) and milk fermented with *Lactobacillus helveticus* R389 for 24 hours (b). Samples were passed on a LKB TSK-G2000SW gel filtration column (600 × 7.5 mm, TosoHaas, United States). The flow rate was 0.7mL/min and the eluted proteins were monitored at 214, 220, 224, and 280 nm using a HP1100 Diode Array Detector. Fractions were collected with a Gilson FC104 Fraction Collector (Gilson, United States), then pooled and concentrated using a Automatic Environnemental SpeedVac® System (AES1010, Savant, United States) and stored at 4°C until their use during in vivo studies. (From Matar, C., Nadathur, S.S., Bakalinsky, A.T., and Goulet, J., *J. Dairy Sci.,* 80, 1965–1970, 1997. With permission.)

is capable of exerting an antimutagenic effect, while milk fermented by its protease-deficient derivative does not.[29] Other studies have shown that a proteinase from *Lb. helveticus* CP790 was able to release an antihypertensive peptide from casein hydrolysates.[30,31] Bioactive sequences of whey-soluble proteins might also be released after fermentation by *Kluyveromyces marxianus* var. marxianus,[32] and from milk fermented by *Lb.* GG and digested by pepsin and trypsin.[33]

Intense proteolysis during cheese ripening was found to affect the presence of various bioactive peptides in a variety of cheeses (for a review, see Smacchi and Gobbetti[34]). These peptides are found after a short or medium ripening period.

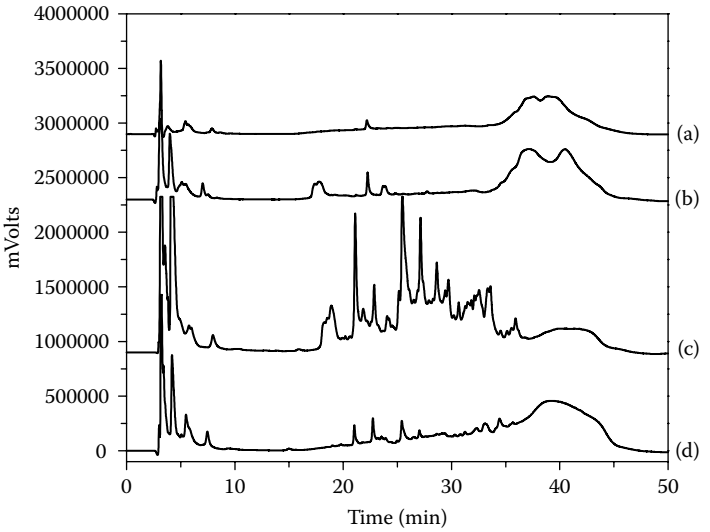

FIGURE 7.2 RP-HPLC profile of unfermented milk (a), milk acidified with HCL (b) and milk fermented by *L. helveticus* R389 with (c) and without (d) pH control.

Casein phosphopeptides were produced during the ripening of Comté and Grana Padano cheeses.[35,36] Angiotensin-converting enzyme (ACE)-inhibiting peptides have also been isolated from several Italian cheeses.[37] The antihypertensive peptides were also found in medium-aged Gouda[38] and in Parmesan cheese.[38] It seems that a long ripening period for cheese could be inactive in these peptides due to further proteolysis. Smacchi and Gobbetti[34] have reported that bioactive peptides might selectively inhibit proteolytic enzymes of LAB as well as the dairy spoilage enzymes in cheese, as those peptides, when exhibiting their physiological activities (immunostimulatory, antithrombotic, and antihypertensive), utilize a common mechanism based on the inhibition of the target proteolytic enzymes.[34] The authors postulated that a similar mechanism may influence the food ecosystem. Bioactive peptides with an inhibitory effect on intracellular peptidases of LAB have been isolated from different types of cheese.[39–41]

7.3 EFFECTS OF THE SUPERNATANTS OF FERMENTED MILKS ON HEALTH AND DISEASE

7.3.1 Immunomodulation by the Supernatant of Milk Fermented by *Lb. helveticus* R389

The gastrointestinal tract is the only part of the body that normally contacts nutrients before they are absorbed. Although nutritional changes ultimately impinge on most organs, it is the epithelium of the gastrointestinal tract that first encounters any variations in nutrient intake.[42] Health benefits derived from the consumption of probiotic bacteria and fermented milks are well documented.[43–48] The beneficial effects of fermented milks can be exerted mainly through two mechanisms: direct effects of live

microbial cells (probiotics) and indirect effects via metabolites of these cells (biogenics). Biogenics are defined as food components derived from microbial activity that provide health benefits without involving the intestinal microflora.[49] The most important biogenics in fermented milks are peptides, which are not present before fermentation. The probiotic effects ascribed to LAB and fermented dairy products arise not only from whole microorganisms and cell wall components, but also from metabolites such as peptides and exopolysaccharides produced during fermentation. Peptides derived from major milk proteins during fermentation are potential modulators of various regulatory processes in the body.[50] It was reported that cell-free yogurt fractions exert antiproliferative effects on cultured mammalian intestinal cells.[51] Matar et al.[52] and LeBlanc et al.[53] also demonstrated immunoenhancing properties for the peptides released from milk by *Lb. helveticus* R389. In addition, peptides released from milk proteins by the same *Lb. helveticus* strain induced a protective humoral immune response after an *Escherichia coli* O157:H7 infection in mice.[54] Peptides derived from milk fermentation appear to survive gastrointestinal digestion, and have been recovered from feces. It seems probable that the peptides generated by the bacteria found in yogurt or other fermented milks affect gut and immune cells directly.[55] Bioactive compounds other than peptides may also be produced by bacterial fermentation.[51] For example, the immunomodulating capacity of kefiran, an exopolysaccharide present in the fermented milk product kefir, has recently been reported.[56](See Chapter 4 for more details on kefiran.)

Lb. helveticus R389 has been used to ferment milk with and without pH control. Milk fermented at a constant pH value of 6 presented a more complex proteolytic profile than milk fermented without pH control, as shown by the reverse-phase HPLC analysis of their supernatants (Figure 7.2). When the fermented milk supernatants (FMS and FMSpH6) were administered to conventional BALB/c mice, an increase in the number of IgA+ cells in the lamina propria of the small intestine was observed. The effects were greater in magnitude in mice that received FMSpH6 than FMS, and were achieved in a shorter period of time (Table 7.2), probably due to the

TABLE 7.2
The Number of IgA+ Cells in the Small Intestine Lamina Propria of Mice Receiving Fermented Milk Supernatant (FMS) Fermented by *L. helveticus R389* with (FMSpH6) or without (FMS) ph Control Compared to PBS Control Mice

	Nº of IgA+ cells in the small intestine lamina propria at different days of feeding		
Sample	**2 days**	**5 days**	**7 days**
Control	76 ± 4^{a}	73 ± 5^{a}	75 ± 6^{a}
Skim milk	78 ± 5^{a}	86 ± 7^{a}	117 ± 11^{b}
FMS	86 ± 3^{a}	114 ± 16^{b}	153 ± 4^{b}
FMSpH6	96 ± 2^{b}	147 ± 7^{b}	197 ± 3^{b}

Note: Values in columns with different superscript are significantly different ($P < 0.05$).

greater content of bioactive peptides in FMSpH6.[57] Moreover, no specific secretory-IgA was produced against the FMSpH6 administered. When the cytokine producing cells were analyzed, it was observed that FMSpH6 induced an unclear-polarized cytokine response, not dominantly Th1 or Th2, suggesting that the enhanced cytokine response was due to an effect on the innate immune cells such as macrophages, dendritic cells, or mast cells. The proliferation of IgA+ cells in the lamina propria could be due to a response to T-independent stimulation of B cells by FMSpH6 that induced the local clonal expansion of B cells.

FMSpH6 modulated the gut immune response as well as gut physiology; mice that received the FMSpH6 for 7 consecutive days showed an enhanced expression of calcium channels (TRPV6) located in the brush border of enterocytes in the small intestine epithelium.[58] An enhanced expression of TRPV6 channels in the duodenum would indicate an improved capacity for dietary Ca^{2+} uptake. Calcineurin is a Ca^{++}-dependent enzyme able to regulate the production of IL-2 and TNFα.[59] Its expression plays a key role in initiating the innate immune response due to the capacity of cytokine TNFα of initiating cross-talk among immune cells.[60] The activation of calcineurin is also involved in key aspects of the adaptive immune response, as calcineurin mediates IL-2 production, which links the innate and adaptive responses.[61] The oral administration of FMSpH6 also enhanced the expression of calcineurin in the small intestine lamina propia, as well as induced a transient increase in the number of mast cells and goblet cells. An enhanced expression of calcineurin able to activate the transcriptional factor NFAT to regulate IL-2 and TNFα production, and to increase in local mast and goblet cells, would indicate an improved state of mucosal surveillance at sites of infection. The oral administration of FMSpH6 transiently affected the gut mucosal immunity and gut functioning. The induction of transient effects would assure temporal effects that would not persist longer than the feeding period. The transient activation of calcineurin and calcium transporters by the FMSpH6 would enhance gut mucosal immunity and calcium availability, reinforcing the immune defense mechanisms and the nutritional status of the host. The enhanced presence of goblet cells would ensure an improved synthesis of mucus, which has important immune and nonimmune functions that would help to protect the epithelial surface. All these events improve the intestinal barrier integrity and function, increasing host protection against infections.

The oral administration of the FMSpH6 was also effective in the diminution of the severity of infection by the enteropathogen *Salmonella enteritidis* serovar Typhimurium in mice.[62] Mice that received the FMSpH6 for 7 consecutive days, and that were then challenged with a single infective dose of *S. enteritidis* serovar Typhimurium, presented lower levels of liver colonization by the enteropathogen on day 7 post-challenge, higher luminal contents of specific anti-*Salmonella* secretory-IgA, and higher percentages of survival after infection. FMSpH6 was also effective in the control of the production of MIP-1α+ cells, a chemokine with a strong capacity for the recruitment of polymorphonuclear neutrophils into sites of infection, thus avoiding excessive tissue damage.

7.3.2 Immunomodulation by the Supernatant of Kefir

Kefir is a fermented milk (drink) produced by the action of LAB, yeasts and acetic acid bacteria, trapped in a complex matrix of polysaccharides and proteins whose chemical, nutritional, and health promoting characteristics are extensively discussed elsewhere (see Chapter 4). Studies where kefir supernatant was orally administered to mice reported that it was able to active cells from the innate immunity pathway isolated from both the peritoneal cavity and Peyer's patches, as well as to induce the proliferation of IgA+ cells and certain cytokine+ cells.[63] Antigen-presenting cells such as monocytes, macrophages, and dendritic cells are responsible for detecting microbes and presenting their antigenic structures to T cells, thus eliciting acquired immune responses. In addition, monocytes and macrophages kill microoganisms by phagocytosis and produce proinflammatory cytokines.[64] It is important to keep this immunosurveillant system in an active state. Both kefir[65] and its cell-free fraction[66] were reported to activate the gut mucosal immune response *in vivo*. The oral administration of commercial kefir to mice diluted in the drinking water for 2, 5, or 7 consecutive days lead to an increase in the number of IgA-producing cells in the small intestine lamina propria, as well as in the number of several cytokine-producing cells. A similar profile of mucosal immune response activation was achieved when mice received the dried cell-free fraction of the same kefir.[65,66]

The effects of the oral administration of kefir supernatant on the cytokines induced were simultaneously compared in the small and large intestine, and in the blood serum and intestinal fluid. The small intestine harbors the Peyer's patches that constitute the principal inductive sites of the immune response after oral administration of an antigen. The small intestine immune system is anatomically connected to the systemic immune system by the lymphatic and blood circulation. An immune response induced in the small intestine can spread through the systemic immune system reaching mucosal and nonmucosal sites. In contrast, the immune response induced in the large intestine is more confined to this site. The greater immune response observed in the small intestine than in the large intestine after the oral administration of kefir supernatant could be due to its soluble nature. In the intestinal fluid, we also observed a significant increase of IL-6, which reached a much higher concentration than the other cytokines measured. This result could be due to the fact that intestinal epithelial cells are an important source of IL-6.[67] The lower levels observed for the other cytokines measured might be due to the proteolytic degradation by luminal enzymes or to the elimination in feces by peristalsis. Lower levels of the other cytokines might have undergone proteolytic degradation by luminal enzymes or elimination in feces. All the cytokines assayed in serum followed a pattern of production that was quite similar to that found for the same cytokines in the small intestine lamina propria, indicating that even though cytokine producing cells were found in the small intestine lamina propria, the cytokines were released into the circulation. These facts, taken together, underline the importance of the small intestine lamina propria, rather than the large intestine, and the blood serum as tools for the study of the immunomodulation of orally administered antigens in animal models.

7.3.3 Effects of the Supernatant of Kefir on the Prevention of Breast Cancer

Considerable advances have been made in recent years towards an understanding of the molecular factors involved in breast cancer development. For women in most western countries, breast cancer still remains a major cause of death. There are genetic and environmental factors that increase the chances of breast cancer, and the most common breast cancer types are estrogen-dependent. Some factors, such as diets rich in cultured dairy products, may inhibit the growth of many types of cancer, including breast tumors.

In addition to LAB, fermented products can possess other nonbacterial components produced during fermentation that contribute to immunogenicity and to other properties, such as their antitumor activities. For example, peptidic fractions liberated during milk fermentation with *Lb. helveticus* R389 stimulated the immune system and inhibited the growth of an immuno-dependent fibrosarcoma in a mice model.[53] Water-soluble polysaccharides, isolated from kefir grains, inhibited 40 to 59% of the growth of an Ehrlich carcinoma in mice.[68]

Kefir and its cell-free fraction (KF) possess several substances that can exert beneficial effects on the immune system, and prevent certain types of cancer.[65,69,70] It was observed that mice receiving 2 days cyclical feeding with whole kefir had diminished tumor growth, and the same cyclical feeding with KF showed the most significant delay of the tumor growth.[71]

The influence of the immune cells on breast cancer has been reported using different models.[72,73] A substantial proportion (up to 50%) of breast tumors comprises cells from the immune system that infiltrate the tumors.[74] These cells produce different biological messengers, such as cytokines, that are implicated in an antitumor response. The important role of cytokines in regulating breast tumor estrogen synthesis stimulated research on these cells and the role of the different molecules produced by them. There are many cytokines implicated in estrogen synthesis in both normal or tumor-invaded breast tissue.[75,76] One of the cytokines most studied in this area is IL-6, which is also a proangiogenic factor.[77] Systemic responses are sometimes important, but it is more useful to study the local cytokine response, because there is local estrogen synthesis in mammary glands, and this could be implicated in tumor growth. In mammary glands, IL-6+ cells increased significantly in the tumor control group over the time course of the experiment. The KF group maintained low numbers of these cytokine+ cells over the course of this experiment, which were lower than in the other groups. Similar results were obtained for cells isolated from the tumors (see Table 7.3). Decreases of IL-6 can explain the observed tumor growth in the kefir and KF groups, but there are other cytokines that are also important to understand the mechanisms involved in the effects observed.

TNFα is known to be a proinflammatory cytokine and to possess tumor necrosis effects.[78] This cytokine is also related to the activation of apoptosis pathways.[79] High numbers of TNFα+ cells were observed in a tumor control group in a breast cancer model. Increases of TNFα at the beginning of the experiment could be important because this observation was also reported for other fermented milk that produced a delay in tumor

TABLE 7.3
Cytokines Positive Cells in Mammary Glands or Breast Tumor

		Mammary glands tissues[1]			Tumor infiltrative cells[2]		
Groups	**Time (days)**	**TNFα**	**IL-6**	**IL-10**	**TNFα**	**IL-6**	**IL-10**
Tumor control	Basal	9 ± 2^{b}	$9 \pm 2^{a,b}$	$6 \pm 2^{a,b}$	ND	ND	ND
	13	$9 \pm 4^{a,b,c}$	$15 \pm 4^{b,c}$	$11 \pm 3^{b,c}$	ND	ND	ND
	20	21 ± 6^{c}	15 ± 5^{c}	13 ± 4^{c}	22 ± 5^{d}	$16 \pm 5^{b,c}$	18 ± 2^{d}
	27	21 ± 5^{c}	33 ± 11^{d}	16 ± 5^{c}	14 ± 1^{c}	23 ± 3^{c}	$8 \pm 2^{b,c}$
Kefir	Basal	$7 \pm 2^{a,b}$	5 ± 2^{a}	$7 \pm 2^{a,b}$	ND	ND	ND
	13	$8 \pm 2^{a,b}$	5 ± 2^{a}	13 ± 4^{c}	ND	ND	ND
	20	14 ± 4^{c}	$15 \pm 3^{b,c}$	14 ± 6^{c}	4 ± 1^{a}	5 ± 1^{a}	3 ± 1^{a}
	27	$11 \pm 3^{b,c}$	18 ± 5^{c}	14 ± 3^{c}	$13 \pm 2^{b,c}$	$6 \pm 2^{a,b}$	$5 \pm 2^{a,b}$
KF	Basal	6 ± 1^{a}	5 ± 2^{a}	5 ± 1^{a}	ND	ND	ND
	13	$9 \pm 2^{b,c}$	6 ± 2^{a}	13 ± 3^{c}	ND	ND	ND
	20	$10 \pm 3^{b,c}$	10 ± 2^{b}	$11 \pm 2^{b,c}$	$9 \pm 1^{a,b}$	3 ± 1^{a}	11 ± 1^{c}
	27	$11 \pm 3^{b,c}$	$10 \pm 4^{a,b}$	$22 \pm 5^{b,c}$	$9 \pm 2^{a,b}$	4 ± 2^{a}	12 ± 3^{c}

[1] For mammary gland tissues, cytokine-positive cells were analyzed using indirect immunofluorescence. Results are expressed as means ± SD of cytokine-positive cells counted in ten fields of vision at 1000× of magnification.

[2] For cells isolated from tumor, cytokine-positive cells were analyzed by immunoperoxidase technique and results are expressed as means ± SD of cytokine-positive cells each 100 counted cells (cells/100). Means for each cytokine and for tissue or isolated cells without a common letter differ significantly ($P < 0.05$).

growth.[80] It is also important that the concentration of this cytokine is maintained or diminished after tumor injection, because it is related to estrogen synthesis.[74]

IL-10 is a regulatory cytokine implicated in the modulation of the immune response. IL-10 and IL-4 are known to be regulatory cytokines, associated with the activated Th-2 lymphocytes.[78] IL-10 can be produced by Th-2 cells and also by other cell populations, such as macrophages and dendritic cells. In different experimental models, TNFα and IL-10 were demonstrated to have opposite effects.[81] The balance between TNFα and IL-10 could modulate the effector function of macrophages and cell apoptosis. In mammary glands, and tumor-isolated cells, IL-10+ cells increased significantly in the KF group at the last sample time. This finding implies that the infiltrative immune cells in the tumor are stimulated, and it was kefir or compounds in the cell-free fraction that induced this activation.

IgA+ cell numbers did not vary in mice injected with a tumor cell line (tumor control) during a recent study (Table 7.4). Two days of cyclical administration of kefir or KF produced increases in the IgA+ cells in mammary glands after tumor injection. An increase was not observed in the animals fed with the fermented milk that did not receive tumor injection, demonstrating that the enhancement of IgA+ cells in mammary glands needs a strong stimulation such as those induced by tumor cells.[82] The biological role of IgA+ cells in response to mammary tumor development is not well understood. It has been suggested that they might be able to bind toxic metabolites produced during tumor development.

TABLE 7.4
Effect of Tumor Injection, Kefir, and Kefir Cell-Free Fraction (KF) on IgA+ Cells and CD4+ and CD8+ T Lymphocytes in the Mammary Glands

		Mammary gland tissues		
Groups	**Time (days)**	**IgA**	**CD4**	**CD8**
Tumor control	Basal	$12 \pm 3^{a,d}$	$9 \pm 3^{a,b}$	13 ± 1^{a}
	13	$9 \pm 3^{a,b}$	$15 \pm 5^{b,c}$	$18 \pm 5^{a,c,d}$
	20	$12 \pm 4^{a,b,d}$	$13 \pm 4^{a,b,c}$	$18 \pm 7^{c,d}$
	27	$11 \pm 4^{a,b,d}$	$15 \pm 4^{b,c}$	$24 \pm 7^{c,d}$
Kefir	Basal	7 ± 2^{b}	8 ± 2^{a}	8 ± 2^{b}
	13	$11 \pm 5^{a,b,d}$	14 ± 2^{b}	$10 \pm 3^{a,b}$
	20	22 ± 6^{c}	$14 \pm 5^{a,b,c}$	$15 \pm 4^{a,c}$
	27	$11 \pm 3^{a,b,d}$	$14 \pm 3^{a,b,c}$	$14 \pm 4^{a,c,d}$
KF	Basal	$9 \pm 2^{a,b}$	8 ± 2^{a}	$9 \pm 3^{a,b}$
	13	$13 \pm 3^{a,b,d}$	$12 \pm 4^{a,b,c}$	$10 \pm 3^{a,b}$
	20	19 ± 3^{c}	27 ± 7^{d}	12 ± 2^{a}
	27	$14 \pm 5^{a,c,d}$	$19 \pm 4^{c,d}$	$13 \pm 3^{a,c}$

Note: For mammary gland tissues, IgA, CD4, or CD8 positive cells were analyzed using direct immunofluorescence. Results are expressed as means ± SD of positive cells counted in ten fields of vision at 1000× of magnification. Means for each cytokine and for tissue or isolated cells without a common letter differ significantly ($P < 0.05$).

The study of the T population is important because tumor antigens recognized by T cells are the principal targets, especially in solid tumors, for protective antitumor immunity. Changes were observed in the balance between CD4+ and CD8+ cells in mammary glands in mice fed 2 days cyclically with KF and injected with tumor cells. They showed increases in the number of CD4+ cells, whereas CD8+ cell numbers remained constant. This was different in the tumor control group that maintained a balance of these cells in mammary glands. An increase of CD8+ T lymphocytes was observed after tumor cell injection (tumor control group), compared to the basal data, but the number of CD4+ T lymphocytes remained constant throughout the experimental period.

Mice that received whole kefir only showed increases in IgA+ cells, but the number of CD4+ cells did not show the same increases that were observed in mice that received the KF. This last observation would confirm the importance of the nonmicrobial substances contained in the fermented milk product on its immunomodulating properties.

These studies, using the model of breast cancer in mice, demonstrated that 2 days of cyclical feeding with kefir or a kefir cell-free fraction delayed tumor development. This effect appeared to be related principally to a decrease in IL-6. KF induced not only a decrease of this cytokine but also a regulatory response, with increased levels of IL-10 in all the samples studied.

The results also demonstrated that the most important effect in this tumor model was due to substances released during milk fermentation to create this product, and not

the microorganisms themselves. The importance of an adequate balance of the local immune response in the mammary glands to avoid the tumor growth was also shown.

7.4 EFFECT OF IMMUNOPEPTIDES ON MUCOSAL IMMUNITY

Intake of milk fermented by LAB has led to a significant increase in various immune responses, such as sIgA-producing cells, macrophage activity, and specific antibody responses during infections.[43,82–84] The stimulation of the immune system by LAB depends on the survival of the bacteria in the gastrointestinal tract, resistance to gastric acid, and ability to adhere to the mucosal surface.[85] However, studies have shown that components of fermented milks other than bacteria also contribute to the immunostimulating effects, specifically,

1. The dialysate and anion exchange fraction of yogurt showed significant inhibitory action against tumors in mice *in vivo*.[86]
2. The soluble compounds produced by LAB during milk fermentation can be used to prevent gastrointestinal disorders and cancer.[87]
3. The supernatant of fermented milk cultured with *Lb. casei* and *Lb. acidophilus* increased the immune response independent of the presence of lactobacilli.[88]
4. Filtered yogurt increases interferon (IFN)-γ production and natural killer (NK) activity of human peripheral blood lymphocytes.[89]

Ng and Griffiths[90] suggested that soluble substances released during fermentation by proteolytic LAB might influence the activity of macrophages. They studied the macrophage cytokine release by cell-free fractions of fermented milk in an *in vitro* model. *Lb. helveticus*-fermented milk and its cell-free fractions enhanced the cytokine release by macrophages. The cell-free supernatant from LAB-fermented milk along with LPS (lipopolysaccharrides) demonstrated an effect on IL-6 production.[90] Peptides released during the fermentation of κ-casein by *Lb. GG*, followed by pepsin/trypsin treatment, enhanced the mitogen responsiveness of *in vitro* cell cultures of human lymphocytes.[91] β-Casein permeate medium fermented with *Lb. helveticus* is able to affect the lymphocyte proliferation *in vitro* of human peripheral blood lymphocytes.[92]

7.4.1 Immunomodulatory Peptides from Caseins

Many studies have reported the presence of immunomodulatory sequences within milk proteins. Several whey and casein-derived peptides, as well as peptides derived from lactoferrin, may play a role in the modulation of the immune system. Bovine κ-caseinoglycopeptide (CGP) is obtained by the chymosin digestion of κ-casein. CGP has been shown to downregulate the immune system by suppressing the proliferation of murine splenic lymphocytes *in vitro* by LPS or phytohaemagglutinin.[93] The dipeptide Tyr-Gly from κ-casein increases cellular proliferation of human peripheral blood lymphocytes activated with concanavalin A.[55,94] Due to its small size, this peptide can, in principle, pass across the intestine in quantitatively significant amounts to reach local lymphocytes.[95] κ-Casein treated with pepsin/trypsin upregulated IL-4 and IFN-γ production, whereas *Lb.* GG degraded casein

downregulated IL-4 production with no effect on IFN-γ.[96] An hexapeptide isolated from the trypsin digest of human caseins, Val-Glu-Pro-Ile-Pro-Tyr, when intravenously injected into mice, improved resistance to *Kleibseila pneumoniae*. *In vitro*, this hexapeptide also stimulated the phagocytosis of sheep red blood cells by peritoneal macrophages of mice.[97]

Enzymatic digests of αs1-casein inhibit the proliferation responses of murine splenic lymphocytes and rabbit Peyer's patch cells[98] and cause the release of the peptide Thr-Thr-Met-Pro-Leu-Tyr. This peptide has been shown to promote antibody formation and phagocytosis *in vitro* and reduce *K. pneumoniae* infection in mice.[99,100] Peptide fragments from β-casein (PGPIPM and LLY) have been demonstrated to stimulate phagocytosis of sheep red blood cells by peritoneal macrophages, and protect against *K. pneumoniae* infections, and the LLY peptide also enhances antigen-dependent T cell proliferation.[101] Depending on the concentration, hydrolysis of the β-casein by pepsin/trypsin either stimulated or inhibited the proliferation of human peripheral blood lymphocytes; however, the pepsin/chymosin digests directly stimulated the proliferation of rat lymphocytes *in vitro*.[55] Pancreatin/trypsin digests of β-casein inhibit murine splenic lymphocyte proliferation and Peyer's patch cells *in vitro*.[98] Coste et al.[102] have obtained the evidence that the peptide 193-209 from bovine β-casein can enhance rat lymphocyte proliferation.

7.4.2 Immunomodulatory Peptides from Minor Proteins in Milk

Casein-derived peptides exert immunomodulating activities, whereas peptiodes derived from whey and lactoferrin exhibit important physiological activities. A pepsin-generated hydrolysate of lactoferrin has been shown to contain immunostimulating peptides, which can enhance the proliferation of spleen cells,[103] and can stimulate the phagocytic activity of human neutrophils.[104] Peptides obtained by tryptic hydrolysis of bovine α-lactoglobulin have been shown to induce oral tolerance in mice.[105] Hydrolyzed α-lactalbumin enhances murine humoral responses to sheep and human red blood cells caused by the modulation of both B lymphocyte and T helper cell activities.[106] Commercial hydrolyzed α-lactalbumin stimulates B lymphocytes in the absence of T cell cooperation due to an enhanced immune response to the T cell-independent antigen TNP-Ficoll.[107] Bovine lactoferricin B, a peptide released from the hydrolysis of lactoferrin, has been found to suppress IL-6 production by human monocytic cells in response to LPS stimulation.[108] Lactoferricin has also been shown to be able to stimulate the release of neutrophil-activating chemokine IL-8 from human polymorphonuclear leucocytes.[109]

Bioactive peptides might exert an indirect effect on the immune system. Opioid peptides, such as β-endorphins, enhance lymphocyte proliferation, NK activity, and neutrophil locomotion.[110,111] These effects could be explained by the presence of opioid μ-receptors on T lymphocytes and human phogocytic leucocytes. β-Casokinin inhibits ACE, causing a decrease of blood pressure, and aldosterone by acting on bradykinin, a hormone with immune-enhancing effects. Bradikinin is able to stimulate macrophages, enhance lymphocyte migration, and increase secretion of lymphokins.[112,113] Thus, this chain of events indirectly produces an overall immunostimulating response.[7]

7.5 EFFECT OF MILK PEPTIDES ON TUMOR GROWTH

Lactic acid bacteria, through the mechanism of fermentation, may release compounds that react with the immune system parameters and induce protective immunity against infections and some tumors.[114] *In vivo* experiments showed that the potentiation of the immune system and inhibition of tumor development is likely related to the proteolysis that occurs during milk fermentation.[52,53] Milk fermented with *Lb. helveticus* R389 increased the number of IgA+ B cells in the small intestine and bronchial tissues (Table 7.5). Increases in the levels of secretory IgA and an activation of the B cells to enter the IgA cycle were not noticeable when milk was fermented by the non-proteolytic variant of this strain (Table 7.6).[52]

Perdigón et al.[115] suggested that the increased numbers of cells secreting IgA in the large intestine of mice given yogurt could contribute to a decrease in the tissue-damaging consequences of a permanent inflammatory response, which occurs during the development of tumors and neoplasia. IgA is considered to be an immune barrier in colonic neoplasia. The increase in the mucosal immunity and the enhancement of the cellular mobilization of the IgA+ cells are properties that can be exploited clinically to prevent infections and development of some tumors in the mucosal network. Those results could be therapeutically used to redirect the immunologic memory and favor a response other than the T helper subset 2 cell, which could prevent inallergic inflammatory disease.[116] *Lb. helveticus*, in our laboratory, was able to hydrolyze milk proteins and cause the release of peptides, as was shown by the level of proteolysis and the HPLC elution pattern of milk after fermentation (Figure 7.2). This same fermented milk increased the phagocytic index of peritoneal macrophages in mice, which was directly correlated with a regression of fibrosarcoma.[52] Further *in vivo* studies

TABLE 7.5
Effect of Oral Administration of Milk Fermented (12 h) by *Lb. helveticus* Wild Type on the IgA Cell Numbers in the Intestine and the Bronchial-Associated Lymphoid Tissues in Mice

	Number of IgA+ B cells			
	Intestine		Bronchus	
Feeding periods	Nonfermented milk[a]	Milk fermented by *Lb. helveticus* wild	Nonfermented milk[b]	Milk fermented by *Lb. helveticus* wild
3 d	80 ± 5.0	180[b] ± 3.5	18 ± 4.0	57[b] ± 5.2
5 d	86 ± 3.2	141[b] ± 1.3	20 ± 3.4	38[b] ± 5.2
7 d	85 ± 2.5	81 ± 3.5	19 ± 1.3	27[c] ± 3.2

Note: IgA^+ B cells were measured by an immunofluorescence test using a monospecific antibody after 3, 5, and 7 d of feeding.[77] Values are means for $n = 4 \pm$ standard deviation.

[a] Controls were animals given uninoculated milk

[b] Significantly different from the corresponding values for controls, $P < 0.01$

[c] Significantly different from the corresponding values for controls, $P < 0.05$.

Source: Matar, C., Valdez, J.C., Medina, M., Rachid, M., and Perdigón, G., *J. Dairy Res.,* 68, 601–609, 2001. With permission.

TABLE 7.6
Effect of Oral Administration of Milk Fermented (12 h) by *Lb. helveticus* Protease (–) (a Nonproteolytic Variant) on the IgA Cell Numbers in the Intestine and the Bronchial Associated Lymphoid Tissues in Mice

	Number of IgA cells			
	Intestine		Bronchus	
Feeding periods	Nonfermented milk (+ 0.4% yeast extract)[a]	Milk (+ 0.4% yeast extract) fermented by *Lb. helveticus* prot (–)	Nonfermented milk (+ 0.4% yeast extract) 2	Milk (+ 0.4% yeast extract) fermented by *Lb. helveticus* prot (–)
3 days	76.0 ± 3.0	71 ± 6.0	25.3 ± 3.1	23 ± 6.0
5 days	92.5 ± 1.0	81 ± 1.4	22.3 ± 2.0	15 ± 1.15
7 days	75.5 ± 1.5	80.5 ± 5.0	22.66 ± 2.2	18 ± 6.0

Note: IgA^+ B cells were measured by an immunofluorescence test using a monospecific antibody after 3, 5, and 7 days of feeding. Values are means for $n = 4 \pm$ standard deviation.

[a] Controls were animals given uninoculated milk supplemented with 0.4% yeast extract. Addition of yeast extract allowed comparable growth in wild-type and protease (–) strains.

Source: Matar, C., Goulet, J., Bernier, R.L., and Brochu, E., in *Probiotics 3 : Immunomodulation by the Gut Microflora and Probiotics*, Fuller, R., Ed., Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000. With permission.

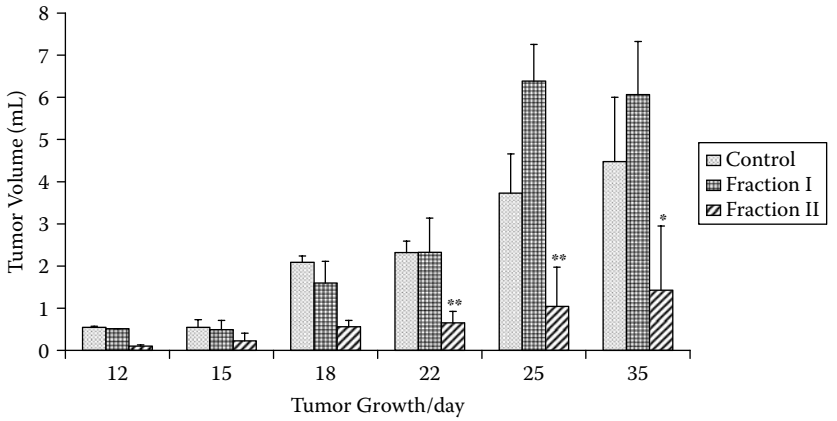

FIGURE 7.3 Effect of previous 7 d feeding of mice with fractions I or II on fibrosarcoma volume (ml). The volume and the growth of the methylcholantrene-induced fibrosarcomas were recorded on days 12, 15, 18, 22, 25, and 35.

designed to analyze the particular effect of the different peptidic fractions in milk after fermention by *Lb. helveticus* have led to the identification of a bioactive fraction. This fraction increased the number of IgA secreting cells in the gut-associated lymphoid tissue (GALT), and caused the regression of subcutaneously implanted fibrosarcomas, an immunodependent tumor (Figure 7.3).[53] These studies thus confirm that peptides

released by bacterial proteolysis might have important implications in modulation of the host's immune response, and have an impact on tumor growth.

7.6 ANTIHYPERTENSIVE PEPTIDES

Antihypertensive agents can be classified in five different categories depending on their modes of action: diuretics, sympatholytic agents, ACE inhibitors, calcium channel blockers, and arterial vasodilators. Antihypertensive peptides are considered, by their mode of action, to be ACE inhibitors. Antihypertensive peptides are one class of bioactive peptides having an inhibitory effect on the ACE.

ACE, found in lungs, blood vessels, and the mucosa wall is an important component of the renin-angiotensin system (RAS), implicated in the regulation of arterial tension, blood volume, and the balance of electrolytes. Renin is an aspartic proteinase of the RAS that hydrolyzes angiotensinogen, releasing angiotensin I, a decapeptide. Then, ACE hydrolyzes angiotensin I to angiotensin II, an octapeptide, by removing His-Leu from its C-terminal. Angiotensin II is a vasoconstrictor—an inhibitor of bradykinin (a vasodilator). This hormone increases the production of aldosterone, which affects ion retention and the excretion of fluids and, therefore, causes hypertension. Angiotensin II directly influences blood pressure. Consequently, any peptide that has an inhibitory action on ACE can be considered an antihypertensive peptide.

Various peptides having ACE-inhibitory activity *in vitro* have been isolated from different proteins including α_{S1}- and β-casein (CN)[112,117;] whey proteins[118,119;] soy proteins[120;] gelatin, fish proteins, and maize[121;] and gluten, zein, and hordein.[122] But milk proteins are the principal sources for such bioactive peptides (Table 7.7).

Different kinds of fermented milk have shown a high hemodynamic regulatory activity. Antihypertensive peptides derived from fermented milk have been studied *in vivo*.[123] LAB have extracellular proteinases that hydrolyze casein to release ACE-inhibitory peptides.[124] Some antihypertensive peptides are released from casein by a purified extracellular proteinase from *Lb. helveticus* CP790 (Table 7.7).[31] Moreover, a dodecapeptide derived from casein enzymatic hydrolysis has been found to have antihypertensive properties *in vivo*.[125] Two other antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, were purified from a sour milk called Calpis fermented with *Lb. helveticus* and *Saccharomyces cerevisiae*.[126] Their amino acid sequences can be found in three positions in bovine caseins: β-CN(f74-76), β-CN(f84-86), and κ-CN(f108-110). As shown in Table 7.7, these peptides have a low IC_{50} and therefore a strong ACE-inhibitory activity.

Several low molecular weight bioactive peptides with ACE-inhibitory activity have also been isolated from ripened cheeses.[55] An antihypertensive casein fragment, β-CN f58-72, which contains the β-casomorphin-7 sequence, has been isolated from Crescenza[37] and cheddar cheeses.[41] Two bioactive peptides have been isolated from the fermentation of whey by *Kluyveromyces marxianus*.[32] The two oligopeptides obtained were the result of the proteolysis of β-lactoglobulin in fragments f98-132 and f4-31. These oligopeptides contain the peptidic sequence YLLF, known to have antihypertensive properties, and are found in β-lactorphin. Consequently, these peptides could have some effects on hypertension. *In vivo* studies to evaluate antihypertensive activity of peptides are usually carried out on spontaneously hypertensive rats (SHR). The anti-

TABLE 7.7
Some Bioactive Peptides with ACE Inhibition Activity

Peptide	Source	Preparation	IC_{50} (μM)[a]	Reference
FFVAPFPEVFGK	α_{S1}-Casein	Trypsin	77	163
FFVAP	α_{S1}-Casein	Proteinases	6	144
TTMPLW	α_{S1}-Casein	Trypsin	16	164
PLW	α_{S1}-Casein	Synthesis	36	164
LW	α_{S1}-Casein	Synthesis	50	164
VAP	α_{S1}-Casein	Synthesis	2	112
FVAP	α_{S1}-Casein	Synthesis	10	112
AYFYPE	α_{S1}-Casein	*Lb. helveticus* proteinase	106	31
KYPVQPFTESQSLTL	β-Casein	*Lb. helveticus* proteinase	93	31
SVLSLSESKVLPVPE	β-Casein	*Lb. helveticus* proteinase	39	31
PPQSVLSLSESKVLPVPE	β-Casein	*Lb. helveticus* proteinase	25	31
RDMPIQAF	β-Casein	*Lb. helveticus* proteinase	209	31
YQQPVLGPVRGPFPIIV	β-Casein	*Lb. helveticus* proteinase	101	31
LPQNIPPLTQTPVVVPPFLQP EVMGVSK	β-Casein	*Lb. helveticus* proteinase	144	31
LLYQQPVLGPVRGPFPIIV	β-Casein	*Lb. helveticus* proteinase	21	31
DELQDKIHPFATQSLVYPFP GPIHNS	β-Casein	*Lb. helveticus* proteinase	4	31
AVPYPQR	β-Casein	*Lb. helveticus* proteinase	15	31
IPP	β-Casein	Fermentation	5	126
VPP	β-Casein	Fermentation	9	126
KVLPVP	β-Casein	Synthesis	5	30
YKVPQL	β-Casein	*Lb. helveticus proteinase*	22	30
WLAHK	α-Lactalbumin	Trypsin	77	165
IVY	Wheat germ	Hydrolyzate	0.48	166
SAYPGQITSN	Zein	Trypsin	7	122
QVSLNSGYY	Hordein	Trypsin	23	122

[a] Concentration of ACE inhibitor needed to inhibit 50% of the ACE activity.

hypertensive effect is observed by a drop in systolic blood pressure after oral administration of the antihypertensive peptide. Milk fermented by *Lb. helveticus* showed strong antihypertensive activity after oral administration to SHR. However, milk fermented by *Lb. helveticus* CP791, defective of proteinase activity, showed no significant antihypertensive activity after oral administration to SHR.[31] Gobbetti et al.[127] used two selected strains of LAB, *Lb. delbruecki* ssp. *bulgaricus* SS1 and *Lactococcus lactis* ssp. *cremoris* FT4, to produce two types of fermented milk that contained ACE-inhibitory peptides. The casein-derived ACE-inhibitory peptides liberated by these two strains were derived mainly from the β-casein. The purified crude fractions that showed the highest ACE-inhibitory activity in milk fermented *by Lb. delbruecki* ssp. *bulgaricus* SS1 contained a mixture of peptides such as β-CN f6-14, f7-14, f73-82, f74-82, and f75-82.[127]

In general, ACE-inhibitory peptides that have been found to have antihypertensive activity in SHR have IC_{50} values lower than 150 μM. However, in some cases, the extent of ACE-inhibitory activity of the peptide is not correlated with the antihypertensive activity.[128] Some peptides show strong antihypertensive activity with a low dose, even though they possess a low ACE-inhibitory activity.[30] For example, Tyr-Pro found in β-CN, κ-CN, and α_{S1}-casein, purified from a yogurt-like product fermented by *Lb. helveticus*, showed a significant antihypertensive activity after oral administration of SHR, even though it has a very low ACE-inhibitory activity (IC_{50} of 720 μM).[129,130] This suggests another possible way of controlling hypertension. Chymase, a major angiotensin II forming enzyme, plays a role in the development of hypertension in the vessels of the heart.[131] The Tyr-Pro peptide may have an inhibitory effect on that enzyme. In addition, antihypertensive peptides also display an effect on bradykinin levels by inhibition of ACE.[132] Further digestion of the precursors of the bioactive peptides themselves by digestive enzymes could affect the activity of the peptides by the enhancement or inactivation of their physiological activity. The liberation of C-terminal amino acid residues of the peptide β-casein f169-175 after *in vitro* pancreatin digestion increases tremendously the ACE-inhibitory activity, and consequently, the antihypertensive effect *in vivo*.[30] It has been demonstrated that ACE-inhibitory peptides, formed in cheese, decreased when proteolysis exceeds a certain level during the storage period.[133]

Most antihypertensive peptides obtained from food proteins have not yet been studied in humans. However, it has been demonstrated that *Lb. casei* cell extract had an antihypertensive effect on hypertensive patients.[134] Val-Pro-Pro and Ile-Pro-Pro have also been tested on humans by daily ingestion of sour milk for 8 weeks. The systolic blood pressure decreased significantly after 4 weeks of treatment.[123] Moreover, no side effects, such as cough and serum lipid metabolism problems usually observed with ACE inhibitors (e.g., Captopril), were observed in patients treated with the tripeptides.[135]

Many peptides with elevated ACE-inhibitory activity are short and possess proline residues at the C-terminus. Another common structural feature of these peptides is the presence of C-terminal aromatic amino acids. Trp, Tyr, and Phe as well as imino acid Pro residues have more affinity for binding to the active site of ACE.[136] The preferable structure for optimal activity must contain principally hydrophobic residues at the three C-terminal positions. The presence of a hydrophobic residue within the ACE-inhibitory sequence is noteworthy. Gobbetti et al.[127] pointed out that ACE-inhibitory peptides derived from caseins contain a high proportion of hydrophobic peptides (> 60%).

7.7 CASOMORPHINS

7.7.1 Antidiarrheal Effect

Oral rehydration solutions are the remedy of choice for the treatment of diarrhea, but increasing numbers of investigations are showing the beneficial uses of opioid receptor ligands for this symptom. The production of opioid receptor ligand peptides in milk-derived products could be a cost-effective remedy for the treatment of

diarrhoea in malnourished children in developing countries. Opioid receptors (μ, δ, and κ) are located in the nervous, endocrine, and immune systems, as well as in the gastrointestinal tract. These receptors can interact with their endogenous ligands as well as with exogenous opioids and opioid antagonists such as milk-derived opioid peptides.[137] Many opioid receptor binding peptides have been obtained by the hydrolysis of milk proteins, or by artificial production using peptidic sequences encoded by these proteins, such as β-casomorphins (f60-70 β-casein), α-casomorphin (αs1-casein), α-lactorphin (α-lactalbumin), and β-lactorphins.[138–140] The major opioid peptides, called β-casomorphins, are fragments of the bovine β-casein sequence 60–70 (YPFPGPIPASL) and have been characterized as μ-type ligands,[140,141] thus exerting a morphine-like activity.[142–144] Opioid peptides are designated opioid agonists. The opioid antagonist peptides called casoxins and lactoferroxins are also found in milk proteins. Their action consists of antagonizing the opioid activity, such as the inhibition of the gut motility.[100,113]

Opioid peptides are characterized by a particular structure that facilitates their interaction with the binding site of opioid receptors. They are characterized by the presence of a tyrosine residue at the amino terminal end, followed by a proline and another aromatic residue, normally tyrosine or phenylalanine, in the third or fourth position (Table 7.8). The removal of the tyrosine residue results in an absence of bioactivity.[145] The proline residue is crucial for maintaining the proper orientation of the aromatic residue side chains.[146]

Opioid peptides have a large spectrum of activity including the analgesic activity[147,148] and stimulation of endocrine response.[149] The mechanism of their antidiarrhoeal properties is due to a potent antisecretory effect at the intestinal

TABLE 7.8
Examples of Opiod Peptides Derived from Bovine Milk Proteins

Bioactive peptide	Sequence	Source (fragment)	Release protease	Reference
		Opioid Agonists		
β-Casomorphin-4	YPFP	β-Casein (f60-63)	LAB protease	26
β-Casomorphin-5	YPFPG	β-Casein (f60-64)	Chyme	138
β-Casomorphin-7	YPFPGPI	β-Casein (f60-66)	Chyme	138
β-Casomorphin-11	YPFPGPIPNSL	β-Casein (f60-70)	Chyme	138
Exorphin	RYLGYLE	αs1-Casein (f90-96)	Pepsin	168
α-Lactorphin	YGLF	α-Lactalbumin (f50-53)	Trypsin	169
β-Lactorphin	YLLF-NH_2	β-Lactoglobulin (f102-105)	Trypsin	119
		Opioid Antagonists		
Lactoferroxin A	YLGSGY	Lacoferrin (f318-323)	Pepsin	124
CasoxinA	YPSYGLNY	κ-Casein (f53-42)	Trypsin	144
Casoxin B	YPYY	κ-Casein (f58-61)	Trypsin	144
Casoxin C	YIPIQYVLSR	κ-Casein (f25-34)	Trypsin	169
Casoxin D	YVPFPPF	$αs_1$-Casein (f158-164)	Pepsin-chymotrypsin	144

Source: Meisel, H. and FitzGerald, R.J., *Br. J. Nutr.*, 84, S27–S31, 2000. With permission.

mucosa and a potent inhibitory effect on gastrointestinal motility. It has already been demonstrated that opioid peptides such as the β-casomorphins are able to alter intestinal electrolyte and fluid movement in the *in vitro* stripped rabbit ileum.[150–153] It has also been shown in *in vivo* studies with rats that these peptides can prolong gastrointestinal transient time by inhibiting intestinal peristalsis and motility.[154] The reduction of the intestinal motility has been shown with the administration of casein in dogs.[155] The effect of the casein was shown to be due to the presence of opioid receptor binding peptides, because treatments with naloxone, a potent opioid receptor antagonist, inhibited this effect. The action of casomorphins at the gastrointestinal level is important, as it can retard the rate of passage of the digesta through the interaction with other components.[156]

It has been suggested that the brush border membrane could contain specific binding sites for β-casomorphins,[157] which could thus act directly at the brush border membrane affecting electrolyte transport. It is also noteworthy that the control of intestinal electrolyte and water transport involves a variety of regulatory sites including enteroendocrine cells, the nervous network, and the immune system. β-Casomorphins, due to their structure and their physicochemical characteristics, could interact with the various control sites altering intestinal electrolyte and fluid transport, and not just exert their effects via opioid receptors.[158] The antidiarrhoeal effect of casomorphins could thus be multifactorial. More research is necessary to understand the complete biochemical mechanisms of their actions.

7.7.2 β-Casomorphins in Fermented Milk

Some reports have noted the presence of the casomorphins and their derivatives in fermented dairy products. Fermented milks containing β-casomorphins resulting from the incomplete proteolysis of caseins by mutants of lactobacilli deficient in proline-specific peptidases was reported by Matar and Goulet.[25] Milk fermented with an x-prolyldipeptidyl aminopeptidase (XPDAP)-deficient mutant of *Lb. helveticus* was able to liberate β-casomorphin-4, because this LAB had lost its ability to hydrolyze the peptidic bond next to the proline residues.[26] As β-casomorphins contains two proline residues (Table 7.8), they would be digested by the proline-specific peptidases, widely present in LAB, if these enzymes were not inhibited or eliminated by mutations.[127] β-Casomorphin-4 amide showed a high potency for endogenous receptors.[145] β-Casomorphin immunoreactive material has been identified in milk incubated with various bacterial species.[159] This milk was incubated with caseolytic bacteria, such as *Bacillus cereus* and *Pseudomonas aeruginosa*. The presence of β-casomorphins in cheese was studied and it was shown that they can be degraded by the enzymes of *Lactococcus lactis* ssp. *cremoris*.[160] It has been demonstrated that trypsin/pepsin hydrolysis of fermented milk by *Lb.* GG led to the release of opioid peptide sequences from caseins.

7.8 HEALTH BENEFITS OF MILK-BASED BIOACTIVE PEPTIDES

It is now accepted that metabolites generated during milk fermentation have more impact on the enhancement of the *in vivo* response and bioactivities than do the

cellular components themselves (cell wall, peptidoglycan, or cytoplasm fraction). Physiologically active peptides are among the most important metabolites that have proven to influence many health parameters in humans and animals. Various strategies have been adopted to develop functional foods containing novel biopeptide preparations. Despite the proven *in vivo* biological activities of many biopeptides, more emphasis should be placed on the specific functionality of these peptides before adding them as single ingredients into food preparations. More research is needed to better understand the interactions of biopeptides with each other, the interaction of biopeptides with other nutrients, the attainment of optimum effects and doses, the mechanisms of action, and their bioavailability. Many observations tend to demonstrate an alteration of biological activities by these compounds when ingested singly.

Shanbacher et al.,[155] in a review on bioactive peptides in milk, noted the importance of the interaction and synergism of the peptides among themselves and with other nonpeptide components. The authors pointed out that peptides, such as casomorphins, might influence the kinetics and the dynamics of other bioactivities in the intestine. Casomorphins, by slowing the passage of the digesta through the gut, increase the time available for the bioactive agents in milk to assert their action on target cells, bacteria, or organs. In addition, the synergic action of bioactive peptides with nonpeptide (lipids, glycoplipids, and oligosaccharides) or peptide agents in milk is necessary for the expression of the bioactivity.[161,162] The major bioactivities in milk are dose-dependent, and act principally at the intestinal site. When a single bioactive peptide was administered, even if its concentration was increased, the biological effect attributed to the single ingredient was still lower than the effect observed with complete fermented milk. The dose and the mode of administration of bioactive substances play tremendous roles in determining the target functionality. An excessive dose might be more harmful than beneficial to the host. The question remains, "Should probiotic preparations or *de novo* generated peptides be administered continually or periodically?" Continuous administration of immunoregulatory substances might initiate downregulatory signals at the level of the immune system, and result in an increase in inflammatory response.[43]

Use of fermentation organisms with enhanced proteolytic activities or engineered probiotic organisms to favor the overproduction of peptides is one option to enrich a food. Applications of bioactive peptides to nutraceutical and pharmaceutical formulations should be submitted to more scientific scrutiny to ascertain whether or not the specific health benefits are achieved. When using such products, possible interaction of peptides with their receptors *in vivo*, the influences of the microflora that differs among individuals, and the dose-response effect should be taken into consideration. The presence of both agonist and antagonist, as in the case for opioid peptides, may have physiological importance and should also be taken into consideration when formulating enriched bioactive peptide products.

7.9 CONCLUSIONS

The role of peptides derived from milk proteins has been reviewed. The multifunctional properties and potential activities on several biological functions of the host,

such as their effects on the tumor and mucosal immunity, antidiarrhoeal, and antihypertensive activity of peptides, were analyzed. The identification of several bioactive sequences in dietary proteins has contributed to the understanding of the role of these molecules as precursors of bioregulating peptides at the intestinal level. During the fermentation process, LAB use a large spectrum of proteolitic enzymes (endopeptidases, aminopeptidases, tripeptidases, and dipeptidases) to fulfill their nitrogen need for growth. During the fermentation of milk, caseins undergo protelysis, generating potentially bioactive peptides.

LAB themselves can modulate the immune response. However, more and more evidence now links the health benefits of fermented products to specific enzymatic activities of LAB and the peptides released during the fermentation process. The mechanisms responsible for the improvement of health are probably multifactorial and involve complex interactions between peptides from milk proteins, LAB, and intestinal cells.

It is very difficult to demonstrate *in vivo* the effect of different LAB administered with the diet, or to show the effects of individual peptides on the gastrointestinal tract, especially related to mucosal immunostimulation. Casein-derived proteins such as human β-casein, bovine and κ-casein human- and bovine-α lactoalbumin, enhance immune functions (macrophage activity, lymphocyte proliferation, protection against infections, etc.). Thus, consumption of fermented milk products may help to prevent intestinal or nonintestinal tumors.

Recent studies[52,114] have shown protection of the mucosal immune system and an inhibition of the development of a nonintestinal tumor (fibrosarcoma) by peptides released from milk fermented with an *Lb. helveticus* proteolitic strain. As similar results were not observed with milk fermented with a nonproteolytic *Lb. helveticus*, a biological peptide obtained using a proteolytic strain is believed to be responsible.

Antihypertensive peptides can be released from casein using a purified extracellular proteinase from *Lb. helveticus*. The ACE-inhibitory activity of this peptide is not correlated with the antihypertensive effect. The presence of hydrophobic residues at the C-terminal positions is necessary for optimal activity.

Peptides derived from milk proteins may play a significant role in the reported beneficial effects of fermented milk consumption. To improve human health, further studies involving molecular biology are needed to better understand the complex network of interactions between food microorganisms and digestive system.

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8 Cheese and Its Potential as a Probiotic Food

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CONTENTS

8.1 INTRODUCTION

For the production of cheese from milk, two key steps are essential:

1. Concentration of the milk casein and fat through coagulating the casein by proteolytic enzymes or lactic acid
2. Drainage of the whey after mechanical disruption of the coagulated casein

Starting with this simple basic technique, more than a thousand cheese varieties are produced today.[1] Variety is brought about by altering different aspects of cheese manufacture: type of starter culture, additional cultures, fermentation conditions, renneting, cutting the curd, scalding, drainage of whey, forming of green cheese, salting, addition of spices, and ripening. The vast variety of cheeses makes cheese production profitable as a small cottage industry as well as for national and multinational food producers, as both gourmet and commodity markets may be served. The variety of the market and the steady growth in cheese production provide opportunity to adopt new food trends. One important food trend in developed countries has been the introduction of health and functional foods during recent years. Especially in the dairy sector, the introduction of the probiotic concept has been successfully accomplished. Although most of the probiotic products developed are based on fermented milks, a few examples are visible in the cheese sector.

In this chapter, the production of the different varieties of cheese and their potential to serve as carriers for probiotic bacteria are discussed. Available data on the beneficial effects of cheeses containing probiotic microorganisms are also presented.

8.2 HISTORY

It is believed that cheese evolved in the area of the former Assyria about 7000 to 8000 yr ago. For people of that time, and probably for those who lived during the following centuries, the most important incentive for cheese production was that cheese constituted a highly nutritious, high energy food with a much longer shelf life than liquid milk. With the increasing knowledge of cheese production and the influence of acidification, salt dehydration, spices, and ripening on shelf life and taste, very different cheese varieties were developed. Whereas some of our present-day cheeses with international recognition were first described more than 1000 yr ago, others are rather recent developments of the last three to four centuries. Table 8.1 lists some major cheese varieties together with their first recorded date of production.[2]

TABLE 8.1
Some Major Cheese Varieties and Their First Recorded Date

Gorgonzola	897	Cheddar	1500	Stilton	1785
Roquefort	1070	Parmesan	1579	Camembert	1791
Grana	1200	Gouda	1697	St. Paulin	1816

Source: From Scott, R., *Cheese Making Practice*, Elsevier Applied Science Publishers, London, 1986. With permission.

Today cheese is recognized as having very high nutritional value due to its generally high content in protein, calcium, riboflavin, and vitamins A and D.[3] Its reputation has not always been that positive, however. In the oldest existing paper dealing exclusively with milk and milk products,[4] the second-century physician Galen from Pergamonisis stated: "There is no cheese I can praise except Oxigalactinus, which is made from sour milk. All others are hard to digest, cause heart burn, and fill the stomach with flatulence." (See Chapter 1 for more details on the history of cheese.) Today, adverse effects due to consumption of cheese are no longer observed, and reports from developed countries on hygienic problems in cheese are extremely rare. However, consumption of some cheese varieties with high fat and salt content may not be recommended for people who have other risk factors such as high blood pressure, smoking, obesity, diabetes, and heredity for a variety of modern diseases.

8.3 SIZE OF PRODUCTION

Cheese is one of the most important products of the dairy industry. World cheese production increased by ca. 10% from 1997 to 2004 to reach ca. 17.8×10^6 tonnes. Europe is by far the largest producing block, followed by North America (Table 8.2).

In the European Union, ca. 50% of the cheeses produced are hard/semihard cheeses, followed by fresh cheese, which accounts for ca. 30% (Table 8.3).

Especially in Europe, dairy companies are very heterogeneous with respect to the amount of milk processed; the largest multinational companies process several million litres and the smallest cottage companies just a few hundred litres per day. The large number of small companies is one of the major reasons for the great variety of cheese produced.

TABLE 8.2
The Production of Cheese Worldwide

The world cheese production trends 1997 to 2004—all kinds[a] of cheese (in thousand tonnes)

Continent/ Countries	Year								Percentage change 1997–2004
	1997	1998	1999	2000	2001	2002	2003	2004	
United States and Canada	4003	4080	4282	4431	4428	4576	4610	4717	17.8
South and Central America	908	898	927	975	951	911	881	896	−1.1
EU 15	6460	6552	6648	6816	7058	7111	7182	7463	15.5
Other Europe	1703	1803	1758	1811	1971	2083	2116	2102	23.4
Africa	605	644	711	711	714	792	915	915	51.2
Asia	926	943	945	990	1009	1026	1042	1038	12.1
Australia and NZ	539	574	574	670	657	738	643	649	20.4
World total	15145	15492	15844	16402	16790	17236	17388	17778	17.4

[a] Includes cheese made from cow's milk, buffalo's milk, goat's milk, etc.

Source: Modified from The World Market for Cheese 1995–2004, *Bulletin of the International Dairy Federation*, 402, 15, 2005.

TABLE 8.3
Cheese Production Trends in the European Union

Production trends 1997 to 2004, by cheese types—the European Union[a] (in thousand tonnes)

	Year								Percentage change
Cheese types	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**1997–2004**
Hard/semihard	3291	3325	3237	3307	3439	3436	3455	3523	7.0
Soft	998	1026	1048	1018	1055	1061	1062	1089	9.1
Blue	134	137	135	138	140	134	136	137	2.2
Fresh	1884	1895	1986	2028	2106	2108	2187	2221	17.9
Not specified	152	150	144	210	228	236	235	253	66.4
Processed	476	481	480	496	489	488	476	486	2.1
Total	6458	6529	6548	6701	6967	6974	7075	7224	11.9

[a] Excluding Luxembourg and Portugal

Source: Modified from The World Market for Cheese 1995–2004, *Bulletin of the International Dairy Federation*, 402, 18, 2005.

8.4 CHEESE GROUPS AND FERMENTATION PROCESSES

8.4.1 Cheese Groups

Cheese manufacture essentially involves concentrating the fat and casein of milk by coagulating the casein enzymatically (rennet cheeses) or by acidic pH (quarg, acid-curd cheeses). Cow's milk is used predominantly in industrial cheese making. In Mediterranean countries, sheep's and goat's milk are used for cheese making to a large extent. Outside Europe, the milk of water buffaloes, camels, and mares is often used for cheese making. Over the centuries, cheese production has led to the existence of an extensive range of cheese varieties. Although more than a thousand individual varieties exist, some of these differ only in size, packaging, place of origin, or name.[5,6]

The classification of cheeses includes an indication of the process used in the manufacture.[5,7] In addition to the moisture content, the method of coagulation of the milk is used for classification. The main difficulty in using classification schemes is that large moisture ranges are allowed for the major cheese groups. According to moisture levels, at least three types of chesses are generally defined: hard cheeses (moisture 20% to 42%), semihard/semisoft cheeses (moisture 45% to 55%), and soft cheeses (moisture > 55%). All three types are consumed after a certain ripening period in contrast to fresh cheeses (moisture > 70%), which are consumed after draining (Table 8.4).

8.4.2 Handling of Milk

Of particular significance for cheese manufacture is the variation of fat and casein levels in the milk. The relative proportion of casein and fat in milk for cheese making are

TABLE 8.4
Major Cheese Groups

Fresh cheeses (Quarg type cheeses, cottage cheese, etc.)

Cheeses with 14%–30% DM and 0%–75% fat i.d.
Acid dominated coagulation (16–48h)
High initial heat treatment (82°C–88°C)
Spontaneous draining by optional slicing our moulding
Acidification and renneting 18°C–28°C (mesophilic lactic starters)
For some varieties more rennet and higher temperatures are used (28°C–32°C) resulting in cheeses with a DM of 25%–33%
Salting: mixing with salt
No ripening, cheeses are consumed after packaging

Soft cheeses (Camembert, Brie, Chaumes, Romadour)

Cheeses with 40%–50% DM and 25%–60% fat i.d.
Produced from thermized or pasteurized milk
Acidification and coagulation (30–90 min): balanced action of rennet and lactic starters
Temperatures 32°C–35°C, favor the action of rennet
Spontaneous draining promoted by optional slicing, moulding, or pressing
Dry salting or brining (NaCl 1.6%–2%)
Short ripening period (14 d)
Very heterogeneous cheese group, appearance, and taste depending mainly on the surface flora (molds or red smear)

Pressed cheeses (semisoft, semihard)
Uncooked: no or slight heating during vat mixing (Gouda, Edam, Cheddar)

44%–55% DM, 30%–55% fat i.d.
Acidification and coagulation rennet dominant (32°C–37°C, 30–60 min)
Draining by mechanical treatments: slicing, mixing, prepressing, pressing
Salting to 1.5%–2% NaCl by bringing
Ripening: 12–60 d (or longer: 6–12 m)
Characteristics: homogeneous dough with few small "eyes"

Pressed cheeses (semihard, hard varieties)
Cooked: Emmental, Gruyére, Comté, Parmesan, Sbrinz

Cheeses with 58%–64% DM, 30–55 fat i.d.
Cheeses with or without propionic acid fermentation
Acidification and coagulation rennet dominant (33°C–38°C, 12–30 min)
Heating during vat mixing (52°C–55°C)
Mesophilic and thermophilic starters (propionic starters)
Use of thermized milk, often raw milk
Draining by mechanical treatments: slicing, mixing (heating), pressing
Surface with or without microflora
Ripening: 6 weeks, >12 m
Characteristics: firm and smooth dough with or without "eyes"

standardized to minimize seasonal variation in milk composition to facilitate the production of cheeses that comply with specific regulations that ensure a uniform aroma and texture required for a single cheese variety. Homogenization is not routinely used in cheese manufacture, but it can be used to improve the texture of cheeses. Homogenization is used mainly in the manufacture of unripened fresh cheeses.[6]

In general, cold storage of milk is necessary before cheese manufacture. The main problems with this step are growth and enzyme activities of psychrotrophic microorganisms, including Gram-negative bacteria (e.g., *Pseudomonas, Enterobacter*) and Gram-positive spore forming bacilli (e.g., *Bacillus cereus*). Psychrotrophic microorganisms have been shown to produce heat-stable proteinases that may be responsible for decreasing cheese yields, and lipases that can cause rancid off-flavors in aged cheeses.[5]

Most cheeses are made from pasteurized milk (72°C for 15 sec). Pasteurization does not affect the physicochemical parameters of the milk significantly, but it destroys most of the pathogenic and spoilage bacteria contaminating the milk. Some nonstarter lactic acid bacteria (*Lactobacillus* spp., *Streptococcus* spp.) and, if present, spore-forming bacteria (*Clostridium, Bacillus*) can survive pasteurization and affect cheese ripening. Heating of milk before cold storage (63°C to 65°C for 15 to 20 sec) may be used for prolonged storage; however, the milk is still pasteurized before cheese making.[6]

8.4.3 Starter Cultures

Acid production during cheesemaking is essential in the formation of the gel from casein. Starter cultures promote rapid acid development during curd manufacture and contribute to distinct textural and flavor properties in the cheese after ripening.[5,8] Bacteria used as starter cultures generally belong to the genera *Lactococcus*, *Streptococcus, Leuconostoc,* and *Lactobacillus*. The typical mesophilic starter culture consists of *Lactococcus lactis* sp. *lactis (L. lactis)* and *L. lactis* ssp. *cremoris (L. cremoris)*. These starters are used for cheeses with moderate scald temperatures (< 40°C, Gouda, Edam, etc.). For cheeses requiring higher scald temperatures, thermophilic starter cultures are used (*Streptococcus thermophilus*, *Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus,* etc.). When "eyes" are required, the gas-forming species *L. lactis* ssp. *lactis* biovar. *diacetylactis, Leuconostoc mesenteroides,* or *Propionibacterium shermanii* can be used.

8.4.4 Clotting Methods

Most of the major international cheese varieties are rennet cheeses. A small percentage of cheeses are acid curd cheeses (cottage cheese, quarg, feta, Harz cheese) or heat/acid coagulated cheeses (ricotta). For rennet-type cheeses, the gel is formed at a higher pH than achieved by acid alone. The rennet gel is more elastic than an acid gel and shrinks and expels moisture in the presence of heat and acid. Rennet is used in cheese manufacture primarily to coagulate milk. However, residual rennet, which is kept in the curd, also plays an important role in the generation of flavor compounds during cheese ripening. In the past, the principal coagulant used was calf rennet, mainly consisting of chymosin and some pepsin or some microbial proteinases from

different mold species. Today a pure recombinant chymosin is increasingly used. Some well-known trade names are Chymogen, Maxiren, and Chy-Max.[5,9,10]

8.4.5 Removal of Whey and Salting

After cooking or scalding, the curd, containing the caseins and milk fat, is separated from the whey, containing lactose, whey proteins, and minerals. In the case of Cheddar cheese, the curd may be textured, milled, and salted. The curd of other cheese varieties may be hooped directly into perforated forms, and then an appropriate pressure is applied for draining. After whey removal, cheeses may be salted by applying dry salt to the cheese surface, or by immersing cheese blocks in brines containing 18% or more NaCl. Brining is performed on small-sized soft cheeses for up to 24 h, and for larger cheeses like Gouda, Edam, or Tilsit (> 2.5 kg). Aged brines develop a typical salt- and acid-tolerant microflora (10^4 to 10^6 colony forming units (CFU)/mL), often with *Debaryomyces hansenii* and *Staphylococcus equorum* as the predominant species. An influence of the brine microflora on cheese ripening is significant for surface-ripened cheeses, especially for smear cheese varieties.[5–7,10,11]

8.5 CHANGES IN THE COMPOSITION OF CHEESES RESULTING FROM FERMENTATION AND RIPENING

8.5.1 Initial Changes during Production

When the casein in milk is coagulated enzymatically or by acidic pH, the fat and casein of the milk are concentrated 6- to 12-fold. Cow's milk contains 3.3% protein (caseins and whey proteins). The primary proteolysis is mediated by chymosin, which specifically hydrolyzes the peptide bond Phe_{105}-Met_{106} of κ-casein. Upon cleavage, κ-casein loses its micelle-stabilizing properties. The solubility of the micelles is reduced, an aggregation occurs, and a coagulum is formed. The texture of the coagulum is dependent on the protein content of the milk, the pH, and the level of calcium ions in the milk. The gel starts to shrink (syneresis) when the coagulum is cut, and the water content of 87% is reduced to values between 20% and 56% in the curd, depending on the cheese variety. Syneresis of the coagulum is controlled by combinations of time, temperature, pH, agitation, and pressure. At the end of manufacturing, all rennet cheeses are essentially very similar. The calcium paracaseinate matrix with various levels of dispersed lipids and a moisture content in the range of 35% to 50% has a rubbery texture and is essentially flavorless.[1,12]

During manufacturing, lactic acid is produced first by starter lactic acid bacteria, and most of the lactose in the cheese is fermented to lactate in the first 24 h of pressing. For Dutch-type cheeses, lactose levels are already undetectable after brining. The rate and extent of acidification have a major impact on cheese texture via demineralization of the casein micelles. About 98% of lactose is removed in the whey during draining. The salt and moisture content of the curd determines the products of postmanufacture residual lactose fermentation. The complete and rapid metabolism of residual lactose by lactic acid bacteria is essential for the production of a good quality cheese.[5,13]

8.5.2 Cheese Ripening

The curd manufacturing process determines the basic composition and structure of cheeses. During ripening, cheeses develop their individuality and unique characteristics. Flavor and texture development is largely controlled by complex biochemical reactions, with glycolytic, proteolytic, and lipolytic activities being the primary events during cheese ripening.[13,14] The extent of protein and fat degradation is determined by the moisture, pH, and salinity in the cheese. Enzymes of various sources result in the production of peptides, amino acids, fatty acids, carbonyl components, and sulfur compounds. All biochemical reactions are usually limited to a certain degree because excessive lipolysis can lead to rancid flavors, and excessive proteolysis may produce bitter off-flavors or altered texture (softening) not characteristic for the specific product. The sources for cheese ripening enzymes are:

Indigenous milk enzymes (proteinases, lipases, phospatases)
Psychrotrophic nonstarter bacteria
Added calf rennet, rennet substitutes, or recombinant chymosin
Starter lactic acid bacteria
Nonstarter lactic acid bacteria
Secondary microflora (molds, smear bacteria, yeasts, etc.)

With high levels of nonstarter lactic acid bacteria (e.g., *Lactobacillus* spp., *Pediococcus* spp.) present in the cheese milk, D-lactate may be formed in addition to L-lactate. During ripening, these bacteria may racemize L-lactate to D-lactate, which may not be significant for flavor characteristics, but may have undesirable nutritional consequences, particularly in infants. In addition, calcium D-lactate is less soluble and may crystallize in cheese, causing undesirable white spots especially on cut surfaces. The nonstarter lactic acid bacteria have oxidative activities, oxidizing lactate to acetate. Acetate is usually present at high concentrations in Cheddar cheese and is considered to contribute to cheese flavor.[13]

The small amounts of citrate in milk can be metabolized by *L. lactis* ssp. *diacetylactis* and *Leuconostoc* spp. that release diacetyl and CO_2. The CO_2 production is responsible for the characteristic eyes of some Dutch-type cheeses. Citrate metabolism is very important for aroma and flavor formation in cottage cheeses and quarg.[13]

Milk contains a very potent lipoprotein lipase. This enzyme has probably no significance for cheeses made from pasteurized milk, as it is completely inactivated by the pasteurization process. It may cause significant lipolysis in raw milk cheeses. Lactic acid bacteria (LAB) (*Lactococcus* and *Lactobacillus*) have low but measurable lipolytic and esterolytic activities. Lipolysis is generally considered undesirable, because even a moderate level of free fatty acids makes cheeses smell rancid. The exceptions to this rule are certain Italian hard cheeses and blue-veined cheeses. The blue cheese flavor is dominated by methylketones and fatty acids.[13]

Phosphatases can play an important role in cheese maturation and cheese development because part of the casein is protected by phosphorylation and can only be degraded by the combined action of proteinases and phosphatases. The origin of acid

phosphatases is the bovine milk and starter lactic acid bacteria. The enzymes are also found in *Penicillium roqueforti*.[13]

8.5.3 Proteolysis

Proteolysis is the most complex of the three primary events of cheese ripening, and is probably the most important for development of flavor and texture. Proteolysis contributes directly to flavor via peptides, amino acids, and derivatives of amino acids (amines, acids, thiols, thioesters, etc.), an increased release of sapid compounds during mastication, a pH increase by formation of NH_3, and changes in texture from degradation of the protein matrix. Proteolysis is responsible for the generation of the smoother softer texture of the mature cheeses. Full flavor is probably caused by correct balance of a mixture of aromatic compounds (*component balance theory*). The products of primary proteolysis, the water insoluble fraction consisting of proteins and large peptides, have no flavor or aroma. Secondary proteolysis includes the water soluble nonvolatile fraction consisting of small peptides, amino acids, and organic acids, which contain most of the compounds responsible for flavor, whereas the aroma is principally found in the volatile fraction.[13,15]

8.5.4 Milk Proteinases and Rennet

Plasmin and plasminogen are associated with the cheese micelles and accompany them into the cheese curd. In milk, the most susceptible plasmin substrate is β-casein. Plasmin activity in cheese and rennet curd increases with cooking temperature, apparently due to the activation of plasminogen. This suggests the importance of plasmin activities is higher for cooked Swiss-type cheeses than for Cheddar or Dutch-type cheeses. Pasteurization increases plasmin activity in milk by inactivation of plasmin inhibitors or increasing the activation of plasminogen.[13]

Between 3% and 6% of the rennet added to the cheese milk is retained in the curd. This is influenced by the pH at whey drainage; with increasing pH, a smaller amount of rennet is retained in the curd. Very little coagulant survives the high cooking temperatures used for Swiss-type cheeses. In the initial stages of maturation, αs_1-casein is degraded by rennet. The amount of intact α_{s1}-casein is related to the firmness of the cheese. If the level of primary proteolysis is excessive, off-flavors due to bitter peptides can occur. β-Casein and α_{s1}-casein are both hydrolyzed by rennet *in vitro*. In Cheddar and Dutch-type cheeses, only α_{s1}-casein is completely degraded. In the cheese environment, the β-casein peptides liberated are different from the rennet specificity, suggesting that plasmin and bacterial proteinases are responsible for the hydrolysis.[13]

Proteolysis by rennet is believed to be responsible for the softening of cheese texture early during ripening via the hydrolysis of α_{s1}-casein to α_{s1}-I. Some rennet-produced peptides are bitter. Rennet-produced peptides can serve as substrate for microbial proteinases and peptidases that produce small peptides, which contribute to the background cheese flavor. All changes in cheese texture appear to influence the general release of aromatic compounds from proteolysis, lipolysis, and glycolysis. This may be the most significant contribution of this primary proteolysis to cheese flavor.[13]

8.5.5 Psychrotrophic Microorganisms

A very important source of potent lipases in milk is the psychrotrophic bacteria that dominate the microflora of the refrigerated milk. Some of these lipases are heat-stable, can absorb on the surface of fat globules, and can act in the cheese environment. The negative effects of the lipases of psychrotrophic bacteria in cheese making are most significant in terms of their effects on proteinases.[5]

8.5.6 Starter and Nonstarter Bacteria

LAB have complex amino acid requirement, and thus have a proteolytic system consisting of proteinases and peptidases that fulfill their needs for a nitrogen supply. Proteinases of starter lactococci are cell wall-bound; all the known peptidases in *Lactococcus* are intracellularly located. A number of peptidases of various specificities were identified in *Lactococcus* and *Lactobacillus* species: aminopeptidases, dipeptidases, tripeptidases, and oligo-endopeptidases, as well as some peptidases with specificities for proline residues like the prolinase and prolidase. Starter bacteria reach maximum numbers shortly after the end of manufacture, and viable cell counts decline quickly thereafter. It can be assumed that part of the bacterial cells lyse after cell death, liberating the intracellular enzymes into the cheese, which promotes the degradation of peptides and proteins.[8,16]

Thermophilic starters are used for a number of cheeses such as the Swiss-type cheeses and Parmesan and Romano; the species *S. thermophilus, Lb. delbrueckii* ssp. *bulgaricus, Lb. delbrueckii* ssp. *lactis*, and *Lb. helveticus* are used. Lactobacilli are generally used in combination with *S. thermophilus* because of mutual growth stimulation, e.g., *S. thermophilus* is stimulated by free amino acids produced by *Lb. bulgaricus*, whereas the production of formic acid and CO_2 by *S. thermophilus* stimulates the growth of *Lb. bulgaricus.* In Swiss-type cheeses, propionibacteria metabolize lactate to propionate, acetate, and CO_2, which is responsible for eye development. They contribute to proteolysis significantly, and are responsible for the high concentrations of proline in Emmental cheese, which contribute to the sweet taste of Emmental.[5,8,13]

Nonstarter lactic acid bacteria (NSLAB) reach populations of 10^8 CFU/g during the ripening of many cheeses. Lactobacilli usually dominate the flora. In pasteurized milk, NSLAB originate mainly as postpasteurization contaminants from the factory. They are a very heterogeneous group, and include thermophilic and mesophilic, heterofermentative as well as homofermentative species. NSLAB have a significant impact on cheese quality. Due to the variability, the number, and the species composition of the NSLAB population, the contribution of these bacteria is difficult to characterize in detail. Sometimes pediococci are the predominant NSLAB species. They are weakly proteolytic and lipolytic, and their main contribution to cheese flavor may be their ability to oxidize lactate to acetate. They are also able to reduce acetaldehyde and propionaldehyde to alcohols, and can produce diacetyl.[5,13]

8.5.7 Secondary Microorganisms

In surface ripened cheese varieties, yeasts and molds metabolize lactate to CO_2 and H_2O causing an increase in pH. Amino acids are also metabolized, which liberates NH_3,

further increasing the surface pH. The elevated pH stimulates the action of plasmin, which together with the coagulant, is responsible for proteolysis in the cheese.[11,14,17]

Molds like *Penicillium roqueforti* and *P. camemberti* possess a strong proteolytic system and are responsible for the liberation of amines resulting from decarboxylation, ammonia, keto acids, carbonyls, alcohols from deamination, other amino acids resulting from transaminations, and hydrogen sulfide, methanethiol, thioesters, and other sulfur compounds from conversion of cysteine and methionine.

Brevibacterium linens, Corynebacterium spp., *Microbacterium* spp., *Arthrobacter* spp., "food-grade" *Staphylococcus* species (formerly described as *Micrococcus* sp.) have active proteolytic systems as well as strong amino acid converting properties. The main contribution of the surface smear bacteria is the liberation of highly aromatic sulfur compounds released by the catabolism of amino acids.

8.6 CHEESE AS A CARRIER FOR PROBIOTIC MICROORGANISMS

8.6.1 General Considerations

When used in marketed probiotic products, probiotic bacteria have to fulfill a number of basic technological requirements when used in commercial probiotic products. Most importantly, probiotic bacteria have to be present in sufficient numbers in the product at the date of consumption, and their properties essential for expressing health benefits after consumption have to be maintained up to that date. In addition, no adverse effects on taste and aroma of the product should be exerted by the probiotic organisms.

For exploitation of the functional properties of the probiotic bacteria, the processes of manufacture of cheese products may have to be modified and adapted to the requirements of the probiotics. When this is not possible, either other probiotic strains may be applied or new products may have to be developed. During recent years, several review articles have addressed the problem of introducing probiotics in cheese.[18–21] Some of the parameters necessary for influencing the application of probiotic bacteria in cheese will be addressed in the following text.

Dairy products containing living bacteria have to be cooled during storage. This applies in particular to products containing live probiotic bacteria. Cooling is necessary to guarantee high survival rates of the probiotics, and to yield sufficient stability of the product.[22,23] In addition, oxygen content, redox potential, and water activity of the product have to be considered,[24] as the target of probiotic bacteria is the intestinal tract. This may be of considerable importance for prepackaged cheese. Cooling of probiotic cheese is also necessary to reduce or inhibit the interaction of the active microorganisms with the components of the food. The degree of interaction depends on the kind and amount of carbohydrates available, degree of hydrolysis of milk proteins and the availability of essential amino acids, and composition and degree of hydrolysis of milk lipids, determining the availability of short chain fatty acids.[13,25] However, the proteolytic[26] and lipolytic properties of the probiotic bacteria may have considerable effects on taste and flavor of the product.[13]

Interactions between probiotics and starter organisms also have to be considered. The intensity of interaction very much depends on the time the probiotics are added

to the product, whether they are present during or added after fermentation. If they are added after fermentation, interactions may be kept to a minimum, since addition is possible immediately before or even after cooling below 8°C, and metabolic activities of starters and probiotics are drastically reduced at these temperatures. Nevertheless, during extended storage, even small interactions may yield measurable effects. These considerations, however, are not entirely new, because application in cheese manufacture of adjunct cultures[25] has already created some experience in the interaction of starter cultures with additional active bacteria.

Antagonism between bacteria is often based on the production of metabolites that inhibit or inactivate more or less specifically other related starter organisms or even unrelated bacteria. Although antagonism caused by bacteriocins, peptides, or proteins exhibiting antibiotic properties[27] has been described as a limiting factor for combinations of starters and probiotics,[28] antagonism caused by other substances also has to be considered. Substances that may be involved are hydrogen peroxide, benzoic acid, biogenic amines, and finally lactic acid.[29–33]

If probiotics are added to the cheese after fermentation, the physiological state of the probiotics may be of considerable importance for survival during ripening and storage.[34–36] This state depends on:

1. The nutritional composition of the growth medium of the probiotics in relation to the nutritional composition of the cheese to which they will be added
2. Harvesting of the culture (whether in logarithmic or stationary phase)
3. Conditions leading to transition to stationary phase 4
4. Treatment of the probiotics during and after harvesting

However, clues on how to handle probiotics may be drawn from current experience in production of commercial starter cultures.[37]

8.6.2 Fresh Cheese as a Carrier

Due to its manufacturing process, fresh cheese appears to be ideally suited to serve as a carrier for probiotic bacteria. One reason is that fresh cheese is an unripened cheese; thus, storage occurs at refrigeration temperatures, shelf life is rather limited, and no prolonged periods of ripening are necessary. As an example of fresh cheese, cottage cheese will be discussed in some detail. For this type of cheese, two options exist for the addition of probiotics:

1. Together with the starter culture
2. Together with cream and salt immediately prior to packaging

For addition together with the starter culture, two problems can be seen:

1. The number of the probiotic bacteria in the final product may be difficult to control, since a considerable number of bacterial cells are lost from the coagulant during drainage of the whey.

2. Survival of the probiotic bacteria in the product may be negatively effected by the rather high scalding temperature of up to 55°C.

An advantage for the producer would certainly be the fact that the size of the probiotic inoculum, and thus the costs would be rather small. However, for cottage cheese, addition of the probiotics together with cream and salt appears to be a desirable alternative. The advantages are that the numbers of probiotics added can be exactly controlled, adverse effects of the high scalding temperature are avoided, and after addition and mixing, the product can be immediately cooled to below 8°C.

To date, two reports on the suitablity of fresh cheese acting as a carrier for probiotic bacteria have been published. In one report,[38] probiotic bacteria (*Lactobacillus acidophilus, Lb. casei, Bifidobacterium bifidum, B. longum*) were added as adjuncts, and survival during refrigerated storage was analyzed. The data showed that although viable counts decreased in 16 days by about one log, final counts after this period were still acceptable. This agrees with data on survival of lactobacilli of the *Lactobacillus acidophilus* group.[39] In contrast, Blanchette et al.,[40] reported an increase of *B. infantis* within the first day after manufacture. However, large losses in viability were observed after 15 days at 4°C.

In Germany, the first cottage cheese called "probiotic" appeared on the market in 1998. The product contained *Lb. acidophilus* La5 and *B. animalis* BB12. However, while the probiotic properties of the bacterial strains added to the product have been demonstrated,[41] the product itself has never been tested for probiotic properties (health benefits).

8.6.3 Ripened Cheese as a Carrier

To apply probiotics in ripened cheese, the same considerations as for cottage cheese must be taken into account with regard to the time of addition of the probiotics and impairment of survival by the scalding temperature. In ripened cheese, however, the long period of ripening causes an additional problem. It is by no means clear to what extent different probiotic strains will survive the ripening period, and to what extent their functional properties will be conserved during this period. Several reports for ripened cheese have been published. In these reports, probiotic strains of the following species were added: *Lb. acidophilus, Lb. brevis, Lb. casei, Lb. paracasei, Lb. rhamnosus, Bifidobacterium animalis, B. bifidum, B. infantis, B. lactis, B. longum, Enterococcus faecium.*

Although several studies showed either unsatisfactory survival of the probiotic microorganisms during ripening or adverse effects on flavor,[42,43] the suitability of the approach of adding probiotics as adjuncts together with the starter culture appeared to be successful in others.[44–57]

Different approaches have been applied to improve survival of the probiotic microorganisms during ripening. These approaches include microencapsulation,[58] immobilization on fruit pieces,[59] addition of growth-stimulating factors,[60] addition of differently prepared cultures,[61] and ripening of vacuum-packaged cheeses.[62]

In 1999, a patent for production of probiotic cheese was granted,[63] and in 2000, probiotic cheese containing *Lactobacillus* GG was introduced into the Finnish market.

Lb. GG is one of the best characterized probiotic bacterial strains with well-established probiotic properties.[64] However, no data exist on its probiotic properties when supplied in a cheese matrix.

8.7 RESULTS OF IN VITRO/ANIMAL TESTS SHOWING BENEFICIAL EFFECTS OF CHEESE

As explained in Section 8.6, fresh and ripened cheeses are believed to be appropriate vehicles for probiotic bacteria. It has been postulated that the embedding of probiotic bacteria in the fat–protein matrix of cheese as well as the buffering capacity and the low acidity of ripened cheese may assist survival of probiotic bacteria during gastrointestinal passage. Vinderola et al.[35] have demonstrated pH tolerance of strains of *B. longum*, *B. infantis*, *Lb. acidophilus* and *Lb. casei* in homogenates of Argentinian fresco cheese using an HCl solution of pH 3. *Propionibacterium freudenreichii* and *P. acidopropionici* suspended in Emmental juice showed survival and tolerance against artificial gastric juice and intestinal fluid.[65,66] In two feeding trials involving three pigs or eight pigs per group, respectively, it was demonstrated, that consuming Cheddar cheese containing *Lb. paracasei* NFBC 338[46,49] or *E. faecium* PR 68[47,48] led to significantly higher mean fecal counts of probiotic bacteria than consuming yogurt produced with the same bacteria.

Furthermore, the long ripening time of many hard and extra-hard cheeses possess no basic hurdle for the production of probiotic cheese. Thus, strains of *Bifidobacterium* sp., *Lb. casei, Lb. paracasei* and *Lb. rhamnosus* survived the 32-week ripening time of Cheddar cheese with counts between 1.6×10^7 and 9×10^8 CFU/g.[67] This is more than the $> 3 \times 10^6$ CFU/g which cheese must contain in order that 1 to 3 slices of hard cheese (~30 g each) provide about 10^8 probiotic bacteria per day; an amount which in many controlled intervention studies was sufficient to exert significant probiotic effects.

To obtain fresh and ripened cheese containing probiotic microorganisms in sufficient numbers, two different approaches have been used:

For one, cheese starter and ripening cultures were screened for potentially probiotic microorganisms. In this process, an acid-tolerant and bile salt-tolerant *L. rhamnosus* was found in Italian Parmigiano Reggiano cheese,[68] and potentially probiotic strains were found among the yeasts in commercial blue cheese.[69] In another approach, bacteria cultures with proven probiotic properties were added during cheese processing, either with the conventional starter culture or separately. Table 8.5 lists those strains which have already been applied for the production of probiotic cheese.

However, most of the propagated beneficial effects of these bacteria were examined in clinical trials, where probiotic bacteria were provided in fermented, yogurt-type milk products or as lyophilized pharmaceutical preparations. Accordingly, up to now, most of the published investigations have been confined to provide proof of survival of probiotic bacteria in the cheese matrix, but clinical studies showing beneficial health effects of the consumption of cheese containing probiotic bacteria have not been carried out, although this is an essential prerequisite for the use of the claim probiotic.[70]

TABLE 8.5
Probiotic Bacteria Strains Used in Cheese Making and Postulated Health-Related Effects

Reference	Strain/Strains	Cheese	Survival in cheese CFU/g cheese (time of storage)	Effects (tested in cheese)	Postulated strain specific beneficial effects (on)[d]
Valio, Finland 2000 (in preparation)	*Lb. rhamnosus* GG	Gefilus cheese: Emmentaler, Edam	3–4 slices of Edamer are equivalent to 150 mL yogurt		Survival of gastrointestinal passage, colonization of the gut[83]; rotavirus-induced diarrhea[84,85]; traveller's diarrhea[86]; antibiotic-induced diarrhea[87,89]; morbus Crohn[90]; obstipation[91]; premature infants[92]; immune modulation[93–95]; allergy, atopic diseases[96,97]; prophylaxis of respiratory and gastrointestinal infections[98]; decrease of cancer promoting enzymes[91]; reduction of caries risk[99]
Hansen, Denmark	*Lb. acidophilus* LA 5 + *B. animalis* BB12	Soft cheese			Survival of gastrointestinal passage[100,101]; rotaviral diarrhea[102]; traveller's diarrhea[103]; antibiotics induced diarrhea[104]; infants[101,105]; modulation of the immune system[106,107]; allergy[96]; cancer[108]; serum cholesterol[109]
Auty et al.[82]	*B. animalis* BB12	Cheddar	$6*10^7$ (2 months)		
McBrearty et al.[83]	*B. animalis* BB12	Cheddar	$\geq 10^8$ (6 months)		
McBrearty et al.[83]	*B. longum* BB536	Cheddar	~10^5 (6 months)		Antibiotics-induced diarrhea[110]
Stanton et al.[46]	*Lb. paracasei* NFBC 338	Cheddar	Survival and growth in cheese >10^8 (6 months)	Better fecal recovery in Cheddar than in yogurt[c]	Bile tolerance[49]
Gardiner et al.[47,48]	*E. faecium* PR 88	Cheddar	Survival and growth in cheese >10^8 (15 months)	Better survival of GI passage of *E. faecium* PR 88 in cheddar than in yogurt[c]	Bile and acid tolerance: Alleviation of symptoms of irritable bowel syndrome[b,111]

(continued)

TABLE 8.5
Probiotic Bacteria Strains Used in Cheese Making and Postulated Health-Related Effects (continued)

Reference	Strain/Strains	Cheese	Survival in cheese CFU/g cheese (time of storage)	Effects (tested in cheese)	Postulated strain specific beneficial effects (on)[d]
Gomes et al.[43]	*B. lactis* Bo + *Lb. acidophilus* Ki	Gouda; goat cheese	Bo: $>10^8$ (9 weeks) Ki: $>10^6$ (9 weeks)		Bile tolerant, Establishment in intestinal ecology[112]; cholesterol control[113]; bactericidal effects on *S. typhimurium* or *C. difficile*[114]
Vinderola et al.[38]	*B. longum, B. bifidus, Lb. acidophilus, Lb. casei*	Argent. fresco	-1Log_{10}/2 months or less	3 h survival in a cheese-HCl-homogenate of pH 3	
Zarate et al.[65] Jan et al.[66]	*Propionibact. freudenreichii/ acidipropionici* isolated from cheese	Emmentaler-like		Survival in cheese juice of bacteria exposed to artificial gastric and intestinal fluid. Bile and acid tolerant	

[a] Feeding 10^9 or 10^{11} CFU/d NFBC 338 in cheddar or yogurt, respectively, to 3 pigs led to a recovery of 10^5 or $10^{4.5}$ CFU/mL small intestinal chyme.

[b] After an initial load by gastric intubation, 17 patients with otherwise incurable IBS received for 4 to 30 months lyophilized *E. faecium*. Weekly examination of fecal samples; assessment of condition scores before and after treatment.

[c] Feeding 1.3×10^{10} or 3.7×10^9 CFU/d PR 68 in Cheddar or yogurt, respectively, to eight pigs led to a fecal recovery of 2×10^6 or 5.2×10^5 CFU/g feces.

[d] Not tested in cheese.

In some cases, the influence on immune parameters and risk factors has been examined rather than the true health effect. Examples are an increase in serum IgG following the consumption of Cheddar containing *E. faecium* PR 68,[48] the maintenance of the antioxidative and antimicrobial activity of *Lb. fermentum* ME-3 after incorporation into an Estonian semihard cheese,[71] or a reduction in fecal coliform count (from 7.5 to 5.0 log CFU/g), and fecal activity of the procarcinogenic enzyme β-glucuronidase in rats fed Edam cheese containing probiotic B. bifidum for over 15 days.[72]

In other incidences, the health effects were, according to conventional definitions, not truly probiotic:

1. The high microbial β-galactosidase-activity in Canestrato hard cheese[44] or Cheddar-like cheese[45] supports lactose digestion, and may, therefore, reduce gastrointestinal complaints in lactose-intolerant people. But this effect does not depend on the viability of the microorganisms, and has not been tested in lactose malabsorbers.
2. Protection of the oral cavity against candida overgrowth[73] is an effect that takes place outside the gut, and is not mediated by the gut-associated immune system (GALT). Therefore, this can be called a probiotic effect only within an extended definition of "probiotic."
3. A so-called bifidogenic growth stimulator (BGS) was produced by *Propionibacterium freudenreichii* in Swiss cheese, which improved the health condition in 12 patients with ulcerative colitis, when administered for 4 weeks in addition to the baseline anti-inflammatory therapy in an open label study.[74] However, this was a prebiotic rather than a probiotic effect.
4. Several bioactive peptides, which are released from α_{s1}-casein, β-casein, β-lactoglobulin, or α-lactalbumin during digestion, or by the proteolytic activity of certain microorganisms and the sequences valine-proline-prolin (VPP) and isoleucine-proline-proline (IPP) from β-casein, have been shown to lower enhanced blood pressure.[75] This is thought to be due to an angiotensin converting enzyme (ACE)-inhibitory activity. ACE has a blood pressure raising effect, by promoting the conversion of Angiotensin I into the vasoconstrictive Angiotensin II, and at the same time inactivating the vasodilating hormone Bradykinin.

For example, aqueous solutions of VPP + IPP inhibit the increase in blood pressure with age in spontaneously hypertensive rats,[76] whereas casein[77] and whey protein hydrolysates[78] or low fat milk fermented by *Lb. helveticus* (~3.5 g/100 mL VPP+IPP) have a clinically proven anti-hypertensive effect (SBP: −6.6 to −11 mm Hg; DBP: −3.6 to −7 mm Hg).[79]

ACE-inhibitory bioactive peptides are also released in cheese during ripening by microbial proteolysis. Semihard cheese, hard cheese and extra-hard cheese, therefore, contain physiologically relevant amounts of VPP + ICP (3.6 ± 4.0, 9.3 ± 5.3, and 12.7 ± 12.8 mg/100g, respectively; mean of 33 cheese samples), and show ACE-inhibitory activity *in vitro*.[80] However, a significant decrease in blood pressure in vivo was only demonstrated in rats fed Festivo cheese.[81] Again, survival of starter

microorganisms in the product and during gastrointestinal transit is not required for the exertion of this health effect, which, therefore, is not truly probiotic.

8.8 CONCLUSIONS

Several reports have been published, in which the production of probiotic cheese has been described. However, designation as "probiotic" relies on the application of bacterial strains for which probiotic properties have been demonstrated. So far, no clinical studies have shown that cheese may in fact serve as a functional carrier for probiotics.

Compared to yogurt, the problem for cheese—especially semihard and hard cheese—acting as a carrier for probiotics results from the high fat and salt content, and the relatively low recommend daily intake. From the latter, it follows that the concentration of probiotics should be about 4 to 5 times higher than in yogurt. However, this does not apply to fresh cheese like cottage cheese, which can easily be adjusted to low fat and salt contents, and for which recommended daily intake is rather high. It may, thus, serve as a food with a high potential to be applied as a carrier for probiotics.

Therefore, in countries, in which yogurt or other fermented milks are less popular, and for lactose-intolerant persons, who do not even tolerate yogurt, probiotic cheese may be a realistic alternative.

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9 Natto

A Soybean Food Made by Fermenting Cooked Soybeans with Bacillus subtilis (natto)

Tomohiro Hosoi and Kan Kiuchi

CONTENTS

9.1 FERMENTED SOYBEAN FOODS IN ASIA

Fermented soybean foods made with *Bacillus subtilis* cells are produced in China, and they are called *dou chi* (or *dauchi*). They include salted, sweet, and nonsalted types. The salted type of dou chi (*xian-dou chi*) contains 10% to 20% salt to inhibit their putrefaction by contaminating bacteria. The most typical sweet dou chi is called *tian-dou chi*: it is used as a seasoning for Beijing duck. Nonsalted dou chi has been developed into various kinds of *natto*.[1] (See Chapter 16 for more information on Chinese fermented foods.) Food of this type is called *itohiki-natto* (hereafter shortened to "natto") in Japan, *kinema* in Nepal and Myanmar, *tua nao* in Thailand, and *chungkuk-jang* in Korea. Natto is produced only with *B. subtilis* (*natto*) (formerly called *B. natto*, see Section 2.1).

These fermented soybeans are consumed in a variety of forms. For instance, tua nao is used as a raw ingredient in salads. *Chungkuk-jang*, which contains cayenne peppers and garlic, is used as an ingredient for a Korean soup called *chige*. In Japan, natto is mixed with soy sauce, sliced Welsh onion (similar to stone leeks), mustard, dried seaweed, and/or raw egg. This seasoned natto is usually eaten with rice in Japan. A looser natto (*hikiwari-natto*) and a dried type of natto are also used. Before World War II, the transportation infrastructure was not well developed in Japan. Hence, dried, hard natto, having a long shelf life, was usually manufactured and consumed. Of the three types of natto, itohiki-natto is the most popular at present in Japan. Hikiwari-natto is used for preparing sushi. Consumption of natto increased compared to other soybean foods during the 1990s, generating sales of 160 billion yen in 1996.[2] (See Chapter 1 for more details on the history of natto.)

9.2 INGREDIENTS OF NATTO

The raw materials required for natto production are *B. subtilis* (*natto*) spores called natto bacilli, soybeans, and water. *B. subtilis* (*natto*) strains are used to initiate fermentation of steamed whole soybeans. The quality of natto is affected by the quality of the soybeans and the *B. subtilis* (*natto*) strains but not the water. Normal tap water is adequate for the production of natto.

9.2.1 *Bacillus subtilis* (*natto*) Spores

In 1913, Dr. S. Sawamura of Imperial University of Tokyo isolated natto bacillus and named it *Bacillus natto*.[3] The first commercially available natto using a pure culture of *B. natto* was sold through the efforts of Professor J. Hanzawa of Hokkaido Imperial

University in 1928.[4] In 1957, *B. natto* strains were included in and documented as *B. subtilis* strains in the 7th edition of *Bergey's Manual of Determinative Bacteriology*.[5] However, only *B. natto* and no other *B. subtilis* strains are used for natto production. This is because natto with desired characteristics can be produced only with *B. natto* strains.[6] Using *B. natto* strains yields natto with a distinctive aroma, a wrinkly bacterial layer on the surface of the soybeans, and a desirable degree of stickiness. When natto of high quality is picked up, it tends to elongate into strings of soybeans and not to lump. In addition, biotin greatly enhances the growth of *B. natto*.[7] Japanese scientists, therefore, usually refer to the natto bacillus as *B. subtilis* (*natto*) to distinguish it from other strains of *B. subtilis,* which are not suited for natto production. Lyophilized spores of *B. subtilis* (*natto*) or spore suspensions in water are sold as a starter for natto by three companies in Japan. Some natto manufacturers have utilized their own *B. subtilis* (*natto*) strains to produce natto of characteristic quality. Contamination of natto products by other microorganisms must be avoided as it largely affects the quality of natto and may cause food poisoning.

9.2.2 Soybeans

The total volume of soybeans consumed in Japan has been approximately 5 million metric tons over the past ten years.[8] Total consumption of soybeans for food production was 1.01 million metric tons in 2000.[9] Japan imports the majority of the soybeans (more than 80%) required for domestic food production. There was a 13% increase in soybean consumption for natto products between 1991 and 2000, and consumption reached 122,000 metric tons in 2000.[9] Domestic soybeans are considered superior to foreign ones for natto production by Japanese natto manufacturers, although the supply of Japanese-grown soybean is insufficient to meet the demands for natto production. Because they are grown in limited domestic regions and their production is small, Japanese soybeans command a high price. Considering the low rate of self-sufficiency in soybeans, and to increase quality using domestic soybeans, a national project has been initiated to increase the production of domestic cultivars of soybeans in Japan. To date, some new cultivars of soybeans have been bred in many districts of Japan and have been used in the production of natto.[10] Names of these soybean cultivars and the location where they were bred have been compiled and published by the Japanese Ministry of Agriculture, Forestry, and Fisheries.[11]

Natto manufacturers prefer certain kinds of soybean cultivars, and their preferences can vary from region to region because of consumer preferences. Numerous processing tests have been conducted in an attempt to elucidate which cultivars of soybeans are most appropriate for making high-quality natto. Popular soybean species for natto production are "suzuhime" and "suzumaru," grown in Hokkaido; "kosuzu" in Iwate, Miyagi, and Akita Prefectures; and "natto-shoryu," in Ibaraki Prefecture.

Desirable qualities of soybeans for natto are generally as follows:

1. Extra small or small size
2. Easily washable
3. Yellow surfaces and hila
4. A suitable degree of stickiness when made into natto

5. Sweet taste
6. Slight changes in constituents during storage

9.2.2.1 Color

Soybeans with a brilliant light yellow or yellow skin and a light yellow hilum are favored. Natto made from brown or black soybeans have traditionally not sold well in Japan due to its appearance and lack of characteristic aroma. However, natto made from black soybeans has recently been sold, and its high content of polyphenols has been emphasized.

9.2.2.2 Size

Soybean size is classified by diameter into four groups in Japan: extra small, less than 5.5 mm; small, 5.5 mm to 7.3 mm; medium, 7.3 mm to 7.9 mm; and large, more than 7.9 mm. Consumers in Kanto and more northern regions of Japan regard the extra small and small soybeans as the most suitable ones for natto. On the other hand, those in Kansai region prefer larger soybeans. Natto processed from small and extra small soybeans tends to have a distinctive natto flavor and a strong taste due to excess fermentation. It was reported that activities of alkaline protease (subtilisin) were higher in natto produced from small soybeans than that from large soybeans.[12,13] However, the activities of neutral proteases (metalloprotease) did not differ with the size of soybeans used.[12] This result suggests that degradation of proteins in natto produced from small soybeans progresses more strongly than that from large soybeans during the latter half of the fermentation period when the pH value of the product is increased by metabolites of the *B. subtilis* (*natto*) cells. This may cause the taste and smell of natto from small soybeans to be stronger than that made from large soybeans. Natto made from large soybeans has a weak smell of ammonia and shows a low degradation rate of proteins, although its nattto-like taste is still perceivable.[12]

9.2.2.3 Protein Content

B. subtilis (*natto*) cells utilize the proteins, peptides, and amino acids in soybeans for their growth. The kinds and quantities of peptides and amino acids produced by the activities of *B. subtilis* (*natto*) during fermentation affect the flavor appeal of natto. Hence, soybeans with high protein content are preferred. By these criteria, "suzuhime" and "zizuka" cultivars, with high protein contents, bright color, and polished appearance, are highly regarded for natto production.

9.2.2.4 Sugar Content

To produce a natto with good quality and flavor, it is important that available carbohydrates be supplied to *B. subtilis* (*natto*), and that the hydrolysis of proteins proceeds appropriately during fermentation. Because extra small soybeans tend to have a higher sugar content compared to larger soybeans, they are regarded as superior for the production of natto. Because the storage life of natto is determined by the ammonia flavor, the content of ammonia is considered to be the important quality control characteristic in natto production.[14] However, it has also been reported that

soybeans with high sugar content are not necessarily best; free sugar content is more important than total sugar content for natto processing.[15,16] This is because *B. subtilis* (*natto*) can utilize certain saccharides such as sucrose, raffinose, and stachyose but not starch for growth.[17]

Small soybeans tend to become softer than medium or large ones do, when they are steamed under identical conditions.[14] A positive correlation between the firmness of steamed soybeans and the ammonia nitrogen level of natto products has been reported.[14] In this report, it was suggested that in hard, steamed soybeans, hydrolyses of the constituents by *B. subtilis* (*natto*) cells probably occurs just under the surface of the soybeans. Degradation and usage of the constituents inside the soybeans by the bacteria does not occur quickly. This means that *B. subtilis* (*natto*) cells can only utilize sugars near the surface in a relatively short time, and then protein degradation begins in the early stages of fermentation. This induces a strong ammonia flavor. Hence, it is important to select soybeans with a high sugar content and to steam them until they become soft. A typical steam treatment is 1.5 kg/cm^2 steam pressure for 20 min.

9.2.2.5 Washing and Storage Methods

Natto is produced all year round, and therefore, harvested soybeans must be stored before they are processed. Soybeans dirty with soil are cleaned, washed, and then stored for months in a cool room. It is desirable that they be stored in a refrigerated room at a temperature below 15°C and a relative humidity about 60%.[18] If soybeans are stored between 25°C and 35°C, the raffinose in soybean increases and the stachyose decreases.[18] Germination rates of soybeans are often examined to check their quality. A low germination rate suggests that the soybeans have been preserved under undesirable temperature and humidity conditions.[19] There was a negative correlation between germination rates and solid matter content of soybeans in soaked water.[19] The quality of natto using such soybeans is generally not acceptable. Therefore, soybeans with low germinative rate are not suitable for natto production. However, germinated soybeans are not used to produce natto.

9.3 NATTO PROCESSING

Natto is processed as shown in Figure 9.1.

9.3.1 Washing and Soaking of Soybeans

Sieves are used to separate small or extra small soybeans at the beginning of the process. Contaminant and foreign substances, such as plant stalks and leaves, soil, and sand, are removed. Soybeans are weighed, washed in a screw wash press, rubbed in a maelstrom flow, polished with burhstone, and finally washed in clean tap water. Soybeans must be softened in water before natto processing because natto produced from unsoftened soybeans is too hard to eat. Soaking should be performed until the weight of soybeans increases approximately 2.2- to 2.7-fold.[14,20] To achieve this, soybeans are soaked in tap water at 10°C for about 20 h. It is important to clean the soaking tanks regularly because these tanks are prone to contamination with lactic acid-producing bacteria. Lactic acid may inhibit the growth of *B. subtilis* (*natto*) and

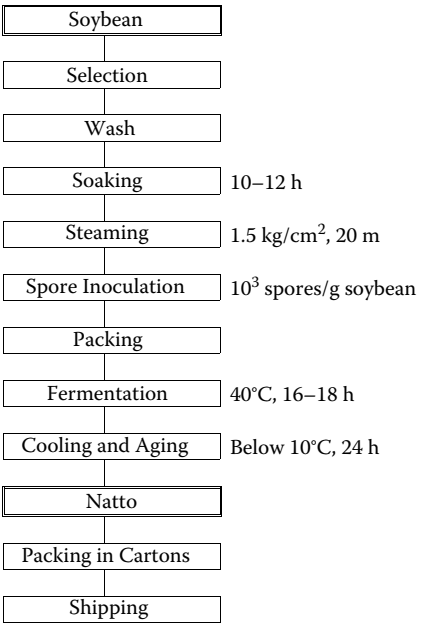


FIGURE 9.1 Procedure of natto processing

impair fermentation. Some processors soak soybeans overnight, flowing tap water through the tanks for cooling. However, too much changing of water during soaking or soaking for too long will reduce the taste of the natto.

9.3.2 Steaming of Soybeans

After soaking, soybeans must be steamed (using steam at 1.5 kg/cm^2 pressure for 20 min) to soften the beans further and denature undesirable soybean proteins such as hemato-glutenin and trypsin inhibitor.[21] At the same time, contaminating bacteria are killed. Steaming vats that can hold 60 to 120 kg of raw soybeans at a time are usually used.

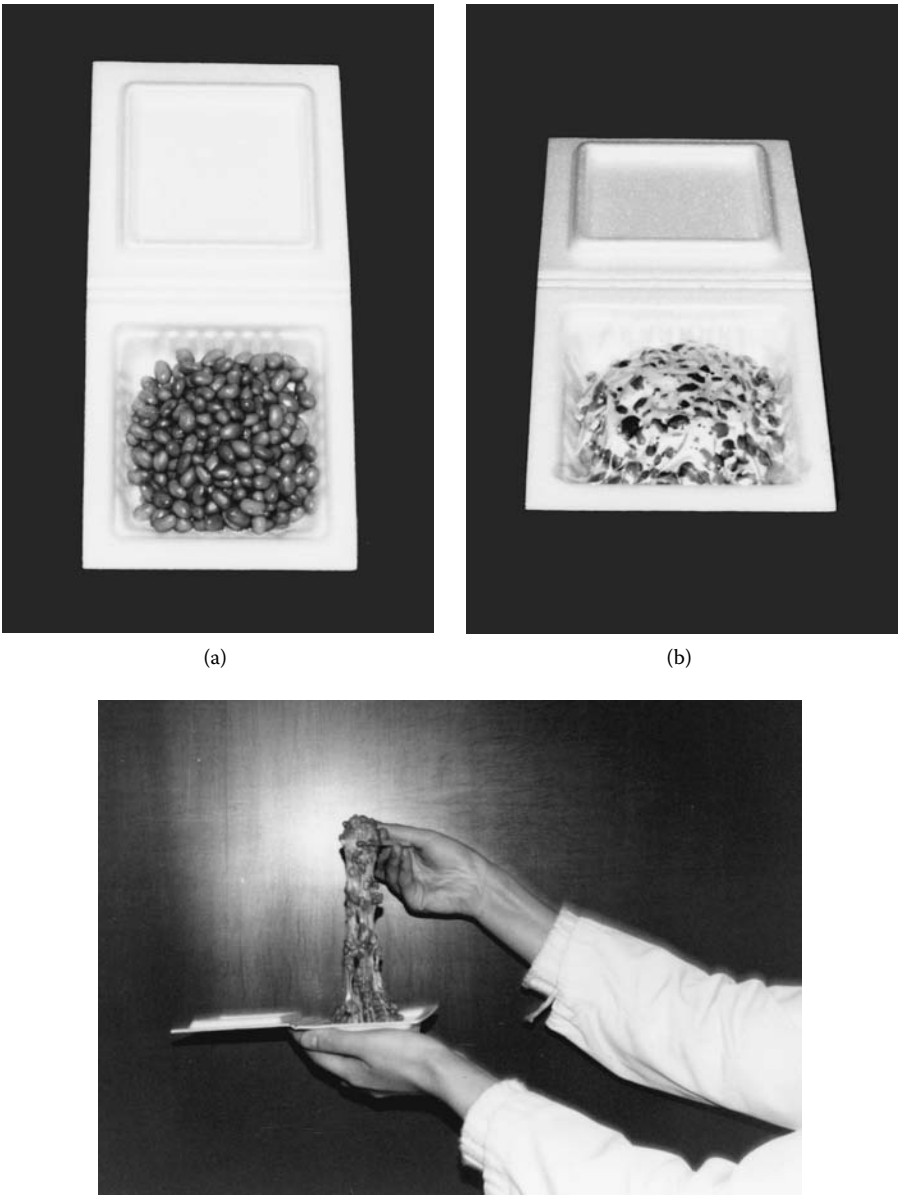
9.3.3 Inoculation with *Bacillus subtilis* (*natto*) Spores

B. subtilis (*natto*) take both vegetative cell and spore form. The spores are more suitable for storage. Therefore, spore suspensions for natto production are sold. Immediately after steaming, while the soybeans are still hot (e.g., 85°C), the soybeans are tipped from the vat and sprayed with a *B. subtilis* (*natto*) spore suspension. The concentration of *B. subtilis* (*natto*) spores in the soybean stock should be approximately 10^3 colony forming units (CFU)/g soybean. Inoculation of *B. subtilis* (*natto*) spores at much higher concentrations inhibits the development of the desired stickiness and appearance.[12]

9.3.4 Packaging

Paper cups or polystyrene paper trays are commonly used to package 25 to 150 g of processed soybeans. The most common size holds 50 g of natto (Figures 9.2a and

9.2b). The historical changes in packaging methods are described in Section 3.7. In recent years, almost all filling processes are mechanized, and manual assistance is not required. In general, 60 g of steamed soybeans inoculated with *B. subtilis* (*natto*) spores are packed as a 50 g package by an automatic filler because moisture



(a) (b)

(c)

FIGURE 9.2 Photographs of natto: (a) natto in polystyrene paper tray package; (b) natto covered with polyethylene film with numerous holes; (c) natto stirred with chopsticks.

loss causes an approximate 20% reduction in weight during fermentation. After filling, soybeans inside the packages are covered with a perforated polyethylene film (Figure 9.2b). Plastic sachets of soy sauce seasoning and other condiments, such as mustard and freeze-dried sliced Welsh onion, can be put on the films. The lid of the package is fixed in place with a bond, using a heat sealer or a rubber ring.

9.3.5 Fermentation

Fermentation rooms are equipped with ventilators and air conditioners in order to control the humidity of the fermenting soybeans. Conditions in the fermentation room are controlled automatically by a computer for optimum fermentation. Packages are placed horizontally in a box and stacked in the fermentation room. To produce high quality natto, a sufficient oxygen supply and a fine adjustment of room temperature, humidity, and fermentation period are essential.[22] Typical fermentation conditions and product status are as follows. The room temperature is set at 40°C, and the initial humidity is controlled between 85% and 90%. The humidity is reduced to 75% between 6 and 16 h and to 55% between 16 and 24 h. Under these conditions, after 8 h of fermentation, a viscous substance begins to be produced, creating the stickiness of natto. After 10 h of fermentation, the internal temperature of the product begins to increase, and it reaches 48°C to 52°C by hour 14. If this optimum temperature of the product is not reached (e.g., too low or too high), the quality of the final natto tends to be lower. The temperature largely affects the stickiness and taste of natto. Thereafter, the temperature of the product is gradually dropped down to 40°C. After 18 to 20 h of fermentation, the temperature of the fermentation room is set so that the product temperature decreases to 10°C at hour 24. At hour 24, the product in the boxes is transferred from the fermentation room to a refrigerated room where the temperature is below 10°C and stored for more than 1 day. It is important to control the condition of the fermentation rooms so that temperature of the product changes as described above. The temperature near the ceiling of the fermentation rooms can easily increase higher than that of lower part near the floor. This difference causes different fermentation conditions and qualities of products. Therefore, it is necessary to adjust the fermentation periods slightly among products at different positions of the room or to regularly change the position of the products in the room during the fermentation. The optimum conditions for fermentation also vary depending upon the strains of *B. subtilis* (*natto*) used.

9.3.6 Packing for Shipment

Automated shrink packaging of two or three polystyrene paper (PSP) trays or cups is the conventional packing method, and an automated carton packer has recently been developed specifically for natto. Cartons are stored in a refrigerated room until they are transported to stores by trucks. Since the odor of natto easily intensifies due to the progressive production of ammonia under warm temperature conditions, natto is treated as a perishable product, and its shelf life is one week if refrigerated below 10°C.

9.3.7 Changes in Packages

The type of packaging affects the fermentation process, but in addition, the following factors are important for natto packaging:

1. Easy to manufacture
2. Easy to distribute
3. Easy to serve
4. Disposable and able to be incinerated

Before World War II, China jars and packages made of rice straw or wood shavings were often used to pack natto in Japan. Rice straw is an excellent packaging material for natto because it promotes growth of *B. subtilis* (*natto*) by maintaining a warm internal temperature and absorbs extra moisture emitted from the steamed soybeans during the initial stages of fermentation. Adequate moisture is required for good growth of *B. subtilis* (*natto*) cells. If moisture inside the packages is lost during fermentation, the resulting natto tends to be dry, with sticky and strong strings. However, these traditional packaging methods did not meet the requirements of being easy to store or the trend towards spoil-proof and mass-produced food after the 1960s. The development of fillers has also promoted the standardization of natto packaging. Paper tips replaced the rice straw first, and then PSP packages were developed. At present, straw packages are used only for souvenirs or special local products. When straw packages are used, natto is packed in thin polyethylene films first and further packed in the steamed and dried straw.

The initial PSP packages containing 100 to 120 g natto were considered to be too large because most consumers want to eat an entire cup of natto at one time. Hence, PSP packages and easily printable paper cups containing 40 or 50 g natto are now mainly being used. Smaller cups containing 20 or 30 g natto are also used for school meals. However, since PSP packaging and paper cups do not regulate temperature and moisture automatically during fermentation, control of the conditions of the fermentation room becomes even more important. Moreover, steamed soybeans mixed with *B. subtilis* (*natto*) spores should be covered with a perforated inner packaging. The purpose of this inner packaging is to assist airflow and maintain the moisture inside the package. It also allows sachets of seasoning to be included in one package. The corners of the perforated lining are pressed down to prevent the steamed soybeans from drying out.

A functional plastic membrane that allows for automatic handling as described above was developed as a packaging material for natto.[23] This was called the "repiramy cup," and it did indeed meet many of the requirements for natto packaging. It functioned to maintain the temperature and allowed for the free movement of oxygen, carbon dioxide, ammonia gas, and other gases, but not of water. This membrane, however, was too expensive to use as a packaging material and is not used at present.

9.4 ASSESSMENT OF QUALITY

9.4.1 Chemical Composition

The chemical composition of natto does not differ greatly from that of soybeans except for the vitamin K content (Table 9.1).[24] *B. subtilis* (*natto*) produces vitamin K_2 (menaquinone-7).[25,26] Domestic soybeans, U.S. soybeans, Chinese soybeans, and natto contain 21, 39, 39, and 2148 μg/100 g dry of vitamin K, respectively. Natto is covered with sticky substances produced by *B. subtilis* (*natto*) during fermentation. These sticky substances are composed of polyglutamic acid and levan (fructan).[27] During fermentation, *B. subtilis* (*natto*) also produces proteases and an amylase.[28] Peptides or amino acids produced during fermentation constitute a part of the natto taste. *B. subtilis* (*natto*) utilizes soybean saccharides and produces the characteristic flavor of natto.

TABLE 9.1
Composition of Soybeans and Itohiki Natto (/100 g dry)

	Dried and raw soybean			
	Japan	**United States**	**China**	**Itohiki-natto**
Energy (kcal)	476	490	482	493
Protein (g)	40.3	37.4	37.5	40.7
Lipid (g)	21.7	24.6	22.2	24.7
Carbohydrate (g)	32.2	32.6	35.2	29.9
Ash (g)	5.7	5.4	5.0	4.7
Vitamin A				
Retinol (μg)	0	0	0	0
Carotene (μg)	7	8	10	0
Retinol equivalents (μg)	1	1	1	0
Vitamin D (μg)	0	0	0	0
Vitamin E (mg)	4.1	3.9	4.9	3.0
Vitamin K (μg)	21	39	39	2148[a]
Vitamin B1 (mg)	0.95	1.00	0.96	0.17
Vitamin B2 (mg)	0.34	0.34	0.34	1.38
Niacin (mg)	2.5	2.4	2.5	2.7
Vitamin B6 (mg)	0.61	0.52	0.67	0.59
Vitamin B12 (μg)	0	0	0	Tr
Folate (μg)	263	249	297	30
Pantothenic acid (mg)	1.74	1.69	1.87	8.89
Ascorbic acid (mg)	Tr	Tr	Tr	Tr

Note: Water contents of Japanese, American, and Chinese dried and raw soybeans, and itohiki-natto are 12.5, 11.7, 12.5, and 59.5 (/100 g wet), respectively; Tr: Trace.

[a] Including menaquinone-7.

Source: Modified from Resources Council, Science and Technology Agency, *Standard Tables of Food Composition in Japan*, 5th rev. ed., Ministry of Finance, Japan, 2000. With permission.

9.4.2 Sensory Tests

The quality of natto is determined by the sensory tests described in the book *Methods of Natto Research*.[29] High quality natto has the following properties:

1. *Even surface layer*—The bacterial layer should be formed entirely on the surface of soybeans.
2. *No lysis and no glistening*—Uneven spots or glistening of soybeans by bacterial cell lysis should not be observed.
3. *No damaged soybeans*—There should be few split, crushed, or peeled soybeans.
4. *Bright color*—The color of soybean surfaces should be brown or light brown and should not be dark brown or blackish.
5. *Good aroma*—The product should have a sweet aroma without an ammonia-like, scorched, undesirable, or acidic odor.
6. *Proper firmness*—The soybeans should be properly soft and have a smooth texture.
7. *Good taste*—Relish, sweet, not bitter, and slightly astringent. The preferred taste is created by amino acids, peptides, and saccharides.
8. *Proper stickiness*—When stirred with a pair of chopsticks, the viscosity of the natto should increase to form strong strings (Figure 9.2c).

Panels can evaluate natto according to these criteria. If any foreign substances are found or tyrosine crystals are formed on the surface of soybeans, they are noted and the natto is given a low quality score.

9.4.3 Changes in Consumers' Preferences

Traditionally, extra small and small soybeans have been used to make natto. However, in 1999, a natto made from medium-sized soybeans won the contest held by the Federation of Japan Natto Manufactures Cooperative Society. Nattos made with large soybeans are also preferred now by Japanese consumers.[30] Present-day consumers tend to buy a natto with a markedly weaker odor and strings.[30] Natto with traditional characteristics (distinctive odor and strong strings) may no longer have mass appeal. Some manufactures have used their own *B. subtilis* (*natto*) stains to produce less aromatic products.

9.5 HEALTH BENEFITS

B. subtilis, *B. subtilis* (*natto*), and natto are considered to have potential as probiotics. Ingestion of bacterial cells probably affects the intestinal microflora and the mucosal immune system. *B. subtilis* (*natto*) cells produce many enzymes and vitamin K_2. A serine protease, subtilisin, can degrade soybean allergens and shows fibrinolytic activity. Ingestion of vitamin K_2 (menaquinone-7) will help coagulant activity and prevent osteoporosis.[31,32] Natto contains the phytoestrogens (isoflavones)

that originate in soybeans.[33,34] Isoflavones seem to have preventive effects on breast and prostate cancer, osteoporosis, menopausal symptoms, and heart disease.[33,34]

9.5.1 *Bacillus Subtilis* (*natto*) Cells

9.5.1.1 Effects on Intestinal Microflora and Feed Efficiency

Lactobacillus spp. and *Bifidobacterium* spp. are mainly used as probiotics for humans and animals.[35] However, other bacteria and fungi can also be used as probiotics. For example, *Bacillus* spp., *Enterococcus* spp., *Streptococcus* spp., and *Saccharomyces cerevisiae* seem to have potential as probiotics.[35] In the screening and selection of certain microbial strains as probiotics, phenotype and genotype stability, carbohydrate and protein utilization patterns, safety, acid and bile stability, adhesion characterization, production of antimicrobial substances, antibiotic resistance patterns, immunogenicity, and viability and properties during processing and storage are considered to be important.[36] *B. subtilis* is an aerobic spore-forming bacteria. *B. subtilis* spores are relatively resistant to oxygen, active oxygen species, acid, drying, and heating compared to other bacteria.[5,27] *B. subtilis* can also grow under O_2-reduced conditions. These characteristics are desirable for potential probiotics. Unfortunately, however, *B. subtilis* is not strongly resistant to bile acid, and is not a predominant bacterium in the human intestine.[37]

Several reports have demonstrated the effects of orally administered *B. subtilis* on the intestinal microflora, body weight gain, and increased feed efficiency of animals and birds.[38–42] These results indicate that ingestion of live *B. subtilis* cells can actually improve the intestinal microflora. When weanling piglets were fed a diet including spores of *B. subtilis* (*natto*), the changes in intestinal microflora varied depending upon the region of the intestine examined.[38] In the jejunum, the numbers of *Streptococcus* spp. and *Bifidobacterium* spp. increased, whereas no differences were observed in the colon, when compared with the control diet group. When turkeys were fed *B. subtilis* culture, body weight gain and cumulative feed efficiency significantly increased, both by 2.5%.[39] When chickens were given *B. subtilis*, the detection rate of the intestinal pathogen *Campylobacter jejuni* decreased in the laboratory portion of the experiment.[40] The cell number of *Salmonella typhilimurium* also decreased. In a field trial, feeding a *B. subtilis* strain decreased the cell number and detection rates of intestinal *Enterobacteriaceae*, *Clostridium perfringens*, and *Campylobacter* sp. When sows and gilts were fed an experimental diet containing *B. subtilis*, the number and detection rates of fecal *Bifidobacterium* spp. and *Lactobacillus* spp. increased, but *Streptococcus* spp., *Enterobacteriaceae*, *Clostridium perfringens*, and *Bacteroidaceae* decreased.[41] The diarrhea rate of the piglets up to 10 days old and mortality rate up to 25 days old also decreased. When mice were intubated with intact and autoclaved *B. subtilis* (*natto*) spores for 8 days, only intact spores changed the fecal microflora, and the patterns of the changes differed depending upon the diets fed.[42] Feeding a diet including egg white decreased fecal *Lactobacillus* spp., although the administration of *B. subtilis* (*natto*) spores inhibited the decrease. On the other hand, feeding a diet including casein and administering *B. subtilis* (*natto*) spores increased only *Bacteroidaceae* but not lactobacilli.

Ingestion of soybean food natto (50 g natto/day) significantly affected the composition and metabolic activity of the human fecal microflora.[43] Ingestion of natto increased the number of *B. subtilis* (*natto*) and *Bifidobacterium* spp. (the latter increased from 15% of the total bacterial count before consumption to 39% after 14 d consumption), although it decreased the number and detection rates of lecithinase-positive clostridia including *Clostridium perfringens*. The concentrations of fecal acetic acid, total organic acids, and succinic acid increased, while fecal concentrations of indole, ethylphenol, and skatol decreased. Fecal ammonia, cresol, and fecal pH values also decreased.

Mechanisms of the above effects have not been clarified. However, germination or some metabolites from *B. subtilis* cells seem to be necessary to explain their effects, because it was shown that in mice, administration of autoclaved spores did not affect the fecal microflora.[42] The possibility of germination of *Bacillus* spp. spores in the intestine has been examined. When *B. subtilis* (*natto*) spores were inoculated in the ligated loops of the ileum of dogs, some spores did germinate, but died after germination.[44] It has been shown that *B. thuringiensis* spores germinate in the gut fluid of the tobacco horn worm.[45] *B. subtilis* spores also germinate in the mice gut.[46] In this report, the number of spores excreted in the feces of the mice was, in some experiments, larger than the number of spores inoculated. However, this is inconsistent with the report that vegetative cells of *B. subtilis* cells could not be detected when spores were inoculated in mice that were left without food for 16 h.[37] Live *Bacillus* cells could be detected only in some organs after ingestion. In general, when foods are ingested, the pH value in the stomach sometimes increases to 3 to 4. Spores of *Bacillus* spp. appear to be resistant to such pH values. Some of the *B. subtilis* spores ingested together with other food may be able to sustain their viability and germinate in the upper intestine once the surrounding pH value is neutralized. This then allows them to produce probiotic activity.

Catalase and subtilisin have been proposed as the active molecules responsible for the effects of *B. subtilis (natto)* on intestinal microflora.[47] The growth of three strains of lactobacilli co-cultured aerobically with *B. subtilis* (*natto*) has been examined. Addition of *B. subtilis* (*natto*) to the culture medium *in vitro* resulted in an increase in the number of viable cells of all lactobacilli tested. Both catalase and *B. subtilis* (*natto*) enhanced the growth of *Lb. reuteri*, whereas *B. subtilis* (*natto*), but not catalase, enhanced the growth of *Lb. acidophilus*. In a medium containing 0.1 mM hydrogen peroxide, its toxic effect on *Lb. reuteri* was abolished by catalase or *B. subtilis* (*natto*). Catalase has been reported to exhibit a growth-promoting effect on lactobacilli.[48] The viability of lactobacilli readily decreases in the presence of active oxygen species. The decrease of viable cell number is partly attributable to the fact that lactobacilli do not generally produce a defense molecule against active oxygen species. However, aerobic bacteria, including *B. subtilis* (*natto*), can produce catalase. Vegetative cells of *B. subtilis* primarily produce catalase-1 in the logarithmic phase of growth, and additionally produce catalase-2 and catalase-3 as growth progresses.[49–51] Intact *B. subtilis* spores contain only catalase-2 in the spore coat. Some other anaerobic bacteria in the intestine, such as *Escherichia coli*, *Bacteroides* spp., and *Eubacterium* spp., also produce catalase.[52–55] It may be important for these bacteria to scavenge hydrogen peroxide to colonize the intestine where

active oxygen species are produced. The addition of a serine protease, subtilisin, from *Bacillus licheniformis* to culture medium improved the growth and viability of *Lb. reuteri* and *Lb. acidophilus* in the absence of hydrogen peroxide.[47] *B. subtilis* (*natto*) secretes two serine proteases, subtilisin NAT with an isoelectric point (pI) of 8.7 and a 90 kDa serine proteinase (pI 3.9).[56–59] Taken together, these results indicate that *B. subtilis* (*natto*) can enhance the growth and viability of lactobacilli possibly through production of catalase and subtilisin.

9.5.1.2 Effects on the Immune System

The mucosal immune system is a first defense line against foreign antigens. The system consists of many kinds of cells, including epithelium, macrophage, dendritic cells, intraepithelial T lymphocytes, B lymphocytes, and neutrophils.[60] The effects of *B. subtilis* cells on some of these intestinal cells have been examined to determine whether *B. subtilis* cells do actually possess immunostimulating effects. A recent study questioned whether human intestinal epithelium-like Caco-2 cells can produce cytokines to *B. subtilis* (*natto*) strains, in addition to other pathogenic and nonpathogenic bacteria.[61] It is not clear whether epithelial cells can respond to nonpathogenic strains of bacterial cells. Live cells of nonpathogenic *B. subtilis* or *B. subtilis* (*natto*) strains, as well as nonpathogenic *Escherichia coli*, pathogenic *Salmonella enterica* and *Pseudomonas aeruginosa,* all induced secretion of interleukin 6 (IL-6) or IL-8, but not of IL-7 and IL-15, and tumor necrosis factor (TNF)-α. The amounts of cytokines induced by *B. subtilis* (*natto*) cells were dependent upon the strain used. Cytokine induction of epithelial cells may differ between bacterial species or strains regardless of their pathogenicity. Some nonpathogenic as well as pathogenic bacteria seem to be able to induce cytokine secretion from normal intestinal epithelial cells when they are orally ingested. Nitrite formation in the macrophage cell line J774.2 in the presence of heat-killed *B. subtilis* cells has been reported.[62] Peptidoglycan from *B. subtilis* induced nitrite formation in macrophages. Lipoteichoic acid from *Staphylococcus aureus* was more potent than lipoteichoic acid from *B. subtilis*. Translocation of *Bacillus* spp. spores or cells has been examined.[37] When mice were fed spores intragastrically, both spores and vegetative cells were detected in the lymph nodes and spleen. In another report, fewer numbers of spores were detected in mesenteric lymph nodes, livers, and spleens. However, the effect of spore ingestion on the increase of bacterial numbers in these organs was not significant.[46] The authors indicated that spores do not appear to translocate substantially across the mucosal surfaces. The effect of oral administration of *B. subtilis* spores on macrophage and natural killer (NK) cells in mice has been examined.[63] The spore administration (0 to 0.25 g/animal) dose-dependently increased the cytotoxic activity of NK cells and oxidative burst activity of macrophages. The effect of *B. subtilis* (*natto*) on T and B lymphocytes in the chicken spleen has also been examined.[64] When chickens were fed 10^7 CFU/g of *B. subtilis* (*natto*) spores, the percentages of T and B lymphocytes in the spleens increased compared to those of control groups. Although *B. subtilis* cells are not predominant in the intestine, these results indicate that ingested bacterial cells can affect the mucosal immune system.

Mechanisms of the effects of *B. subtilis* cells on the immune system remain unclear, although interaction between bacterial cellular components and Toll-like[65] or Nod-like[66] receptors seems to be essential to their effects. Many bacterial components, including peptidoglycans, lipoproteins, lipoteichoic acid, flagellin, and unmethylated CpG dinucleotides in bacterial DNA are known to bind to Toll-like receptors and induce cytokine responses.[65] *B. subtilis* and *B. subtilis* (*natto*) cells would presumably also contain some of these active substances. In addition, it has been shown that *Bacillus* spp. including *B. subtilis* (*natto*), their peptidoglycan-related molecules, and food "natto," but not Gram-positive *Lactobacillus* sp. and several Gram-negative bacteria stimulate both Nod1 and Nod2.[66] *B. subtilis* and natto might stimulate the host immune system in a distinctive manner, which is different from that of other fermented food and related microbes.

9.5.1.3 Anti-Allergy Effect of Subtilisin

About 15 soybean proteins have been shown to be recognized by sera of soybean-sensitive patients with atopic dermatitis.[67] Three major allergens were identified and designated as *Gly m* Bd 60K, *Gly m* Bd 30K, and *Gly m* Bd 28K, respectively. *Gly m* Bd 60K is an α-subunit of β-conglycinin.[68] *Gly m* Bd 30K is a soybean oil-body-associated glycoprotein homologous to Der p (or f) 1, a major allergen of house dust mite.[68] *Gly m* Bd 28K is a vicilin-like glycoprotein, which is a minor component fractionated into 7S globulin fraction.[68] *B. subtilis* (*natto*) produces a serine protease of subtilisin NAT during its growth.[56–58] Subtilisin NAT appears to degrade *Gly m* Bd 28K.[69] Various nonfermented soybean products, such as soybean protein isolate (SPI), tofu, kori-dofu, and yuba contain *Gly m* Bd 28K at high concentrations, although fermented soybean products, such as natto, soy sauce, and miso, do not.[69]

Recent studies have implied that the intestinal microbial flora are important to the interrelationship between infection and allergy.[70] Endogenous flora of the gut stimulate the immune system. It is likely that *Lactobacillus* spp. administration may have preventive and therapeutic effects on allergic diseases.[71,72] It has also been indicated that allergic children are less colonized with *Lactobacillus* spp. compared with nonallergenic children.[73] In addition, *Lactobacillus* spp. differently modulate expression of cytokines and maturation surface markers in murine dendritic cells dependent upon the strains.[74] Dendritic cells, present throughout the gastrointestinal tract, play a pivotal immunoregulatory role in the Th1 and Th2 cell balance.[70] Th2 cells produce IL-4, IL-13, and IL-5, which coordinately regulate the allergic response.[70] Recently, concerning *B. subtilis* (*natto*) and natto, it has been shown that levan (β-2, 6-fructan), a major fraction of fermented soybean mucilage, but neither γ-PGA nor killed *B. subtilis* (*natto*), exert strong activity to induce production of IL-12 p40 and TNF-α by macrophage cell lines *in vitro*.[75] TLR4 was involved in pattern recognition of levan. Oral administration of the levan significantly reduced the serum levels of ovalbumin (OVA)-specific IgE and the Th2 response to OVA in mice immunized with OVA. These results suggest that ingestion of live *B. subtilis* (*natto*) and natto have possible preventive and therapeutic effects on allergic diseases.

9.5.1.4 Fibrinolytic Activity of Subtilisin

Circulating platelets and blood-derived proteins (fibrin) are essential for the formation of blood clots, which prevent bleeding long enough for healing to occur.[76] However, excess coagulation prevents normal physiologic blood flow, which causes thrombotic disorders. Thrombolytic therapy is the most direct means of restoring blood flow.[77] *Bacillus* spp. produce serine proteases called subtilisins, which are known to have fibrinolytic activity.[56–58, 78–81] Subtilisin NAT produced by *B. subtilis* (*natto*) (also called nattokinase) is 99.5% homologous to subtilisin E.[58] Oral administration of subtilisin or natto induced mild and frequent enhancement of fibrinolytic activity in plasma and production of tissue plasminogen activator.[81] Euglobulin fibrinolytic activity, degradation products from fibrin/fibrinogen, and the amount of tissue plasminogen activator increased by administration of subtilisin. However, whole blood clot lysis time did not significantly decrease. In addition, ingestion of natto decreased euglobulin lysis time and increased euglobulin fibrinolytic activity.[80] The mechanisms of fibrinolytic activity of subtilisin NAT are not fully understood, although subtilisin NAT appears to digest fibrin directly and cleave and inactivate plasminogen activator inhibitor-1 (PAI-1).[82] Elevation of PAI-1 in plasma is found in patients with thrombotic disease.[83] High PAI-1 activity is related to impaired fibrinolysis, and low activity is associated with bleeding disorders.[84–86]

9.5.1.5 The Role of Vitamin K2 (Menaquinone-7) in the Prevention of Osteoporosis

Vitamin K has important roles in blood coagulation and bone metabolism.[31,32] The most abundant forms of vitamin K are phylloquinone (vitamin K_1) and menaquinone (vitamin K_2). Menaquinone refers to a series of vitamin K homologues with polyunsaturated aliphatic side chains of varying length. These compounds are generally referred to as menaquinone-*n* (MK-*n*), where *n* is the number of isoprenoid residues of which the side chain is composed. A marked deficiency of menaquinone-7 and menaquinone-8 has been demonstrated in patients with osteoporotic fractures.[87] An effect of vitamin K_2 treatment on spinal bone mineral density (BMD) in postmenopausal women has been demonstrated.[88] In addition, low serum and bone vitamin K status in patients with long-standing Crohn's disease has been reported.[89] Some bacteria including *B. subtilis* (*natto*) produce vitamin K_2 (menaquinone-7) (see Section 4.1).[25,26] Ingestion of 100 g of natto increases vitamin K levels in serum.[25] There were no significant differences in serum vitamin K_1 levels after intake of natto, although vitamin K_2 levels and total vitamin K levels 24 h after intake significantly increased from 0.90 to 6.21 and from 1.95 to 7.14 ng/ml. A large geographic difference in serum vitamin K_2 levels in postmenopausal women in Tokyo and Hiroshima in Japan and in England has been reported.[90] A trial using a bacterial strain with a high productivity of vitamin K_2 has been performed, where vitamin K_2 production was compared to that of a commercial strain.[91] The results showed that the selected bacteria strain was capable of producing twice the concentration of vitamin K_2 produced by the original commercial strain.

9.5.2 Phytoestrogens—Effects on Cancer and Osteoporosis

It has been hypothesized that ingestion of phytoestrogens (isoflavones) contained in soybeans play an important role in the prevention of cancer, osteoporosis, menopausal symptoms, and heart disease.[33,34] Many epidemiologic studies have examined the relationship between soybean consumption and cancer risk,[33,34] although no significant conclusion has been reached. Early exposure (during the neonatal or prepubertal period of life) to soybean products is thought to be essential for cancer protection.[92–95] Some soybean products with a high isoflavone content have been developed and sold in Japan by using selected soybean varieties and specific strains of *B. subtilis* (*natto*).

Isoflavones are a subclass of the more ubiquitous flavonoids. The primary isoflavones in soybeans are genistein and daidzein and their respective β-glycosides, genistin and daidzin. Much smaller amounts of glycitein and its glycoside, glycitin, are also present in soybeans.[96,97] Isoflavones in nonfermented soybean foods appear mostly as the conjugate, whereas in fermented food such as natto and miso, the aglycones dominate.[98] Hence, levels of genistein in fermented soybean products are higher than those in soybeans and nonfermented foods. The calculated daily intake levels of genistein and genistin ingested from soybeans and related soybean products by the Japanese are 1.5 to 4.1 and 6.3 to 8.3 mg/person, respectively.

To clarify the mechanisms of the effects of isoflavones, many studies have been performed. Intestinal microbial cells can convert daidzein into several different products, including the isoflavonoids equol, dihydrodaidzein, and *O*-desmethylangolensin.[99] It has been proposed that genistein was metabolized to dihydrogenistein and 6′-hydroxy-*O*-desmethylangolensin in humans.[99] Estrogenic activities of isoflavones are quite weak when compared to those of physiologic estrogens.[100] However, consumption of soybean foods increased blood isoflavone concentrations to several orders of magnitude higher than those of physiologic estrogens.[101] Isoflavones can also have anti-estrogenic effects when placed in a high-estrogen environment.[102,103] Genistein inhibits several enzyme activities involved in signal transduction and DNA topoisomerases I and II.[104–110] In addition, genistein increases the *in vitro* concentrations of transforming growth factor-β (TGF-β), an inhibitor of epithelial cell growth.[111] This effect is thought to be an important contributor to the anticancer effect. Isoflavones have been shown to bind to estrogen receptors (ERs). Recently, a new receptor, ERβ, has been discovered, to which genistein can bind, but the binding is weaker than for natural estrogen, 17β-estradiol.[112] It is possible that this finding, and the fact that ERβ is expressed in differing amounts depending on the types of cells, support the observation that isoflavones have antiosteoporotic but weakly antiuterotrophic effects.[113,114]

9.6 CONCLUSIONS

Natto is a simple, low-priced, popular soybean food made by fermenting cooked soybeans with *B. subtilis* (*natto*) in Japan. Natto has characteristic aroma and stickiness. The consumption of natto products has increased during the 1990s in Japan. Natto contains many nutrients originating from soybeans and the metabolites of *B. subtilis*

(*natto*) cells, which show many physiological functions. In 1999, the Food and Drug Administration (FDA) in the United States approved the use of health claims for soy protein related to the reduction of the risk of coronary heart disease by lowering blood cholesterol levels when 25 g of soy protein per day are consumed. In addition, many physiological functions of *B. subtilis* cells have recently been reported. Although *B. subtilis* (*natto*) is not a predominat bacterium in the human intestine, it is thought to have the potential as a probiotic. Some pharmaceutical products containing *B. subtilis* spores have been sold and utilized in Japan and European countries. Both scientific researchers and consumers are paying attention now to natto and *B. subtilis* cells. However, their effects and mechanisms are not clearly understood yet. Further careful studies are necessary to obtain data on their effects, safety, and efficacy. We hope that the studies on the nutrition and physiological function of natto products, *B. subtilis* cells, and related products will progress steadily and that such products will become popular all over the world.

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10 Fermented Meat

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CONTENTS

10.1 INTRODUCTION

A number of reviews and even monographs have dealt with the properties and characteristics of fermented meats.[1–5] In this chapter, the emphasis is on the nutritional and health-related aspects of the fermentation process or, more specifically, the products obtained with the aid of this process. Points to consider are the inherent properties of the nonfermented matrix (i.e., the nutritive value, hazards, and shelf life) as well as the effects of the fermentation process on these criteria.

10.1.1 Nutritional Role of Meat in the Human Diet

Meat has traditionally been considered an essential component of the human diet to ensure optimal growth and development. With a limited range of foods available

in societies throughout history, meat was important as a concentrated source of a wide range of nutrients. Anthropological research shows that the length of the gut in primates and humans became shorter with the introduction of animal-derived food. Smaller quantities of food of high digestibility required relatively smaller guts, characterized by simple stomachs and proportionally longer small intestines, emphasizing absorption.[6,7] It is perhaps due to the fact that meat has been eaten as much for enjoyment as for its nutritional qualities that consumption of meat and meat products have increased with the affluence of the consumer. This conclusion is consistent with the meat consumption data in selected countries. As shown in Table 10.1 it is predicted that these values will not change substantially within the next decade.

The meat consumption and production figures published by the United States Department of Agriculture and the European Union do not distinguish between fresh meat and processed or fermented meat products. Therefore, only estimates can be provided concerning the size of production of fermented meat products. Approximately 5% of the total meat production (carcass weight) is further processed by fermentation. The major producers of fermented meat products in the European Union are Germany, Italy, Spain, and France.[8] In these countries, 20% to 40% of processed meat products can be classified as fermented meat products. Fermented meat products include products listed in Table 10.2.

The fat content of meat as consumed is around 2% to 5%, even though total fat content varies with species, feeding regimes, and age. The principal fatty acids in meat are saturated fatty acids, including palmitic acid (C16:0) and stearic acid (C18:0). Around 40% of the fat in meat is monounsaturated, of which oleic acid (C18:1) is one of the main contributors.[9] Protein of high biological value and micronutrients such as iron, zinc, vitamin B_1, niacin equivalents, and vitamin B_{12} significantly contribute to the nutritional value of meat.[10] Hambraeus[11] reported that the requirement for iron is one of the most difficult nutritional requirements for humans to be met, because iron deficiency is caused not only by a low intake but is also the result of low bioavailability. Increased iron requirements may result from physiological variables or clinical

TABLE 10.1
Meat Consumption in 2006 and Predicted Values for 2015 (kg per person per year)

Country	2006	2015
United States	100.63	103.36
Canada	77.13	81.74
Australia	85.64	85.00
European Union	69.06	72.11
Japan	34.12	36.64
China	46.24	55.33
India	4.29	4.94
African countries[a]	10.57	10.82

[a] Republic of South Africa not included.
Source: OECD (http://stats.oecd.org).

TABLE 10.2
Fermented Meat Products

I. Comminuted fermented meats[a]

Group	Water activity	Examples
Dry		
Mold ripened	< 0.9	Classical Italian salami such as Tipo Milano, Felino, Narzi, etc.; French saucisson sec; Hungarian salami (additionally smoked); Spanish chorizos
Smoked		German Katenrauch, Dauerwurst, etc.
Semidry		
Mold ripened	0.9 – 0.95	Various French and Spanish fermented sausages
Smoked		The majority of U.S. and Northern European fermented sausages (e.g., the Netherlands, Scandinavia, Germany)
Nondried		
Spreadable	0.94 – 0.96	German Mettwurst, Teewurst, Spanish Sobrasada

II. Whole meat products

Group	Examples
Classical ham made from thigh of hog with or without bone	Prosciutto di Parma or San Daniele (Italy); Jambon de Beyonne (France); Jamón Serrano (Spain); Kraški pršut (Slowenja); Virginia ham (United States); Yunnan ho-twe and Tshingwa ho-twe (China)
Cuts of meat	
Pork	Bacon (United Kingdom, United States); Pancetta (Italy), Capocollo (Italy); Culatello di Zipello (Italy); Schwarzwälder, Westphälischer, Lachs-Schinken (Germany); Tirolean Speck (Austria)
Other animal sources	
Beef	Bresaola (also equine, and venison) (Italy); Bündner Fleisch (Switzerland); Pastirma (Near orient); Biltong (also antilope, South Africa)
Mutton	Fenelar (Norway)

Note: Sausages produced from varying size of meat particles, obtained from various animals (most common pork), in various casings of varying diameter and containing additives and spices of varying compositions.

problems. Red meat contains 50% to 60% of its iron in the heme form (from hemoglobin and myoglobin), which is absorbed in humans by a more efficient mechanism than nonheme iron, the source of iron in plant foods.

An important role of meat and meat products in everyday food culture and consumer health may be questioned by the fact that the populations of vegetarians living in rich countries are characterized by lower rates of cancer and cardiovascular disease.[12–14] The analysis of dietary patterns, as a possible approach to examining diet–disease relations, identified two major eating patterns defined by factor analysis using dietary data collected from food frequency questionnaires.[15] The first factor, the "prudent dietary pattern," was characterized by a high intake of vegetables, fruits, legumes, whole grains, and fish or other seafood, whereas the second factor, the "Western pattern," showed a high intake of processed meat, red meat, butter,

high-fat dairy products, eggs, and refined grains. A study has been published involving Seventh-Day Adventists, a well-characterized population, in which the effect of dietary intake of nutrients on biochemical parameters in blood and urine were compared to vegetarian and nonvegetarian subjects.[16,17] Remarkably, the dietary intake of cholesterol was higher in nonvegetarian subjects (560 to 710 mg/d) compared to vegetarians (< 20 mg/d) and was associated with elevated serum cholesterol levels in the nonvegetarian population. These results demonstrated a correlation between dietary intake of certain food components (e.g., cholesterol) relevant for diseases (e.g., coronary heart disease) and their blood concentrations.

10.1.2 The Relation between Meat in the Diet and Disease

Numerous studies have compared the health status and mortality of vegetarians to those of omnivores. The results show a strong correlation between per capita consumption of meat and the incidence of colon cancer among various countries.[18] In more detailed case-control and cohort studies, in which lifestyle factors were better controlled for, the consumption of red meat was associated with a high risk of colon cancer.[19–21] Results from a meta-analysis by Howe et al.[22] including 13 of the case-control studies, indicated that total energy intake was positively associated with a higher risk of colon cancer. Surprisingly, the intake of fat, protein, and carbohydrates were not related to cancer risk, independent of their contribution to total energy. Compared with Western vegetarians, nonvegetarians have a higher mean body mass index (BMI) by about 1 kg/cm^2, suggesting that higher total energy intake and meat consumption might be associated with the "Western diet pattern."[23]

The mechanisms that increase the risk of colon cancer are not yet clear. Several in vitro studies suggest that DNA damage in human cell lines can be caused by food ingredients or their metabolic products.[24,25] High meat consumption, for example, leads to higher levels of bile acids and *N*-nitroso compounds in the feces. Bile acid and *N*-nitroso compounds, as well as their metabolites, potentially promote colon cancer development.[26,27] Animal studies show that large intestinal *N*-nitrosation does not occur in germ-free rats, but it has been shown to occur in the presence of a conventional flora.[28] The effect of diet on the composition of the intestinal microflora was shown by Finegold et al.[29] Subjects eating a Western diet were compared with subjects eating a Japanese diet. The subjects eating the Japanese diet had a lower risk for colon cancer and had significantly higher numbers of *Enterococcus faecalis*, *Eubacterium lentum*, *E. contortum*, *Klebsiella pneumoniae*, and various *Lactobacillus* species in their feces. The Japanese diet has been associated with low incidence of large bowel cancer. Japanese people who migrate to the United States and then adopt the Western diet develop this cancer with increased frequency, approaching that of native-born Americans.[30,31] The high-risk group with the Western diet patterns had increased counts of species of the genera *Bacteroides*, *Bifidobacterium*, *Peptostreptococcus*, and *Clostridium* in their fecal flora. Goldin and Gorbach[32] reported that rats fed with a high-fat (meat) diet had higher enzyme activity of the fecal bacterial enzymes β-glucuronidase, nitroreductase and azoreductase, than rats on a low-fat (no meat) diet. These results were confirmed and extended in subsequent animal and human studies demonstrating that a high-fat diet, independent of the meat content,

elevated the activity of these bacterial enzymes, which are implicated in the generation of mutagens, carcinogens, and various tumor promoters.[33]

Regular consumption of meat is also associated with increased risk of death from coronary heart disease (CHD) mortality. The most compelling evidence comes from studies with Seventh-Day Adventists. It was found that men and women who consumed red meat daily had around 60% greater chance of dying from CHD than those who consumed red meat less than once a week.[16,34] A review of studies of the association between blood homocysteine concentrations and atherosclerotic disease showed that 16 of 21 investigations reported significantly higher homocysteine concentrations in case subjects compared with control subjects.[35] Because red meat is a major source of methionine in the diet, and methionine is the direct metabolic precursor of homocysteine, a higher intake of red meat may be involved in cardiovascular disease initiation and progression.

In summary, a high dietary intake of energy, saturated fat, and red meat, all associated with the Western diet pattern, are likely to have adverse effects on chronic disease risks, particularly those of colon cancer and coronary heart disease. On the other hand, little evidence indicated that the consumption of moderate amounts of meat or meat products is harmful in regard to either cancer or cardiovascular disease.

10.2 THE HISTORY AND CULTURE RELATED TO FERMENTED MEAT

Meat is extremely susceptible to microbial spoilage. Virtually all ecological factors characterizing meat as a substrate are optimal for the growth of bacteria, which are the most efficient agents in remineralization of organic matter. For example, in meat, water activity and pH are 0.96 to 0.97 and 5.6 to 5.8, respectively, and nutrients and growth factors are abundantly available. Any storage of this nutritionally rich food and preservation of the nutrients contained therein requires the suppression of microbial growth or the elimination of microorganisms and prevention of recontamination.

The traditional methods employed for prevention of microbial spoilage are still in use, though with a different meaning in the various products. These methods comprise reduction of water activity (drying, salting) and pH (fermentation, acidification), smoking, storage at refrigeration or freezing temperatures, and use of curing aids (nitrite and nitrate). Commonly, these methods act together in different combinations, building up hurdles against microbial growth. With regard to fermented sausages, these hurdles are low water activity (0.85 to 0.95) and pH (5.6 to 4.5), the use of nitrite (nitrate) and smoke. In addition, during fermentation and ripening, ecological factors, such as reduced redox potential and low temperatures (10°C to 12°C, at least for dry sausages), together with antagonistic compounds produced by the fermenting flora exert a selective effect against the growth of undesirable microorganisms. Basically, the same antimicrobial hurdles are effective in achieving the microbial stability of ham, except for the effect of a low pH. As no lactic fermentation takes place, the reduced water activity is the most effective hurdle against microbial growth in ham. The understanding of these ecological factors and their control is not only a prerequisite in quality assurance, but also provides a basis for understanding to what extent these food matrices might be used to serve as probiotic foods.

The production of dried and cured meat (ham) can be traced back to prehistoric times. It cannot be excluded that among the sausages that are mentioned in historical literature (e.g., Homer's *Iliad*) fermented products were included, although we do not have sufficient knowledge of their production processes to permit a conclusion that a fermentation step was part of the technology. The origin of fermented sausages can be traced back with accuracy to ca. 1730, when salami was first mentioned in Italy.[36] The art to produce fermented sausages spread from Italy to other European countries, and was established in Germany in 1735 and Hungary in 1835. Today in various parts of the world, a large number of different types of fermented sausage exist. For example, 330 different types are produced in Germany.[37] A large number of ham varieties are also produced.[38] This very high consumption of fermented meats is an indication that such products have a long tradition of being safe. However, some specific safety aspects deserve consideration.

The high fat content commonly found in fermented sausages (usually around 50% of dry matter) has been of nutritional concern, and more lean products are now available (some as low as 5%). The sensory quality of the traditional high-fat sausages is, however, unique and a standard for the gourmet. The body fat content of pigs has been already drastically reduced by breeding, but with regard to ham, it is left to the consumer to cut off the fat layer before consumption.

Mold-ripened varieties of both sausages and ham (e.g., Tirolean speck, Jamon Serrano, and Bündner Fleisch) exist. The production of such products free of mycotoxin is a concern, because of the potential of fungi for contamination. In addition, a carryover from animal feed to the meat may be a source of mycotoxin contamination.[39] This hazard is the target of general meat inspection and control. In rare cases, mycotoxins have been detected in fermented sausages and ham. One way to overcome this hazard is the use of competitive mold strains that have a proven absence of a mycotoxigenic potential.[40,41] These starter cultures usually contain strains of *Penicillium nalgiovense* or *P. chrysogenum*, and are already widely in use in Europe. For ham production, the absence of mycotoxins is still a matter of rigorous quality control.

Meat may also contain bacterial food pathogens. Because fermented meat products usually do not undergo a physical treatment to eliminate pathogenic microorganisms, the meat has to be of high quality with regard to hygiene and microbial counts. The control of pathogens is achieved by appropriate fermentation technology, including the use of starter cultures.

10.3 THE FERMENTATION PROCESS

The traditional aim of the fermentation process is to transform the highly perishable substrate meat into a shelf stable and safe product ensuring an optimum nutritive value and sensory quality. The factors affecting the process are the nature of the raw materials, and the activity of microorganisms, as well as endogenous enzymes and process technology. For all fermented meat products, the raw material is meat with a variable amount of fat that has not been subjected to a thermal or any other germ-reducing process. Meat is the flesh (muscle tissue) of warm-blooded animals, but fermented specialities from poultry (sausages as well as cured and smoked fermented poultry) are also available. Two groups of products can be differentiated on

the basis of the microbial populations involved in the fermentation process—foods from a comminuted matrix and whole meat products.

10.3.1 Fermentation of a Comminuted Meat Matrix

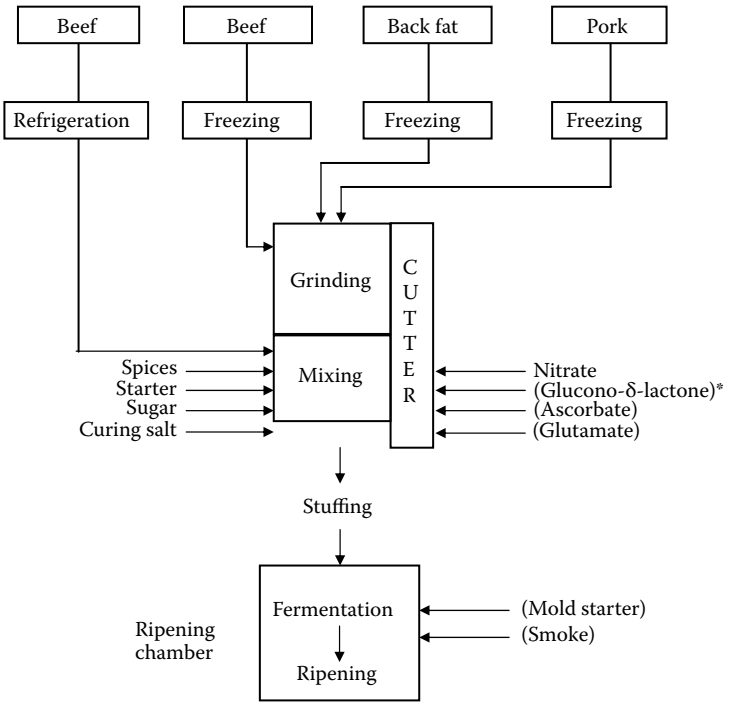
10.3.1.1 Variables in Sausage Production

The comminution of the muscle tissue to particles varying in size between 1 and 30 mm, together with the homogeneous distribution of fermenting organisms, is the prerequisite for a fermentation process taking place throughout the matrix. The content of fat and the origin of meat, e.g., from pork (most common), beef, mutton, reindeer, horse, donkey, or venison, affect color, flavor, and texture of the sausages. Curing salt, nitrate, ascorbic acid, and, in some cases, sodium glutamate and glucono-δ-lactone are added to the particles together with spices. In addition, a carbohydrate source is added, which serves as the fermentation substrate for lactic acid bacteria and its concentration is a means to direct the pH drop to a desired level. Glucose is commonly used, but sucrose or maltodextrin are also in use. The addition of lactose or milk powder is a means to reduce the initial water activity and thereby the potential growth of undesired bacteria in that critical process phase. Each of these compounds exerts a more or less strong effect on the growth and performance of the fermentative flora.

The fatty tissue should be as fresh as possible, as any initiated oxidative process will strongly affect the shelf life by causing early rancidity. The whole comminution process of chopping or grinding together with a mixing procedure requires temperatures below 2°C to 3°C. Thereafter, the temperature is raised usually to > 20°C and < 28°C to initiate the fermentation process. Semidry sausages of the U.S. summer sausage type are fermented at even higher temperatures (32°C to 38°C). The many types of fermented sausages are the result of the formula including the ingredients described above, and of a great variety of process conditions. These conditions include as variables the following:

- The particle size of the comminuted meat and fatty tissue
- The selection of ingredients including additives
- The temperature/humidity conditions prevailing in the course of fermentation until the final ripening
- The diameter of the sausages
- The nature of the casings
- Smoking
- Heating after fermentation
- Supporting the development of mold growth on the surface or establishing a special tight surface film (e.g., by coating with a titanium dioxide film)
- Dipping in antifungal preparations (sorbic acid or pimaricin)[3,42]

A flow scheme of the process of production of common fermented sausages is depicted in Figure 10.1. The fermentation process ensures that the original highly perishable raw materials turn into a spoilage-resistant, flavor-rich product with a defined texture and stable color. Great variation exists with regard to texture that



* The ingredients shown in parenthesis may be used but are not essential in traditional processes.

FIGURE 10.1 Flow scheme of the process of production of dry fermented sausage of a common German type.

may extend from spreadable to sliceable, from soft to a very hard appearance. With regard to the microbial effects, the first days are of great importance. During that time, the organisms multiply, reduce the pH to values ranging from 5.4 to 4.5, exhibit enzymatic activity, and interfere with undesired microorganisms, which constitute the indigenous flora of the meat. In the course of ripening, the pH usually rises again. This rise in pH does not constitute a safety hazard, because at the same time the water activity is decreased to levels that discourage bacterial growth.

The study of the fermentation processes has revealed that lactobacilli and micrococci play a decisive role. These organisms develop under specific prevailing ecological conditions and were sometimes inoculated by back slopping, i.e., adding chopped fermented sausage back to a new meat mixture. Especially between 1950 and 1960, microorganisms were isolated and turned into preparations of starter cultures, which are now commonly used as they exhibit numerous advantages when compared with the classical indigenous fermentation. For example, the reproducibility with regard to process time and product quality is greatly enhanced and microbial risks are reduced in such a way that the application of starters plays a role in the Hazard Analysis and Critical Control Points (HACCP) concepts for quality assurance. In Table 10.3 microorganisms are listed that can be found as components in some starter cultures. Many

TABLE 10.3
Current Status of Species Employed in Meat Starter Cultures

Bacteria

Lactic acid bacteria

Lactobacillus acidophilus,[a] *Lb. alimentarius*,[b] *Lb. paracasei*,[a] *Lb. rhamnosus*,[a] *Lb. curvatus, Lb. plantarum, Lb. pentosus, Lb. sakei, Lactococcus lactis, Pediococcus acidilactici, P. pentosaceus*

Actinobacteria

Kocuria varians[c]
Streptomyces griseus
Bifidobacterium spec.[a]

Staphylococci

Staphylococcus xylosus, S. carnosus ssp. *carnosus, S. carnosus* ssp. *utilis, S. equorum*[b]
Halomonadaceae
Halomonas elongata[b]

Fungi

Penicillium nalgiovense, P. chrysogenum, P. camemberti

Yeasts

Debaryomyces hansenii, Candida famata

[a] Used in probiotic cultures.
[b] Used in premarket studies at industrial scale (Laboratorium Wiesby, Niebüll and Rudolf Müller and Co. Gießen, pers. comm.).
[c] Formerly *Micrococcus varians*.

of the bacteria used are lactic acid bacteria (LAB), which are of primary importance, but included in this table are also nonlactic acid bacteria that are used mainly in combination with LAB and contribute to the fermentation process as they have unique properties. For example, *Kocuria* spp. and *Staphylococcus* spp. exhibit nitrite and nitrate reductase activity, respectively, which is important for the reddening of the sausages, i.e., the formation of the stable red color of nitrosomyoglobin. In addition, these organisms exhibit catalase activity that counteracts the formation of hydrogen peroxide and thus color defects and rancidity. Yeast and fungi contribute mainly to the development of flavor and to a minor extent also to color stability.

10.3.1.2 Sausages as Possible Probiotics

Sausages have been developed and are marketed with the claim that they contain probiotic bacterial strains. The use of fermented sausages to create a probiotic food that can fulfill the requirements necessary to justify a claim related to the health of the consumer has numerous obstacles. These have been discussed by Hammes and Haller.[43] It was argued that the sausages should contain the probiotic bacterial strain at counts that are needed to be effective in the intestines. They have to be in a state that supports survival and metabolic activity in the intestinal tract, and they should be consumed at adequately high numbers on a daily basis. Finally, adequate human

studies with the probiotic food should have been performed that substantiate any health claim. Intestinal isolates as well as potential probiotic strains of lactobacilli isolated from food sources and bifidobacteria had been used for sausage production, and it could be shown that they were present in finished sausage or model sausages; some even contribute to the fermentation process comparable to commonly used starters.[44–47]

In studies of Bunte et al.[48] and Jahreis et al.,[49] a food isolate (*Lactobacillus paracasei)* was used as a starter culture for production of a moist type of fermented sausage with a defined shelf life. Human studies were performed in which the microbiology of the sausages and occurrence of the test strain in the feces were investigated. Volunteers had consumed 50 g sausage containing ca. 10^8 colony forming units (CFU)/g of the test strain for a period of 4 weeks. The analyses of blood samples from these volunteers revealed that in those persons with high counts of the test strain in their feces, certain blood parameters were affected during the challenge period. In particular, the values of CD4 T helper cells were elevated and the phagocytosis index increased. Furthermore, the expression of CD54 (ICAM-I) decreased. This glycoprotein is otherwise upregulated in response to a variety of inflammatory regulators. Finally, an increased titre of antibodies against oxidized low-density lipoprotein (LDL) was measured. In this case, the starter strain brought about the "probiotic effect." In this example, the probiotic strain served as a starter culture because of its technological and sensory effects. Thus, in principle, it is possible to produce probiotic fermented sausages. However, sausages have to be designed such that the probiotic bacterial strain can exhibit its beneficial effects. It can be assumed that any large reduction of pH (e.g., pH < 5.0), extended ripening (e.g., > 1 month), drying or excessive heating (e.g., summer sausage) has the potential to make the probiotic effect questionable, as most strains of bacteria may be damaged or killed under these conditions.

10.3.2 Fermentation of Whole Meat Products (Ham)

Immediately after slaughter, enzyme-catalyzed reactions start to act on the physical and chemical nature of muscle, turning it into meat. These reactions continue even when technological/processing measures are imposed such as cool storage and lowering the water activity by drying or salting are imposed. However, the reactions proceed in a predictable and controlled way. This process provides the foundation of ham production. With the exception of examples given below, microorganisms do not play a role in the fermentative processes taking place in ham.

By far the majority of fermented raw ham is made from pork, but in some regions beef (Bresaola, Bündner Fleisch, Pastirma) and even meat from game, reindeer, or bear is used to produce similar products.[1,38] The traditional ham in ancient Greek and Roman times, as well as in China, was made from the bone containing ham of hogs. This type of ham is still considered the gold standard of quality and is produced in many countries, e.g., Prosciutto di Parma (Italy), Jambon de Bayonne (France), Jamón Serrano (Spain), Kraški pršut (Slovenia), Virginia ham (United States), and Yunnan ho-twe and Tshingwa ho-twe (China).

The process of ham production follows a rather simple principle.[38] It consists of curing by salting (with or without the use of nitrite or nitrate) to achieve a water activity of < 0.96, which is equivalent to 4.5% sodium chloride. At low temperatures (5°C), the salt will diffuse to the deepest part of the meat, thus overcoming the hazard of food poisoning through *Clostridium botulinum* contamination. After a phase of equilibrating the salt concentration and flavor development, the temperature is raised to 15°C to 25°C to ripen the ham. This phase lasts at least 6 to 9 months and may be extended even to 18 months to achieve the optimum flavor. At the end of the ripening step, the moisture has been reduced by about 25% and a salt concentration between 4.5% and 6% results. In some countries (e.g., Germany) in addition to a nitrate cure, smoking is used to obtain a characteristic flavor and to suppress surface growth of molds. When ham is produced from only one or a few muscles, numerous methods are applied to accelerate production time and to control flavor development and water content. For example, the curing is performed in brine or by injection of curing salt and, above all, the ripening period is drastically reduced. Microorganisms may cause spoilage by growing in the inner muscle parts before the water activity is reduced to safe levels. However, microorganisms also contribute favorably to the process as they are involved in nitrate curing in brine by the formation of the reactive nitrite, which affects color by reddening, flavor, and microbial safety. The microorganisms involved are Gram-negative bacteria such as vibrios and *Halomonas* spp. Unlike fermented sausages, ham has no apparent potential to be used as a probiotic food.

10.4 COMPOSITION AND CHANGES DURING FERMENTATION

The physical, biochemical, and microbial changes during sausage fermentation are summarized as follows: growth of LAB and concomitant acidification of the product, reduction of nitrates to nitrites and formation of nitrosomyoglobin, solubilization and gelification of myofibrillar and sarcoplasmic proteins, degradation of proteins and lipids, and dehydration.

10.4.1 Fermentation Microflora

The initial microbial population of sausage minces is highly variable and strongly depends on the microbial load of the raw materials. The ecological conditions of sausage minces favor the growth of Micrococcacea and lactobacilli. Lactobacilli generally grow to cell counts ranging from 5×10^8 to 10^9 CFU/g, and these numbers remain stable throughout ripening. Micrococcacea (predominantly *Kocuria varians*, *Staphylococcus carnosus*, or *S. xylosus*) generally grow to cell counts of 10^6 to 10^7 CFU/g, when a nitrate cure is applied. The growth of these organisms is inhibited by the application of a nitrite cure as well as the decrease of pH. Higher cell counts are reached at the outer layer of the sausages, where higher oxygen partial pressure occurs.

Because of their high salt tolerance, the predominant microorganisms found in dry cured ham fermentation belong to the classification that was formerly included in the family Micrococcacea. The species most often isolated are *Staphylococcus*

xylosus, *S. equorum*, *S. sciuri*, but *K. varians* were also found at appreciable cell counts.[38,50,51] Growth of staphylococci occurs primarily at the surface of hams.[52]

The growth of yeasts and fungi on mold-ripened products is restricted to the surface of the product, where cell counts reach numbers of 10^5 to 10^7 CFU/cm^2 within 4 weeks of ripening. Many of the traditional fermentation processes involving fungal ripening rely on inoculation by the "house flora" associated with the building or equipment used for fermentation and maturation. The occurrence of yeasts and other fungi on fermented meats was reviewed by Cook.[53] *Penicillium* constituted 96% of the microflora; the nontoxigenic species *Penicillium nalgiovense* was most frequently isolated. Long ripened ham such as Country Cured Ham in the United States or Jamón Serrano in Spain are characterized by low water activity and contain primarily aspergilly of the *A. glaucus* (teleomorph *Eurotium*) group on their surfaces. On the Spanish ham, the xerophilic *A. rubrum* does not produce mycotoxins and forms a so named "bishop's cape" of purple color.[38] The halotolerant yeast *Debaryomyces hansenii* is the predominant yeast in meat fermentations.

10.4.2 Acidification, Dehydration, and Microbial Antagonism

Acidification to the isoelectric point of meat proteins (pH 5.3 to 5.4) and the increase in the ionic strength induces gel formation of the proteins and thus confers important structural changes. The high levels of sodium chloride and lactate in fermented sausages contribute to the development of the characteristic taste of the product. Rapid acidification and subsequent drying are of paramount importance for the inhibition of the growth of pathogens and their subsequent inactivation during ripening.[54] As there exists nondried fermented sausage of a spreadable type (e.g., in Germany known as Streichmettwurst, Teewurst, Rohpolnische) the highest hygienic standards of the raw materials and production facilities are key to product safety. In addition to low pH and water activity, specific microbial metabolites such as diacetyl or short chain fatty acids exert an inhibitory effect towards pathogens. Several meat starter cultures produce bacteriocins—small, heat stable peptides with antimicrobial activity.[55] The use of bacteriocinogenic starters has been shown to contribute to the elimination of *Listeria* during sausage fermentation.[56] However, because of the resistance of Gram-negative organisms, including *Salmonella* and *Escherichia coli* O157:H7 strains, the contribution of bacteriocins to the overall hygienic safety of fermented meats is limited.[57,58]

Growth and metabolism of LAB result in a fast drop of pH during the first days of sausage fermentation, followed by a slight increase during the ripening period. Lactic and acetic acids are the major fermentation products, and the molar ratio of lactate to acetate ranges between 7 and 20.[59,60] The product pH depends on the buffering capacity of the meat, the metabolic activities of the fermentation microflora, and the addition of fermentable carbohydrates. In Northern Europe and U.S. summer sausage, the pH typically ranges from 4.6 to 5.2, corresponding to a content of 200 mmol lactate/kg dry weight. In Mediterranean-type products involving longer ripening periods of up to several months, the final pH typically ranges from 5.4 to 5.8. In mold-ripened products, the sausage pH may increase to levels close to 6.0 due to lactate consumption and the formation of ammonium. The dry matter content of

fermented sausages ranges from 50% to 75% or more, corresponding to water activity (a_w) values ranging from 0.86 to 0.92 upon ripening.

10.4.3 Proteolytic and Lipolytic Degradation during Fermentation

The proteolytic events during fermentation of raw sausages and dry cured ham had been reviewed by Toldra and Flores, and Ordónez et al.[61,62] In the course of ripening, peptides and amino acids accumulate to levels of about 1% dry matter.[63,64] Peptides and amino acids themselves may contribute to the characteristic taste of dry cured products and act as flavor enhancers and synergists. Excess proteolysis may result in bitter and metallic off-flavors because of the presence of bitter peptides. Furthermore, amino acids and peptides are utilized by microorganisms for the conversion to flavor volatiles. Several food proteins, mainly milk proteins, but also meat contain peptide sequences with numerous bioactive potentials. The release of these bioactive peptides is known to be influenced by lactic fermentation. Arihara reported that the extract of European fermented sausage contained levels of angiotensin-I converting enzyme (ACE) inhibitory activity exceeding those of nonfermented porcine meat products.[65]

Bovine hemoglobin, porcine myosin, as well as actin and myosin, from chicken encompass peptides that inhibit ACE. The ACE inhibitory activity of these peptides was demonstrated after protein hydrolysis with thermolysin or with chemically synthesized peptides.[66] The *in vitro* activity of peptides does not correlate well to their activity after oral ingestion because the peptides are further hydrolyzed by pancreatic proteases. However, small peptides with a C-terminal Pro residue such as NMP and NPP from porcine myosin, or LAP from chicken muscle, have a higher resistance to degradation by digestive enzymes.[67] ACE regulates blood pressure as well as the fluid and salt balance in humans. Food derived ACE-inhibitory peptides were suggested to treat hypertension;[67] however, it remains unknown whether those ACE-inhibitory peptides that are encrypted in meat proteins accumulate to active levels during fermentation of meat products.

The hydrolysis of muscular proteins to peptides is mainly achieved by the activity of endogenous enzymes. The endopeptidases cathepsins—B, B+L, and H—were shown to remain active throughout the fermentation of dry cured ham and fermented sausages, whereas tissular calpains were inactivated during fermentation and do not contribute significantly to overall proteolysis. Furthermore, muscle exopeptidases contribute to peptide conversion to amino acids. The proteolytic system of lactobacilli consists mainly of cell wall-associated proteinases that convert proteins to oligopeptides. Oligopeptide transport is the main route for nitrogen entry into the bacterial cells, and virtually all peptidases are located intracellularly.[68,69] The proteolytic activity of starter cultures is weak compared to that of tissular enzymes. Correspondingly, the inoculation of sausage minces with starter cultures leads only to a minor increase in amino acid levels of the sausages compared to aseptic control batches.[63] The proteolytic activity of *Kocuria varians* is inhibited by environmental conditions prevailing during sausage ripening, yet the peptidase activity may contribute to the formation of amino acids.[70] It had been shown that *Lactobacillus casei* utilizes peptides released from pork muscle sarcoplasmic and myofibrillar proteins under conditions of sausage ripening.[71,72] Amino acid accumulation is particularly

relevant in the case of glutamate that is related to the savory (umami) taste of foods. Glutamate accumulation during sausage fermentation is not only attributable to proteolysis but also to glutamine deamidation by starter LAB.[73,74] Glutamine conversion to glutamate was observed for *Lactobacillus sakei* and *Lactobacillus plantarum* during growth in sourdough, and their glutamine deamidase activity should also contribute to glutamate accumulation during sausage ripening.[75] *Lb. sakei* additionally exhibits asparaginase activity during sausage ripening.[76] This activity improved the performance of *Lb. sakei* in sausage fermentations, but aspartate is less relevant for the taste than glutamate

The fat content of fermented sausages typically ranges from 40% to 60% of dry matter. During fermentation, long-chain fatty acids are released from triglycerides and phospholipids. Typically, an increase in the levels of free fatty acids up to approximately 5% of the total fatty acids has been found.[64] The fatty acid composition of fat varies considerably depending on the previous feeding regime of the animal. The specific release of polyunsaturated fatty acids is higher than that of monounsaturated or saturated fatty acids. This may reflect a preference of microbial and meat endogenous enzymes for the sn3 position of the tryglyceride most frequently occupied by unsaturated fatty acids, or a preference for the polar lipid fraction.[64,62] Lysosomal muscle acid lipase and adipose tissular lipases remain active throughout several months of dry cured ham ripening.[61] Comparisons of aseptic fermented batches of sausages with batches inoculated with starter cultures has shown that lipolysis during fermentation is attributed mainly to meat endogenous enzymes.[77–79] Lactobacilli are considered to be weakly lipolytic. Strains of *K. varians* and *S. carnosus* or *S. xylosus* have been found to exhibit lipolytic activity, which is, however, inhibited at pH values below 6.[80,77] In mold-ripened products, lipolytic activities of the surface mold flora contributed to the generation of long-chain fatty acids.[81]

10.4.4 Generation of Flavor Volatiles

Flavor compounds are generated during sausage fermentation by the following general routes:

1. Flavor volatiles are generated by lipolysis and hydrolysis of phospholipids, followed by oxidation of free fatty acids.
2. Microorganisms produce organic acids; convert amino acids and peptides to flavor active alcohols, aldehydes, and acids; and modify products of lipid oxidation, e.g., by esterification of acyl moieties or reduction of aldehydes.
3. Depending on the product formula and maturation conditions, the sausage aroma is determined by the addition of spices, smoking, or surface-ripening with yeasts or molds.

An overview of mechanisms for generation of flavor compounds during sausage fermentation is shown in Figure 10.2. Despite the differences in the process technology and the fermentation microflora, it may be assumed that the generation of flavor during fermentation of dry cured ham is governed by the same principles.[82,61]

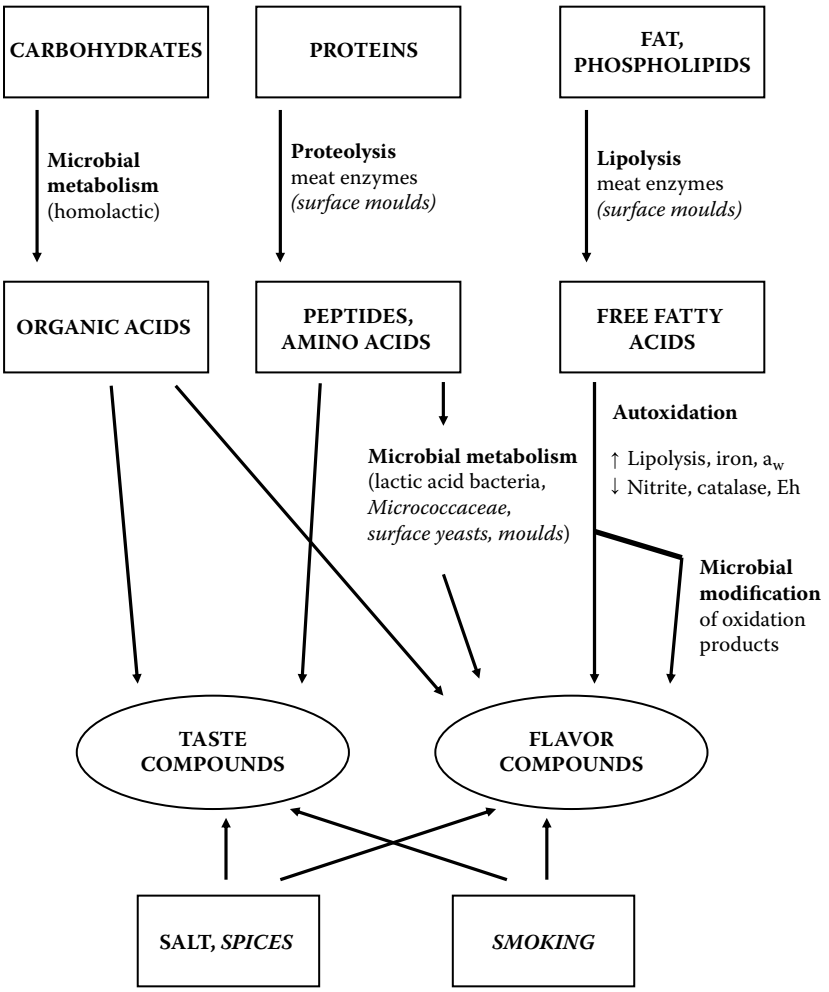


FIGURE 10.2 Schematic overview of mechanisms for generation of flavor compounds during sausage fermentation. *Note:* The relative importance of the biochemical, chemical, and microbial transformations on overall taste and flavor remains to be determined, and is expected to differ considerably depending on regional or product-related variations in the product formula and ripening conditions. Smoking of sausages, or ripening with a surface mould flora is used for a significant proportion of fermented sausages depending on regional preferences.

Data are available from aroma extract dilution analysis showing the identification of those volatile compounds that have a significant impact on sausage flavor.[83] The most important odor compounds in French, Italian, and Spanish salami are those originating from added spices, i.e., sulphur compounds (e.g., diallylsulphide) originating from garlic, and eugenol from nutmeg. Products of lipid oxidation, fatty acids, as well as fermentation volatiles, such as acetic acid, diacetyl, and phenylethanol, further contribute to overall flavor. The comparison of flavor volatiles and

sensory attributes of sausages of various origins reveals that a high level of fatty acids negatively affected the sausage aroma.

Unsaturated fatty acids are prone to autoxidation in the presence of oxygen. Many of the products of lipid oxidation are highly volatile and have a low odor threshold. Hexanal, nonenal, 2(Z)octenal, 1-octen-3-ol, and 1-octen-3-on were identified as the most potent flavor volatiles resulting from autoxidation of linoleic acid, and these compounds are present in fermented sausages.[54,64,83,84] Aldehydes originating from lipid oxidation are subjected to further modification by microbial reductases.[85,86] The rate of lipid oxidation is greatly enhanced by heme or nonheme iron. The increase in NaCl concentration during ripening also favors lipid oxidation. Nitrite present as part of the sausage formula or formed from nitrate by microbial nitrate reductase acts as an antioxidant. The removal of peroxides by catalase, pseudocatalase, or manganese-dependent superoxide dismutase activities of the Micrococcaceae and lactobacilli is crucial to limit the extent of fatty acid oxidation and to prevent off-colors.[60,85,87]

Amino acid catabolism by staphylococci and lactobacilli yields volatile products contributing to meat flavor. Insight into the relevant metabolic pathways has primarily been obtained from LAB used in cheese manufacturing.[88–90] Amino acid degradation is initiated by pyridoxal-5′-phosphate (PLP)-dependent transamination, followed by decarboxylation. A second pathway for transformation of methionine and cysteine involves β-γ-elimination and subsequent formation of methanethiol, methional, and low molecular weight sulfhydryl compounds. It has been shown that comparable pathways occur in meat starter cultures. *Staphylococcus xylosus* and *Staphylococcus carnosus* were shown to produce a large variety of flavor volatiles originating from amino acid degradation during growth on sausage minces.[91] The formation of flavor volatiles is strongly affected by the growth parameters salt, nitrate, glucose, and oxygen.[92,93] Degradation of leucine was also shown for strains of *Lb. curvatus*, *Lb. sakei*, and *Lb. plantarum*.[94] The addition of proteolytic enzymes and the use of proteolytic starter cultures does not enhance the microbial conversion of amino acids to flavor-active derivatives.[80,95]

10.4.5 Biogenic Amines

The content of histamine, tyramine, phenylethylamine, tryptamine, putrescine, and cadaverine in fermented meat products ranges from not detectable to levels exceeding 100 mg/kg.[60,96] At concentrations above that level, the pressor amines histamine and tyramine may constitute a health hazard for patients under treatment with monoamine oxidase inhibitors.[97] Biogenic amines in fermented meat products are mainly derived from bacterial decarboxylation of amino acids. Putrescine and cadaverine are produced by the Gram-negative spoilage flora of raw meat, and high levels of these compounds indicate poor hygienic quality of the raw materials for sausage production. LAB used for sausage fermentation have the potential to decarboxylate tyrosine, histidine, and ornithine to the corresponding amines tyramine, histamine, and putrescine.[98] LAB are assumed to be the main source of tyramine in fermented sausages. The formation of tyramine during sausage fermentation occurs during the initial phase of the fermentation, in which LAB are metabolically active rather than during the ripening phase. Proteolytic events during sausage fermentation do not

appreciably affect levels of tyramine and histamine.[99] The use of starter cultures with low tyrosine decarboxylase activity to rapidly inhibit metabolism of Gram-negative bacteria and to suppress growth of the indigenous fermentation flora with potentially higher decarboxylase activities, has been shown to effectively reduce tyramine levels in fermented sausages.[100] A reduction of tyramine was observed in sausages where both tyramine and high levels of tyramine oxidizing *K. varians* were added. Only a limited effect of microbial tyramine oxidases was found in experiments where the same strain was applied in combination with a tyramine producing strain of *Lb. curvatus.*[101,102]

10.5 POTENTIAL BENEFICIAL HEALTH EFFECTS OF BACTERIA INVOLVED IN MEAT FERMENATION

It is characteristic of LAB that certain species are found not only as members of the human intestinal microflora but also as part of the man-made ecosystems present in fermented food.[103,104] Denaturating gradient gel electrophoresis (DGGE) of DNA fragments generated by polymer chain reaction (PCR) with 16S rDNA targeted group-specific primers has been used to demonstrate that food-associated bacteria and especially meat fermenting bacteria such as *Lb. sakei*, *Lb. curvatus*, *Leuconostoc mesenteroides,* and *Pediococcus pentosaceus* are present but not culturable in human feces.[105] Bunte et al.[48] demonstrated that *Lb. paracasei* LTH 2579 ingested as a component of dry-fermented sausage can be recovered from the human feces, suggesting that food-associated microorganisms may contribute to the microbial ecosystem of the gastrointestinal tract (see Section 3.1.2).

Although many variables can determine the degree to which bacteria survive the gastrointestinal transit, the survival in, and temporary colonization of, the human digestive tract by some LAB have been predicted with the use of in vitro experiments. Studies by Pochart et al.[106] and Marteau et al.[107] showed that by using intestinal perfusion, the recovery of viable bacterial cells in the small intestine correlated with their tolerance to in vitro acid/bile treatment. It has recently been shown that certain strains of bacteria important in meat fermentation such as *Lb. plantarum*, *Lb. paracasei*, *Lb. sakei*, *Lb. curvatus* and *Staphylococcus carnosus,* have a similar capability to survive low pH (1.5 to 2.5) and bile (10 mM), to hydrolyze bile salts, and to attach to enterocyte-like CaCO-2 cells when compared with bacteria of intestinal origin or probiotics.[108] These results may suggest that the properties of microorganisms that determine survival and function in the gastrointestinal tract may also be present in food-associated bacteria. However, the gastrointestinal survival rate of bacteria as a sole criterion for probiotic functions is not a valid argument, but requires additional studies that are adequate to substantiate specific health claims. Colonization studies in animals have shown that murine *Lactobacillus* spp. were permanently re-established in mice that had been rendered free of lactobacilli by antibiotic treatment, and that germ-free mice were susceptible to permanent colonization with bifidobacteria.[109,110] However, in human trials, the recovery of probiotic or food-associated strains soon stopped after their oral administration was discontinued.[111] Consequently, the probiotic function of microorganisms may be determined by their ability to directly interact with the gut-associated tissue or to

affect metabolic parameters of the indigenous microflora while passing through the gastrointestinal tract.

It has to be emphasized that the development or substantiation of health claims for fermented food in general, and for fermented meat especially, requires not only adequate human trials, but also a profound understanding of the molecular and cellular mechanisms of bacteria–host interactions with the gastrointestinal tract as a sophisticated interface between a rapidly changing luminal environment and specific host-target cells. The intestinal epithelium is a highly selective barrier between the luminal environment and gut-associated immune cells. Intestinal epithelial cells (IEC) constitutively express, or can be induced to express, co-stimulatory molecules,[112] components of the major histocompatibility complex [113,114] (MHC), pattern recognition receptors,[115,116] inflammatory and chemoattractive cytokines,[117] as well as antimicrobial peptides.[118,119] Remarkably, most of these molecules are at least in part transcriptionally regulated by the transcription factor NF-κB.[120] The intestinal epithelium is considered to be a constitutive component of the mucosal immune system. In fact, IEC contribute to the initiation and regulation of innate and adaptive defense mechanisms by directly interacting with lamina propria dentritic cells (DC), lamina propria lymphocytes (LPL), intraepithelial lymphocytes (IEL), as well as mediators of the immune and the enteric nerve system.[121–123] Together with nonimmunological barrier functions such as intestinal motility, mucus secretion, and cell turnover, the regulation of IEC integrity is a key element for the mucosal defense system. Specific attention has been focused on the interaction between IEC and DC as a mechanism to polarize colitogenic T cell responses towards Th1/Th17 and Th2 effector functions under conditions of chronic intestinal inflammation including Crohn's disease and ulcerative colitis, respectively.[124–126]

Experiments in germ-free animals have shown that lymphoid populations in the lamina propria are considerably reduced in the germ-free intestine, but assume its normal appearance of physiological inflammation after bacterial colonization.[127–129] This is consistent with the concept that intestinal epithelial cells upon activation with proinflammatory mediators or enteropathogens, express higher levels of molecules responsible for antigen presentation such as HLA class II molecules, classical class I, and nonclassical HLA class Ib molecules, the adhesion molecule ICAM-1, complement factors, cytokines and cytokine receptors.[130]

Epithelial cell transduction of information from the luminal environment to the mucosal immune system is not limited to pathogenic microorganisms. Commensal bacteria including the meat starter strain *Lb. sakei* LTH 681 and nonpathogenic *E. coli* were shown to elicit a characteristic cytokine response in leukocyte sensitized intestinal epithelial cell lines (CaCO-2 and HT-29) *in vitro* and to deliver a discriminative signal to underlying immunocompetent cells.[131,132] In an experiment to demonstrate the dynamic interaction between enteric bacteria and host recognition, the colonization of reconstituted lactobacilli free (RLF) mice with *Lb. reuteri* triggered a transient activation of a NF-κB-dependent proinflammatory gene program even in the presence of an already established microbiota. These findings show that the intestinal epithelium integrates numerous signals from both enteric bacteria and immune cells in order to maintain gut homeostasis.

Rescigno et al.[133] showed that dendritic cells, an important antigen presenting cell population in the intestinal mucosa, specifically open tight junctions not only between the epithelium and sample pathogenic bacteria, but also nonpathogenic bacteria from the gut. The epithelial compartment and the lamina propria are thus potential sites where bacteria of the intestinal microflora may directly encounter cells of the specific and nonspecific immune system. Several studies have shown that lactobacilli isolated from the human gastrointestinal tract activate human mononuclear cells, and are potent inducers of monocyte derived cytokine IL-12.[134–136] On the other hand, *Lb. sakei* LTH 681 and the intestinal isolate *Lb. johnsonii* La1 showed a similar capability to induce natural killer (NK) cell activation, suggesting that the immunogenic activity of lactobacilli are also present in food fermenting microorganisms.[137,138]

The complex interaction between nonpathogenic bacteria, the epithelium, and professional immune cells in the mucosa is a prerequisite for the development of mature immune function and defense mechanisms in the gut. To trigger the development and maturation of the gut-associated immune system, enteric bacteria mediate proinflammatory processes that are tightly controlled by the host and are often referred to as physiologic inflammation. There is evidence that the intestinal luminal microenvironment is responsible for the initiation or perpetuation of chronically relapsing inflammatory bowel disease (IBD). Since the break in intestinal epithelial barrier function precedes the onset of chronic immune-mediated histopathology in IBD patients, and animal models of IBD, the loss of epithelial cell homeostasis seems to be critical for the development of chronic intestinal inflammation.[139,140] Bacteria- and host-derived signals converge at the epithelial cell level and play a critical role in the initiation and regulation of chronic intestinal inflammation. The specific role of enteric bacteria as a requirement for immune-mediated chronic intestinal inflammation is strongly indicated by experiments in rodent models.[141,142]

It was shown that the monoassociation of germ-free wild-type mice with colitogenic *Enterococcus faecalis* transiently induced toll-like receptor 2 (TLR2)-mediated NF-κB-dependent gene expression in native IEC at early time points of bacterial colonization, whereas persistent activation of the TLR/NF-κB pathway was observed in chronically inflamed interleukin 10 deficient (IL-10$^{-/-}$) mice.[143] Consistent with this "self-limiting" induction of TLR2-mediated NF-κB signaling preceding any histological evidence of colitis, the level of TLR2 protein expression in IEC dramatically decreased after the initial colonization of the germ-free host. Interestingly and in contrast to wild-type controls, the presence of persistently active TLR/NF-κB signaling in IL-10$^{-/-}$ IEC was associated with the development of clinical and histological signs of intestinal inflammation at late stages of bacterial colonization, suggesting a pathological role for bacteria-epithelial cell signalling under chronic inflammation. Mechanistically, this showed that TGF-β-activated Smad2 signaling induced rapid TLR2 degradation[143,144] and inhibited CBP/p300-mediated histone phosphorylation in IEC.[145] In addition, this showed that prostaglandin J2-induced protein phosphatase 2A (PP2A) activity triggered NF-κB RelA dephosphorylation,[146] and IL-10 mediated p38 signaling inhibited endoplasmic reticulum (ER) stress responses in the intestinal epithelium. The latter one was mediated through mechanisms that inhibit nuclear recruitment of the ER transcription factor ATF-6 to the ER-derived glucose-regulated protein (grp)-78 promoter.[147] These findings strongly support the

possibility that host-derived immunosuppressive mediators significantly contribute to regulatory feedback mechanisms towards the luminal bacterial challenge at the epithelial cell level.

Madsen et al. demonstrated that two-week-old SPF IL-10$^{-/-}$ mice displayed changes in bacterial colonization with increased aerobic adherent and translocated bacteria, in conjunction with reduced levels of lactobacilli. Rectal administration of the endogenous *Lb. reuteri* strain enhanced mucosal barrier function and attenuated the development of colitis at 4 weeks of age.[148] Similar protective effects were demonstrated in a rat model of methotrexate induced enterocolitis after oral administration of *Lb. plantarum*.[149] Treated animals were characterized by a decrease in body weight loss, intestinal permeability, and myeloperoxidase levels. In addition, *Lb. rhamnosus* GG ATCC53103 inhibited cytokine-induced apoptosis in IEC lines by activating the Akt/protein kinase B signaling pathway,[150] supporting the concept that probiotic bacteria may help to maintain the barrier function of the intestinal epithelium. A mechanistic role for loss in barrier function in the pathogenesis of mucosal inflammation is shown in *N*-cadherin dominant negative mice,[151] and mice with disruption of the multidrug resistance gene 1a (mdr1a$^{-/-}$).[152] An additional molecular mechanism of probiotic activity was shown at the level of epidermal growth factor (EGF) receptor signalling. Resta-Lenert and Barrett revealed that *Lb. acidophilus* ATCC 4356 and *Streptococcus thermophilus* ATCC 19258 restored EGF receptor phosphorylation/activation in IEC cultures after the infection by the enteroinvasive strain *E. coli* O29:NM.[153] These effects were associated with increased expression of tight junction proteins, and with improved barrier function in the model epithelium.

Convincing evidence for protective activities of certain LAB in human irritable bowel disease (IBD) was reported in studies by Gionchetti et al., who showed that the combination of eight different LAB including lactobacilli, bifidobacteria, and streptococci (VSL#3) inhibited relapse of chronic pouchitis (15% recurrence rate in the VSL#3-treated group versus 100% in the placebo group), with inhibition of mucosal TNF, and upregulation of IL-10 in the treated pouches.[154,155] In ulcerative colitis (UC) patients too, the VSL#3 probiotic mixture has been associated with remission of the disease.[156] Evidence for the beneficial effects of VSL#3 was also shown in IL-10$^{-/-}$ mice. VSL#3 treatment of SPF IL-10$^{-/-}$ mice resulted in the normalization of physiologic colonic function, barrier integrity, and histopathology, in conjunction with a reduction of mucosal TNF and IFNγ secretion.[157] Interestingly, VSL#3 induced cytoprotective heat shock protein expression in IEC and blocked proteasome function followed by the inhibition of the pro-inflammatory nuclear transcription factor (NF)-κB.[158] Moreover, VSL#3 ameliorated Th1-mediated colitis in trinitrobenzene sulfonic acid (TNBS) treated mice by inducing IL-10 and IL-10-dependent TGF-β-bearing regulatory T cells.[159] Additional mechanistic evidence for the protective effects of VSL#3 was shown at the level of Toll-like receptor (TLR) 9 signaling using CpG sequences from bacterial DNA for the treatment of DSS-induced experimental colitis in TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR9$^{-/-}$ mice.[160]

10.6 CONCLUSIONS

In summary, LAB as part of a traditional human diet or probiotic therapy may influence the homeostasis between the intestinal microflora and the host, but their efficacy in the prevention or even treatment of certain disease remains to be clarified. Since mechanisms by which LAB confer therapeutic effects may be multiple, the use of a multitude of bacterial species in various food matrices including those present in fermented sausages should be of advantage when used as part of the normal diet. It may be possible in the future to find bacterial strains with probiotic activities that are capable of producing a fermented meat product with all the sensory qualities preferred by consumers, but at the same time sustain their beneficial effects in the complex matrix of the fermented meat product.

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11 Miso

Production, Properties, and Benefits to Health

Yukiko Minamiyama and Shigeru Okada

CONTENTS

11.1 INTRODUCTION

The history of fermented foods and drinks dates back more than 4000 yr. Wine already existed around 5000 B.C., and the original forms of soy sauce and fermented milk existed around 3000 to 2000 B.C. Microorganisms obtained from the environment were put to use for the fermentation and maturation of fermented foods. Regional differences in starting materials, climate, culture, and other environmental factors have developed unique fermented products in various parts of the world. At the same time, regional and racial differences have had a big effect on whether some fermented products are considered good tasting or bad. This chapter describes the traditional Japanese fermented soybean product *miso*, and its effects on human health and metabolism.

11.2 MISO

Miso is a fermented soybean paste, one of the essential seasonings in Japanese cuisine. It tastes and aroma resemble soy sauce. Miso is made from steamed soybeans mixed with salt and *koji*. Koji is mold-treated rice, barley, or soybean that acts as a fermentation starter.

Miso soup is usually served for breakfast in Japan; it contains seasonal vegetables, and *tofu*. Tofu contains no cholesterol and is generally low in saturated fats, which have been linked to coronary disease. Miso can also be used in marinades, dressings, stews, dips, and casseroles. There are regional differences in flavor and taste; *shiro* (white) miso paste has a mild taste and is low in salt, whereas *aka* (red) miso is very salty and has a different, stronger odor than shiro miso. The total commercial production of miso per year amounts to 600,000 tonnes in Japan. There are over 1,500 miso factories in all parts of Japan today.

11.2.1 The History of Miso

Miso is one of the most traditional and characteristic fermented foods of Japan and an important soybean product. Soybean has also been used for soy sauce, soy milk, soy curd (tofu), sticky bean (*natto*), bean sprouts, and for extracting oil. (See Chapter 9 for more details on natto.) The soybean was introduced to Japan most likely from Northern China through Korea, between 200 B.C. to 300 A.D. The first Korean book on miso is thought to be *Taiho-ritsuryo*, compiled in 702 A.D., but by that time, a type of miso was already known in Japan. The technique of miso production was introduced either from Korea or China. One theory proposed that miso was developed from *jan* of Chinese origin, which is also a fermented food from rice or soybean. It is used in China as a seasoning. About the time when the Japanese came to know about jan (about 550 to 600 A.D.), it was a food made in Buddhist temples. It is believed that a Chinese Buddhist named Ganjin, 688 A.D. to 763 A.D., came to Japan in 753, promoted Buddhism in Japan, and at the same time brought jan to Japan.

11.2.2 Manufacturing Miso

Various types of miso are produced that have differences in their soybean/salt/koji starter ratio, aging periods, and other parameters. Figure 11.1 shows the simple manufacturing process for miso. Microorganisms called *koji mold* (fungi *Aspergillus oryzae*) in Japan are used for the fermentation process. Yellow koji mold, *A. oryzae*, is also used in the brewing process of Japanese sake. Enzymes (amylases) in the koji convert the rice starch to sugar. The koji used for soy sauce and miso is also high in proteases and peptidases, which convert proteins to amino acids.[1]

Miso can be classified based on its raw ingredients. Japanese miso products are introduced in Table 11.1 and Figure 11.2. If the salinity is similar, the miso containing a large percentage of malt is sweet. Miso grows rich in color as the ripening period gets longer. Rice miso, the most common type of miso, is made with rice koji starter. Barley miso and soybean miso are made with barley koji and soybean koji starters, respectively. *A. oryzae* is used in the fermentation of all three types of miso.

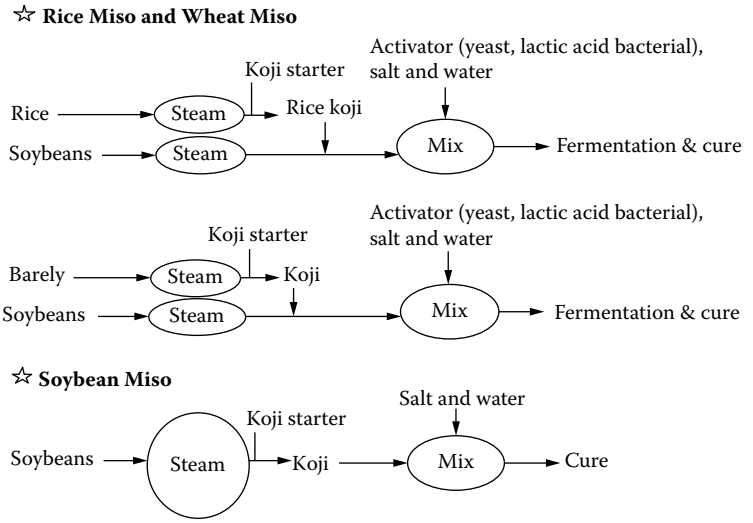

FIGURE 11.1 The miso manufacturing process.

TABLE 11.1
Japanese Miso Introduction

Material	Taste	Color	Producing District	Comments
Rice		White	Kinki district, Okayama	Rice miso: 80% of national production
	Sweet		Hiroshima, Yamaguchi, Kagawa	
		Red	Tokyo	
	Mild sweet	Pale	Shizuoka, Kyusyu district	
		Red	Tokushima, etc.	
	Dry	Pale	Kanto and Koshinetsu districts	Shinsyu miso (Nagano): 35% of national production
			Hokuriku district, etc.	
		Red	Kanto and Koshinetsu districts	
			Tohoku district, Hokkaido district, etc.	
Barley	Mild sweet		Kyusyuu, Shikoku, and Chyugoku districts	
	Dry		Kyusyuu, Shikoku, Chyugoku, and Kanto districts	
Soybean			Aichi, Mie, Gifu	

Kinki district, Kyoto, Osaka, Shiga, Hyogo, Nara, Wakayama; Kyusyu district, Fukuoka, Saga, Nagasaki, Kumamoto, Ohita, Miyazaki, Kagoshima: Kanto district.

The main ingredients of miso are soybeans, rice, salt, and koji. Although many kinds of soybeans exist in the world, there are only a few cultivars of soybean of high enough quality for miso production in Japan. Salt made from seawater is recommended for miso production because it contains metal elements such as magnesium and calcium that positively affect the fermentation. Traditionally in Japan, people

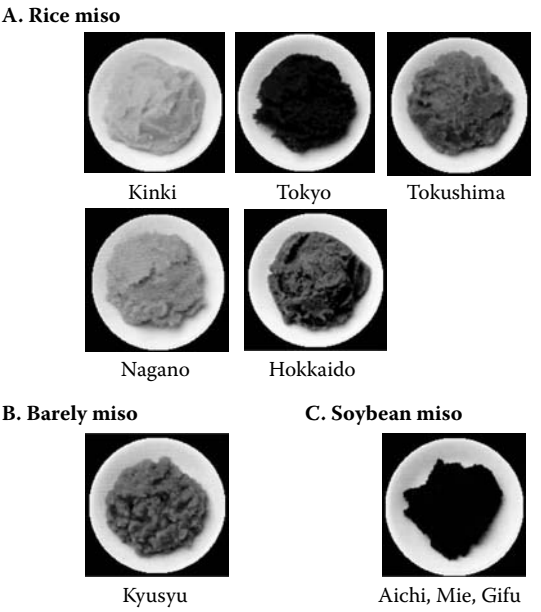

FIGURE 11.2 Major miso in Japan.

used koji from previous batches as a starter culture. However, because there was no way to ensure the identity and quality of the fungus, batches of miso varied in quality (smell and taste). For many years, the supply of koji was controlled by miso manufacturers, and not commercially available. Recently, public brewing institutes in many Japanese prefectures have started research on the selection of better strains of miso koji, and this has resulted in the production of several standardized miso products. Fungus strains are now available through the Internet or at miso shops in Japan.

11.2.3 Home Production of Miso

Japanese consumers have often made their own homemade miso. To make koji, rice (sometimes barley or soybean are substituted) is first steamed. After the temperature of the cooked rice cools down to 25 to 30°C, a small amount of koji fungus is added and mixed well with the rice. After about 10 h, the mixture produces its own heat, which is evidence that the fungi are growing. The depth of the rice pile is controlled to maintain the temperature between 35 and 40°C for about 40 h. Feather-like fungi fibers grow on the rice particles as the fermentation continues.

Soybeans are boiled and mashed while they are hot. The temperature of the mashed soybeans must be cooled down to 35 to 40°C before mixing with the koji. The mashed soybean (40 to 60%), rice koji (25 to 40%), salt (12 to 20%), and warm water (usually the rest of soybean boiling water, ca. 15%) are mixed well until the mixture has the appearance and texture of soft dough. The mixture is then placed in a plastic container and sealed with a plastic sheet on the surface to prevent exposure to the air. A lid is added and weighed down. The container is covered with wrapping

TABLE 11.2
Constituents of Miso

	Content	Metabolic activity
Nonnutritional substances (mg/kg soybean miso)		
p-coumaric acid	1.3	Antioxidant
Ferulic acid	1.0	Antioxidant
Daidzein	14.5	Antioxidant (hydrolysis during fermentation)
Genistein	5.3	Antioxidant (hydrolysis during fermentation)
8-OH-daidzein	1.2	Potent antioxidant (hydrolysis during fermentation)
8-OH-genistein	1.5	Potent antioxidant (hydrolysis during the fermentation)
Syringic acid	3.1	Potent antioxidant
Vanillic acid	8.5	
p-Hydroxybenzoic acid	1.0	
Nutritional Compounds	**Content (mg/100 g on soybean miso)**	
Protein	17000	
Fat	11000	
Carbohydrates	15000	
Ash	13000	
Salt	12000	
Iron	4.0	
Calcium	150	
Sodium	4300	
Water	45000	
Vitamins		
Ascorbic acid	0	
Tocopherols	2.4	
Retinol (IU)	0	
Carotene	0	
Vitamin B6	0.13	

paper, tied in place with strings, and then stored in a cool, dark place. After a month, the fermentation is checked. Miso can be stored and used for several years, and during this time it continues to smell good and retain its yellow-brown color. The composition, including nutrient content, of miso is shown in Table 11.2.[2]

11.2.4 Antioxidative Effects of Miso Ingredients

Free radicals are implicated in many diseases.[3] Therefore, antioxidative defense (intake of antioxidants) is important in biological systems to prevent free radical injury. Miso also has strong antioxidant properties.[4–6]

The brown-colored substance in aged miso has been identified as melanoidin. It strongly suppresses the production of peroxides derived from fatty acids in the body and prevents aging of the body.[7] Vitamin E, daidzein, saponin, and the brown

pigment contained in miso all work as antioxidants, which prevent peroxides from accumulating in the body. Experiments using rats have shown that the increase of peroxides is remarkably inhibited by feeding saponin and the brown pigment.[8]

Hirota et al.[9] reported that nine compounds were isolated from soybean miso that have DPPH radical-scavenging activity. Of these, 8-hydroxydaidzein, 8-hydroxygenistein, and syringic acid had as high DPPH radical scavenging activity as that of α-tocopherol. Another report said that the livers of rats fed with a brownish miso or a peptide-glucose reaction mixture showed lower thiobarbituric acid (TBA) and chemiluminescence values than those of the control.[7] From these results, it was clear that miso and the peptide-glucose reaction products also exhibited antioxidative effect in vivo. In order to explain this mechanism, the scavenging activity against reactive oxygen species (ROS) of various Maillard reaction products (MRPs) was studied. It was confirmed that peptide-glucose reaction products, Amadori rearrangement products, melanoidins, modified protein and its hydrolysate, brown pigments isolated from miso, and other foodstuffs showed strong scavenging activity against hydroxyl radical and superoxide anion. Scavenging activity of MRPs against ROS played an important role in the antioxidative effect of MRPs in vivo.

11.2.5 Effects of Miso on Health

It is believed that the Japanese diet and methods of food preparation have contributed to the longevity of the Japanese people. Among Japanese foods, the outstanding medicinal qualities of miso have been supported by scientific research. In 1981, Hirayama of Japan National Cancer Center[10] conducted an epidemiologic study and reported that those who eat miso soup daily suffer significantly less from stomach cancer and heart disease. In 1972, Ito discovered an alkaloid in miso that removes heavy metals from the body,[11] and recently, Yoshikoshi et al. at the Tohoku University of Japan isolated substances in miso that neutralize the effects of some carcinogens.[10] It has been known from antiquity that miso keeps the body in good condition, and it is said that miso is "a detoxicating drug in the morning" or "a doctor killer." Major ingredients of miso and their expected effects on health and metabolism are listed in Table 11.3.

11.2.5.1 Gastrointestinal Diseases

A study showed that people who eat miso soup regularly (daily) are less susceptible to stomach diseases such as gastritis and gastric and duodenal ulcers, than those who seldom or never eat it.[12] A more detailed survey on the eating habits of people by age indicated that daily miso eaters in their sixties and older have a lowered risk of stomach diseases.[13] Recently, *Helicobacter pylori* has been identified as the causative organism for gastric inflammation and peptic ulcers, and is associated with gastric cancer. Among the isoflavones of miso, genistein, which has an inhibitory activity of tyrosine kinase, especially showed a potent anti-*H. pylori* activity.[14–18] A large portion of the proteins contained in soybeans are degraded by enzymes and microbes in the fermentation process of miso. In addition, miso contains highly active enzymes, which help digestion and absorption of other nutrients.

TABLE 11.3
The Major Active Ingredients of Miso and Their Expected Health/Metabolic Effects

Nutrients (per 100 g)	Origin	Expected effect
Protein (10–20 g)	Soybeans	Reduced blood cholesterol; maintain elasticity of blood vessels; prevent cerebral apoplexy
Vitamin B2 (0.1 mg)	Aspergilli	Promote oxidization reduction in the body
Vitamin B12 (0.1 mg)	Bacteria	Help blood formation; reduce mental fatigue
Vitamin E (0.3–2.4 mg)	Soybeans	Inhibit lipid peroxidation; antiaging
Enzymes	Koji, yeast, lactic acid bacteria	Help digestion
Saponin	Soybeans	Inhibit lipid peroxidation; reduce blood cholesterol; prevent hardening of the arteries; prevent hepatopathy
Trypsin inhibitor	Soybeans	Anticancer; prevent diabetes
Isoflavon	Soybeans	Deoxidization; alleviate stiff neck and shoulders; anti-variant prevent breast cancer
Lecithin	Soybeans	Reduced blood cholesterol; prevent hardening of the arteries; prevent senile deterioration
Colin	Soybeans	Prevent fatty liver; antiaging
Prostaglandin E	Linoleic acid in soybeans	Prevent high blood pressure
Brown pigment	Soybeans	Inhibit lipid peroxidation; antiaging
Dietary fiber	Soybeans	Reduce blood cholesterol; prevent colon cancer

It is believed that plant fibers in miso help "clean the intestines." Microbes in miso antagonize putrefactive bacteria in the intestines and decompose harmful substances in the body.[19]

11.2.5.2 Cancer Prevention

Epidemiologists have known for years that vegetarians and other people who eat diets rich in plant products have relatively low rates of various cancers.[20–24] It is now widely known that anticancer effects are associated with an intake of miso on a regular basis.[25] Miso contains such ingredients as unsaturated fatty acids, isoflavones, yeasts, and lactic acid. Soybeans also contain a trypsin inhibitor and genistein, which have antimutagenic properties.[26]

Japanese women at home and abroad have a very low incidence of breast cancer as long as they maintain their native diet, but the incidence becomes higher if they adopt a relatively soy-free diet.[27] A similar relationship holds for Japanese men and prostate cancer.[28] In both cases, the intake of soy makes the difference, not the intake of fat. According to the results of the epidemiologic research conducted by Dr. Hoshiyama,[29] a negative correlation was found between miso soup consumption and the incidences of death from stomach cancer. People who do not eat miso soup at all are at 50% higher risk of dying of stomach cancer than those who eat it every day.

Researchers at the National Cancer Institute of Japan have identified certain types of phytochemicals in soybeans that have anticancer properties.[30] The group of chemicals called isoflavones exhibits the most powerful anticancer effects. Soybeans are the only commonly consumed food that provides isoflavones in the diet. Certain sugars, in particular oligosaccharides, in soybeans promote the growth of the beneficial bacteria called bifidobacteria in the colon.[31] High levels of bifidobacteria in the intestines have been associated with a lower risk of colon cancer.[32] The level of oligosaccharides is higher in soybeans than in any other food.[31,32]

11.2.5.3 Elimination of Radioactive Materials

Miso export to European countries increased after the accident at the Chernobyl nuclear power plant in 1986 because it was believed that miso consumption could reduce the effects of radiation exposure.[33] Researchers working on microbe activities have shown that the consumption of miso helps to eliminate radioactive substances from the body.[34] After the Hiroshima and Nagasaki atomic bombing at the end of World War II, it was observed that miso factory workers were less affected by radiation than others in the general population. The reason for this protective effect of miso is not known. However, some experimental evidence indicates that rats fed with miso eliminate radioactive materials more rapidly from the body than animals not receiving miso.[35]

11.2.5.4 Effect on Metabolic Syndrome and Aging

Eating soya foods appears to markedly lower blood cholesterol, thereby reducing the risk of heart disease.[13] Miso contains several important substances such as linoleic acid, plant sterols, and vitamin E among others, that have been shown to be cardioprotective. Clinical and experimental studies have shown that substituting soy protein for animal protein or simply adding soy protein to the diet significantly reduces cholesterol levels, regardless of the type or amount of fat in the diet.[13,36–38] For example, 15 healthy nonvegetarian premenopausal women were studied over 9 months. A significant reduction in total cholesterol was found in those consuming 50 g miso/d (45 mg conjugated isoflavones).[37] Kohno et al.[39] reported that soybean β-conglycinin (5 g consumed twice a day) could lower serum triglyceride levels in humans, as well as reduce visceral fat, as observed with a computed tomography (CT) scan around the umbilicus, in a randomized controlled trial. In cholesterol-fed rabbits, the level of cholesteryl ester hydroperoxide (ChE-OOH) induced by $CuSO_4$ in plasma in the high isoflavone group was significantly less than that in the control group.[38]

Scientists are also looking at soy products in connection with both osteoporosis[40–44] and kidney disease.[45,46] A study showed that rats excreted 50% less calcium in their urine when they replaced the animal products in their diet with soyfoods.[47] The mechanism responsible for the calcium retention is not known at this time.

11.2.5.5 Protection of Hypertension

He et al.[48] reported the results of a well-conducted randomized trial of soybean protein (40 g/d) in 302 Chinese adults 35–64 years of age with prehypertension

or stage 1 hypertension. The main finding was a highly statistically significant net reduction in blood pressure of 4.3 mm Hg (systolic) and 2.1 mm Hg diastolic after 12 weeks. The effect was present at 6 weeks but seemed to increase further. Their report was not complete, but higher protein intake may prove to be healthful.

The Dietary Approaches to Stop Hypertension (DASH) trials, initiated by the National Heart, Lung, and Blood Institute, studied the effects of dietary patterns designed around both food groups and nutrients. They agreed that soybean protein may be recommended as part of the DASH diet. Watanabe et al.[49] reported that a miso diet including 2.3% NaCl, and a control diet containing 0.3% NaCl, did not increase blood pressure in Dahl rats, whereas rats consuming the 2.3% NaCl control diet (and no miso) did have increased blood pressure. These results show that blood pressure was not increased by the miso diet.

11.2.5.6 Protection from Harmful Effects of Tobacco

"Miso soup is for smokers" is a Japanese saying from old times. The saying may have originated from the habit of using miso soup to clean pipes plugged with tars, which was a practice in the Edo era (1603 to 1867). Miso soup has a superior cleansing ability for nicotine when it is poured into a nicotine-stained pipe, compared to that of hot or cold water. Miso soup contains B vitamins, which are believed to protect the smoker's throat by neutralizing harmful substances of tobacco.[50]

11.3 CONCLUSIONS

A daily intake of miso soup is recommended by many researchers. The beneficial effects of miso have attracted worldwide attention, and studies are being conducted in various institutes to provide further experimental evidence on the health benefits of consuming miso. Many of the beneficial effects of miso appear to be due to bioactive compounds found in the soybeans themselves. However, the fermentation used in the production of miso imparts added benefits.

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12 Korean Fermented Foods

Kimchi and Doenjang

Jeonghee Surh, Young-Kyung Lee Kim, and Hoonjeong Kwon

CONTENTS

12.1 INTRODUCTION

Koreans have been known for their taste for fermented foods and their skill in making them for more than 1500 yr.[1] The fermentation products span a whole spectrum of raw materials and use various methods of preparation. Major fermented food items, excluding alcoholic beverages, consumed today in Korea may be divided into three categories (see Table 12.1). The first category is *kimchi*, which is consumed the most. Kimchi has napa cabbage and/or radish as its main ingredient, although virtually

TABLE 12.1
Fermented Food Consumption in Korean Population (grams per person per day)

			Age						
Food Items		**Overall**	**1~2**	**3~6**	**7~12**	**13~19**	**20~29**	**30~49**	**50~64**	**> 65**
Kimchi[a]	Scallion	1.3	0.0	0.0	0.0	0.5	1.0	2.1	2.0	0.9
	Kodulbbagi	0.6	0.0	0.0	0.0	0.2	0.5	0.9	0.8	0.6
	Mu (radish)	24.3	1.9	4.4	11.8	24.4	29.0	29.4	30.6	20.8
	Mool (mostly liquid)	13.6	2.0	4.2	5.6	4.6	9.1	15.7	21.7	34.0
	Baechu (Napa cabbage)	83.8	9.9	21.7	50.5	78.6	89.7	105.3	94.5	85.6
	Sobagi (cucumber)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
	Got (mustard leaf)	0.7	0.0	0.0	0.1	0.1	1.1	1.1	1.1	0.3
	Total	124.3	13.8	30.3	68.0	108.4	130.4	154.5	150.8	142.3
Soy based	Ganjang (soysauce)	6.6	1.8	3.7	5.8	6.4	6.7	8.0	6.8	5.3
	Gochujang (hot pepper–soybean paste)	3.7	0.2	1.2	3.0	3.4	4.0	5.2	3.5	1.9
	Doenjang (soybean paste)	5.6	0.9	1.6	3.5	3.0	5.0	7.1	8.0	7.2
	Jajang (blackbean paste)	1.1	0.4	1.2	1.8	0.9	1.2	1.3	0.7	0.3
	Chongkukjang (soybean paste, quick fermented)	1.0	0.2	0.7	0.5	0.4	0.6	1.3	1.6	2.1
	Mixed bean paste	1.0	0.0	0.2	0.3	0.7	1.3	1.5	1.0	0.4
		19.0	3.5	8.6	14.7	14.8	18.8	24.4	21.6	17.2
Jeotgal (salted fish and shellfish)		2.7	0.2	0.2	0.9	1.7	1.9	3.8	3.7	3.9
Vinegar		0.5	0.1	0.1	0.3	0.4	0.6	0.8	0.6	0.3

[a] Classified based on the main ingredient.

Source: Adapted from the Ministry of Health and Welfare, Report on 1998 National Health and Nutritional Survey (Dietary Intake Survey), Korea Health Industry Development Institute, Seoul, 1999.

any kind of vegetable can be used. Fermentation is achieved in a comparatively short time. Second in consumption are soy-based products, which include *ganjang* (soy sauce), *doenjang* (soybean paste), *chongkukjang* (quick fermented soybean paste), and *gochujang* (hot pepper–soybean paste). This category comprises major condiments in Korean cuisine. Traditionally, most households prepare these condiments once every year, and keep and use them for many years. Fermented products based on fish and shellfish comprise the third category. These can be used as one of the ingredients in kimchi or eaten as they are. Although dozens of fermented foods are consumed in Korea, scientific research regarding their health effects is mainly concentrated on kimchi and doenjang, the two fermented food items consumed in large quantities. Therefore, the focus in this chapter will be on these two food items.

12.2 KIMCHI

Kimchi is mentioned in the book *Samkuksaki*, published in 1145 A.D., and is thought to be a simple fermentation product of a vegetable in brine prepared in a stone jar.[2] Since then, kimchi has developed into many different products (more than 50) that use different vegetables such as *baechu* (napa cabbage; *Brassica rapa* L. ssp. *pekinensis* [Lour.] Han), radish, cucumber, or scallion as the major ingredient, with various methods of preparation. The essence of kimchi making is to brine the major raw vegetable; drain; mix with minor ingredients such as scallion, garlic, ginger, red pepper, fermented fish, salt, and sugar; and store the mixture for fermentation. Most people consider it to be well ripened when kimchi has been kept for 3 weeks at 4°C or for 4 d at 15°C, although it may be eaten at any stage of storage until it becomes too sour. Raw kimchi is eaten like salad, often mixed with sesame seed oil and sugar, while overripened kimchi is made into *jigae* by boiling with meat. The average daily consumption of kimchi for the Korean population is 124.3 g, and 154.5 g for the age group (30–49 yr old) consuming the most (see Table 12.1). Many households still make kimchi at home, but commercially prepared kimchi has been gaining in popularity. Typically, ingredient composition of commercial baechu-kimchi is as follows: baechu, 85.9%; radish, 2.8%; garlic, 1.4%; red pepper powder, 2.9%; scallion, 1.5%; fermented fish, 1.9%; ginger, 0.7%; salt, 2.5%; sugar, 0.8%; flavor enhancers, 0.3%.[3] As can be predicted from the numerous ingredients, several components of kimchi have been implicated for various functionalities. Compounds from the ingredients such as ascorbic acid, carotenoids, chlorophylls, capsaicin, sulfur-containing compounds, polyphenols, fibers, and compounds generated from the fermentation process such as lactic acid, glycoproteins, bacteriocin as well as lactic acid bacteria (LAB), have been claimed to contribute to laxation and have antioxidation, antiaging, antimutagenesis/antitumor, antiobesity, and antibacterial properties.[4, 5]

12.2.1 Changes During Fermentation

The microoganisms mainly responsible for the fermentation process in the production of kimchi are LAB, although fermentation by aerobic bacteria, yeasts, and molds occur simultaneously.[2] Numerous biochemical changes occur during fermentation, forming flavor compounds and changing the texture of the vegetables. Kimchi is an important

source of vitamins and their precursors, such as the vitamin B group, β-carotene, and ascorbic acid, which change in concentration during fermentation. For example, the ascorbic acid content of baechu-kimchi (3.5% salt) was 15.2 mg% in a freshly made sample, decreased gradually by 10% over a 12-d period, increased to 18 mg% on day 18, and then decreased again when kimchi was kept at 7°C.[4] The vitamin B_{12} content went through similar changes in baechu-kimchi (salt 3.25%) when kept at 2 to 7°C. The percentage change in the vitamin B_{12} concentration was much more pronounced (about a 60% increase) than for ascorbic acid. Vitamin B_1, B_2, and niacin increased in concentrations without showing any reduction during the initial stage. The maximum concentration for the B and C vitamins are observed after around 3 weeks of aging, when kimchi is considered well ripened by most people. In contrast, ascorbic acid contents in kimchi made with other main ingredients such as cucumber or young radish tops showed different patterns, increasing from the beginning of the fermentation to reach maximum concentrations in 4 to 5 d at 10°C.[6]

No matter how long it takes to achieve maximum level, acceptable ripeness, shown by the sensory evaluation, matches the increase of the vitamin content. Vitamin B_1 shows the greatest percent increase (137%), followed by B_2 (117%), B_{12} (60%), and niacin (53%). Unlike the B vitamins, and vitamin C, the β-carotene concentration does not increase, but rather decreases to about 50% of the initial concentration when kimchi is well ripened, which suggests that there is no biosynthesis of β-carotene by microorganisms found in kimchi.[7] Changes in the concentration of these particular nutrients during fermentation may have important implications in terms of the antimutagenic and anticarcinogenic properties of kimchi, because several of these vitamins are believed to prevent certain cancers.[8–10] Kimchi is also a good source of dietary fiber, which may prevent colorectal cancer, diabetes, obesity, atherosclerosis, high blood pressure, etc.[11–13] The concentration of various fiber fractions seems to change as kimchi ripens. When, for example, concentrations of the pectic substances were studied in relation to texture softening upon aging, water-soluble pectins and pectic acid increased by 127% and 31%, respectively, whereas the alcohol-insoluble solids and protopectin decreased by 45% and 61%.[14] Both the total fiber contents and the crude fiber contents, however, remained relatively constant. The total dietary fiber contents of raw kimchi (0 week) and fermented kimchi (3 weeks at 5°C) were 20.7% and 24.0%, respectively, on a dry weight basis, and crude fiber contents were 8.2% and 9.3%.[15]

12.2.2 Cancer

12.2.2.1 Epidemiology

In 1985, Lee et al.[16] reported that patients with stomach and other cancers consumed unusually large amounts of kimchi. It was suggested, however, that the positive correlation might not be due to kimchi per se, as green vegetables, which are used as the main ingredient, are believed to reduce rather than increase the incidence of cancer.[8,11,17] A case-control study in 1995 also has cited kimchi as one of the risk factors associated with stomach cancer, together with doenjang and gochujang.[18] It was concluded, however, that the salt in those foods was the most important contributing factor. Park et al.[19] investigated the effect of several different fermented vegetables—

baechu-kimchi, danmuji (pickled radish), and cucumber kimchi—on the incidence of stomach cancer. Of the three food items, only consumption of cucumber kimchi showed a positive correlation with cancer incidence. Although consumption of regular baechu-kimchi did not show any effect, those kimchi made with a high level of fermented fish had a positive correlation. This may also be explained on the basis of a salt effect, as fermented fish made in Korea is high in salt (15 to 18%). In Asian communities, high salt consumption seems to be an important contributing factor to stomach cancer.[18,20–23] Kimchi normally contains about 2.5% salt, although the salt content can be as high as 9%. Traditionally, people in the southern part of Korea made saltier kimchi because of the warm weather. With the availability of refrigerators, kimchi now tends to be made with less salt. Thus, when epidemiological studies involving kimchi are planned, the salt level needs to be considered carefully in order to minimize confounding and to properly reflect the effect of kimchi as it is currently manufactured and consumed.

For colorectal cancer, where salt has not been implicated as an etiological risk factor, the protective role of kimchi is more apparent. In a recent case-control study, food intakes of 136 patients with either colorectal cancer or large bowl adnomatous polyps, were compared with those of the control subjects. The highest tertile of kimchi intake had a 68% lowered risk of the colorectal cancer ($p = 0.0079$).[24]

12.2.2.2 Anticarcinogenic and Antimutagenic Activities in Animal and in *in vitro* Models

Vegetables used for kimchi contain high levels of vitamin C, β-carotene, dietary fiber, flavonoid, indoles, sulfur-containing compounds, and chlorophylls, which are believed to be effective in preventing cancer.[5,8,11,17] Kimchi also contains LAB, which have been reported to show antitumor activity.[25,26]

Some of the raw vegetables used for kimchi, such as baechu, parsley, perilla leaf, green pepper leaf,[27] garlic,[28] and red pepper[29] have been tested and shown to be antimutagenic with an in vitro assay system. A methanol extract of kimchi was found to be antimutagenic against aflatoxin B_1 and *N*-methyl-N′-nitroso-N-nitrosoguanidine (MNNG) in the Ames assay and the SOS chromotest.[30] It was also found to be antimutagenic in the wing hair spot test on *Drosophila melanogaster*.[31] When several organic solvents were used for extraction and the activities compared, the dichloromethane extract showed the highest antimutagenicity.[32] These authors suggested that flavonoids, steroids, fatty acids, and terpenoids extracted into the dichloromethane might be responsible for the observed antimutagenicity.

Park et al. varied the ingredient compositions in kimchi and reported that kimchi containing high levels of red pepper powder and garlic showed a stronger antimutagenic activity in the bacterial systems studied.[33] They also determined the effect on the growth of AGS human gastric cancer cells and observed a higher inhibitory effect for the same type of kimchi. The antimutagenic and anticarcinogenic activities of capsaicin in red pepper and sulfur-containing compounds in garlic are well documented in the literature.[34,35] Kimchi made with organically grown vegetables showed greater effects also, perhaps because no residual pesticides that could offset the antimutagenic and anticarcinogenic activities were present.[36]

Several LAB isolated from kimchi suppress mutagenicity induced by 4-NQO, MeIQ, and Trp-P-2 in the Ames test and SOS chromotest.[37] *Leuconostoc mensenteroides* was especially effective, although other organisms such as *Lactobacillus brevis, Lb. fermentum, Lb. plantarum,* and *Pediococcos acidilactici,* were also as effective as *Lb. acidophilus* from yogurt. Yogurt administered to mice has been shown to reduce Ehrlich cancer, and extracts of microorganisms from yogurt have been shown to inhibit growth of Ehrlich carcinoma and sarcoma 180. More recent works on the anticarcinogenicity of yogurt have been reviewed by Teitelbaum and Walker.[25,26,38] Kimchi also contains LAB. A cell lysate of *Lb. plantarum* isolated from kimchi has been shown to inhibit the cancer induced by the sarcoma 180 in mice.[39] It has been suggested by the authors that the cell wall components play a major role by stimulating immune systems and/or by inhibiting the production of carcinogens by other resident microorganisms in the gastrointestinal tract.

The antimutagenicity of kimchi depends on the length of fermentation. Well ripened kimchi (3 weeks old) was found to be more effective than the raw (0-week-old) or the 6-week-old over-ripened kimchi.[40] These findings suggest that the chemical components present in the raw materials, and compounds produced during the fermentation process, and destroyed in the later stages of fermentation, may also be responsible for the antimutagenicity of kimchi.

Various mammalian cell cultures have been utilized to investigate the effect of kimchi on the growth of cancer cells. Kimchi extract was shown to inhibit growth of AGS, HT-29, HL-60, K-562, MG-63, and sarcoma-180 cells.[32,33,40–42] Growth inhibition involved the induction of apoptosis in HL-60 human leukemia cells as evidenced by the result of a DNA fragmentation assay.[32] Both aqueous and organic extracts were effective. As was noted for the antimutagenicity assays, the degree of inhibition depended on the length of fermentation. The supernatants of kimchi kept at 5°C for 0, 3, and 6 weeks were used to determine the effect on the growth of K-562 (human leukemia) and MG-63 (human osteosarcoma) cell lines. In both cases, the 3-week-old kimchi showed a higher inhibition rate than the 0 and 6 weeks samples.

When Cho et al.[41] fractionated kimchi extract by using various organic solvents, the dichloromethane fraction showed the highest inhibitory effect on the growth of AGS and HT-29 cancer cells, although all other fractions (hexane, methanol, ethyl acetate, butanol, and aqueous) had some inhibitory effects. The dichloromethane fraction was also the most effective in reducing the cytotoxicity incurred by MCA and DMBA on mouse embryonic C3H/10T1/2 fibroblast cells.[43] On the transformation of cells treated with methyl cholantherene (MCA) and 10-dimethyl-1,2-benzanthracene (DMBA), the same fraction that inhibited the formation of type 2 and 3 foci, reduced the number of type 3 most effectively.

The anticarcinogenicity properties of kimchi may be due to an enhancement of the immune system of the host. Phagocytic activity of the peritoneal macrophages of mice was significantly augmented by kimchi extract when tested both in vitro and in vivo.[42] Alternatively, the anticarcinogenicity of kimchi may be due to its ability to affect the metabolism of mutagens. In guinea pigs administered with polycyclic aromatic hydrocarbons, kimchi increased the activities of liver enzymes responsible for the removal of foreign compounds.[28] When rats were given a single injection of diethylnitrosamine (DEN) and an oral administration of 2-acetylaminofluorene (2-AAF)

with or without kimchi extract for 6 weeks, the numbers of glutathione *S*-transferase placental form-positive (GST-P$^+$) foci in the livers of the kimchi-treated group were decreased to 8.8/cm^2 from 13.8/ cm^2 for the control group, indicating a protection against these two hepatocarcinogens.[44] To date no human data are available.

12.2.3 Cardiovascular Disease

Several ingredients of kimchi are thought to reduce the incidence of cardiovascular disease. Baechu has considerable levels of β-sitosterol and *S*-methylcysteine sulfoxide, both of which have been reported to lower blood cholesterol in animals.[45–47] Red pepper contains capsaicin, which inhibits platelet aggregation and induces blood vessel expansion.[48] Garlic was reported to reduce plasma cholesterol and triglyceride levels, and ginger was observed to reduce serum cholesterol levels in animals.[49,50] LAB appear to lower blood pressure and prevent the increase of plasma cholesterol concentrations.[51]

Recently, a clinical study involving 102 healthy Korean men was carried out to determine if kimchi is hypolipidemic for humans.[52] The men were divided into four groups, each group consuming an average of 68 g, 118 g, 208 g, and 383 g of kimchi daily. Kimchi consumption was positively correlated with high density lipoprotein (HDL) cholesterol and negatively correlated with low density lipoprotein (LDL) cholesterol (see Table 12.2). Preference for hot taste was negatively correlated with systolic blood pressure (correlation coefficient; −0.15 with $P < 0.05$). Supplementation with a freeze-dried kimchi pill for 6 weeks lowered plasma triglyceride (TG) level by 16.8%, as well as the atherogenic index, compared to the placebo group.[53]

To study the effect in animal models, male Sprague–Dawley rats were fed with diets containing 0%, 3%, 5%, and 10% freeze-dried kimchi for 6 weeks.[54,55] Plasma cholesterol was lowered in rats fed with all three levels of kimchi, but TG levels were decreased only in the highest dose group of animals. More specifically, kimchi intake reduced very low density (VLDL) cholesterol, and LDL TG at all levels of kimchi consumption. Kimchi consumption also increased serum HDL-cholesterol. Intake of 5% and 10% kimchi also lowered cholesterol, TG, total lipid, and apolipoprotein B

TABLE 12.2
Biochemical Parameters of Korean Adult Men with Respect to Daily Kimchi Consumption

	Quartile of kimchi intake (g/day)			
	1st (68.13 ± 24.2)	**2nd (118.5 ± 11.4)**	**3rd (208.5 ± 19.0)**	**4th (382.3 ± 71)**
HDL-C (mg/dl)	58.5 ± 21.9	60.2 ± 15.2	63.5 ± 13.5	66.5 ± 13.6
LDL-C (mg/dl)	116.0 ± 54.2	107.2 ± 30.1	99.4 ± 25.5	93.4 ± 35.3
TG (mg/dl)	143.6 ± 82.2	149.4 ± 92.9	158.3 ± 77.3	152.8 ± 62.9

Note: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride

Source: Adapted from Kwon, M.-J., Chun, J.-H., Song, Y.-S., and Song, Y.-O., *J. Korean Soc. Food Sci. Nutr.*, 28, 1144, 1999, With permission.

in the liver, whereas it increased fat, cholesterol, TG, and apolipoprotein A-1 levels in feces. Kimchi intake elevated the T3 levels in rats but had no effect on the thyroxine (T4) level. These results suggest that intake of kimchi in rats decreases plasma and hepatic lipid levels by increasing fecal excretion of triglyceride and cholesterol, elevating T3 level, and altering lipoprotein metabolism.[55] The antioxidative component identified in kimchi by the authors as 3-(4-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid (HMPPA), has been synthesized and tested in animals. When HMPPA was injected into hypercholestrolemic rabbits, the LDL-cholestrol and TG plasma levels dropped by 5.48% and 7.06% compared to the control group, and HMG-CoA reductase activity in liver was increased by 48.4%. The effect of HMPPA was comparable to that of simvastatin, a known hypolipidemic drug.[56] HMPPA also exhibited antiartherogenic effects by reducing the thickness of aorta arch in rabbits fed cholesterol.[5] It seems that the antiartherogeic effect of HMPPA is mediated by the inhibition of the inflammatory response of macrophage. It has also been shown that the nitric oxide (NO) production and COX-2 expression were inhibited in murine macrophage cells.[5,57]

To assess the effect of kimchi on thrombosis, plasma fibrinolytic activity was measured in rats fed with kimchi.[55] Fibrinolytic activity of plasma from rats consuming diets with 10% kimchi was higher than that of controls, whereas the activities for animals consuming 3% and 5% were the same as those of controls. Fibrinolytic activities were also measured for water and methanol extracts of kimchi using the fibrin plate method. Fibrinolytic activity of the methanol extract of kimchi was much higher than that of the water extract. The active chemical components in the methanol extract have not yet been determined.[55]

12.2.4 Antibacterial Activity

The sulfur containing compounds in the ingredients, as well as the LAB, have been shown to possess antibacterial property.[5] The implication of the human health is the inhibition of pathogenic microbes in digestive tract. *Helicobactor pylori* infection is one of the major etiological factors in gastritis and stomach cancer. The LAB isolated from kimchi blocked the adherence of *H. pylori* to a human gastric epithelial cell line and inhibited the growth of *H. pylori* in vitro.[58] However, human study with *H. pyroli* infected volunteers did not show statistically significant inhibition of *H. pyroli* urease.[59] In the same study, kimchi intake lowered the fecal β-glucosidase and β-glucuronidase activities as well as fecal pH. The results suggest that the LAB has survived the low gastric pH, and successfully competed with the pathogenic bacteria in the lower digestive tract, partly explaining the epidemiological observation of risk reduction in colorectal cancer.[24]

12.2.5 Formation of Undesirable Compounds

Nitrosamines are known carcinogens for animals and suspected to be carcinogenic for humans as well.[60] There have been concerns about the possible formation of nitrosamines in kimchi, because baechu-kimchi contains high levels of both nitrate and fermented fish product that contain amines. When kimchi was analyzed for nitrate, nitrite, and nitrosamines during fermentation, the results showed very little nitrite

(undetected up to 0.5 ppm) and nitrosamines (undetected up to 0.05 ppb) throughout the process.[40] It was thought that the reaction of phenols and ascorbic acid with nitrite were at least partly responsible for these results. Recently, Kim et al.[61] reported that up to 91 ppb of *N*-nitrosodimethylamine (NDMA) could be formed at low pH, high temperature and low salt concentration (pH 4, 16°C, 1.5%). However, it would be rather difficult to meet these conditions under the usual practice of kimchi making.

Ethyl carbamate, which is mainly present in fermented foods and beverages, has been associated with cancer for a long time. Ethyl carbamate has been measured in some Korean foods; kimchi values ranged 0 to 16.2 ppb.[62]

Biogenic amines—histamine, putrescine, spermidine, cadaverine, and tyramine—are the microbial decarboxylation products of amino acids, and are often a concern in fermented foods. The concentration of the biogenic amines has been determined in total of 24 commercial samples of eight different kimchi, and they appear to be well below the levels for human health concerns, with the highest level of 151mg/kg cadaverine in a sample of baechu-kimchi.[63] Under the conditions of commercial kimchi production, formation of undesirable compounds should not be a problem. However, considering the large daily intake of kimchi by Koreans, it would be wise to identify the specific conditions under which undesirable compounds could be produced, in order to be able to make recommendations on how to avoid them.

12.3 DOENJANG

Doenjang is a soy-based food consumed daily in Korean households. It is a soup base used for regular broth-type soup (kook) and much thicker stew-type soup (jigae), both of which are eaten with rice. Traditionally, most soybean-based fermented foods are prepared once a year and kept up to 2 to 3 yr. The preparation starts in the late fall with the natural fermentation of *meju*, which are solid blocks (size varies by household, but approximately $20 \times 15 \times 10$ cm) made from steamed soybeans. The fermentation lasts for about 2 weeks. The product is then dried over the winter and subjected to further fermentation in brine during the spring for 6 to 8 weeks. At the end of the wet fermentation, doenjang (soybean paste, solid material) is separated from ganjang (soy sauce, supernatant). It can be consumed after 30 to 50 days of maturation; however, longer maturation is considered to produce a better flavor.[1]

12.3.1 Cancer

12.3.1.1 Epidemiology

Doenjang has had to endure a rather negative reputation because it has been linked to stomach cancer in Korea. A positive association between doenjang intake and stomach cancer was first reported by Crane et al.[64] in 1970. In this case-control study, the authors simply speculated that the causal factor might be contamination by aflatoxin. However, this mycotoxin is presently considered as a liver but not a stomach carcinogen. The main reason for the speculation was that one of the main fungi involved in the fermentation of meju is *Aspergillus oryzae*, which is in the same genus as the aflatoxin-producing *A. flavus.* However, the production of the toxin during and after

natural fermentation of meju was found to be insignificant, even though *A. flavus* could grow quite well.[65]

Several other groups have reported positive correlations between the intake of fermented soyfoods and stomach cancer. [18,66,67] In a case-control study conducted in Korea, three fermentation food dishes were cited as risk factors for stomach cancer; doenjang (soybean paste) jagae, gochujang (hot pepper soybean paste) jigae, and kimchi. It was also reported that the frequent consumption of tofu made from soybeans decreased the risk.[18] This study pointed out the importance of cooking methods as well as ingredients and analyzed food consumption by prepared food type, rather than raw materials. The authors singled out salt as the most important contributing factor.[15] It is noteworthy that kook (broth-type soup) made from doenjang and gochujang did not show any positive correlation with the incidence of cancer. Later, the same group pooled and analyzed the epidemiological data available in Korea and again reached the same conclusion that salt, rather than soy components or fermentation byproducts, was the risk factor for stomach cancer.[20] In a recent meta-analysis of 14 pooled epidemiological studies, Wu et al. cautiously suggested that fermented soyfoods may increase the relative risk for stomach cancer to 1.26. In the same work, nonfermented soyfoods were thought to lower the risk to 0.72, based on the analysis of the data of ten pooled studies. The authors, however, warned of possible confounders such as salt and fruits-vegetable intake.[66] Responding to the Wu et al.[66] study, Ji and colleagues reanalyzed 1,124 original data from the 1998 case-control study,[22] which had showed a decreased cancer risk in men with increased soy intake, and a small insignificant increased risk in women. The reanalysis showed that adjustment for salt intake and salt preference lowered the odd ratio slightly, while nonfermented soyfoods lowered the cancer risk in men, but not in women. It was also shown that fermented bean curd was responsible for the increased risk among women.[67] A prospective study involving a Japanese immigrant population in Hawaii reported that miso (a Japanese fermented soybean product) was mildly associated with gastric cancer, while salt consumption showed a stronger association.

Considering the results of many biochemical and animal studies with various soy components, which indicate protection against cancer, it may indeed be the salt, that constitutes as much as 20% of doenjang, that increases the stomach cancer risk. Other studies have also indicated salty foods as etiological factors apart from fermented foods.[21–23] A cohort study in Japan showed self-reduction of salty food consumption decreased the risk of the progression of precancerous lesions to gastric cancer to a relative risk of 0.56 (95% CI, 0.32 to 0.96).[68]

Ever since the differences in diets and in certain cancer rates were noted for Western and Asian populations, consumption of soyfoods has been suggested as a health promoting factor.[69] In cancers other than stomach cancer, the role of soyfood is more hopeful, especially in breast cancer, although epidemiologic data has been mostly inconsistent and inconclusive.[70] Also no conclusive epidemiological evidence links the consumption of legumes and the reduction of cancer.[71] It is interesting to note two recent population-based case-control studies conducted in Shanghai. Originally, breast cancer risk was shown to be a weak inverse relationship to overall soyfood intake, albeit not statistically significant one.[72] The same Shanghai study, however, was analyzed for the soyfood intake during adolescence and the later development

of breast cancer, and a strong inverse correlation was observed, in both pre- and postmenopausal women.[73] There appears to be an age specific protection mechanism against breast cancer, which was not recognised in most of previous studies (see Section 12.3 of this chapter for futher discussion).

12.3.3.2 Anticarcinogenic and Antimutagenic Activities in *in vitro* and Animal Models

In contrast to epidemiological studies, most of the laboratory studies have shown a positive effect of doenjang on the prevention of the development and growth of cancer. One possible explanation may be that studies have used extracts (usually of organic solvents), which are virtually devoid of salt. Many reports documenting the antimutagenicity properties of doenjang extracts. [74–79] In classical bacterial antimutagenicity tests to see whether the test material inhibits mutagenesis induced by known compounds, doenjang showed the highest overall antimutagenic activity among methanolic extracts of the four Korean soy fermented products—*doenjang, ganjang, chongkukjang,* and *gochujang*—even though the effect varied among the mutagens employed. One noteworthy observation in this study was that homemade samples of these foods showed higher antimutagenicity than their commercial counterparts or raw materials, in all four items tested.[79] Commercial fermentation typically utilizes a shortened process time by employing an inoculation of a defined mixture of microbes at an elevated but controlled temperature. Chemical conversions needed to produce the antimutagenic ingredients may have slow kinetics and/or multi-step processes that require a variety of enzymes from different microorganisms. These complex conversions may be more common in slower fermentations carried out in home production systems, which typically display a more complex microbial composition than commercial production does. Fractionation experiments showed that these antimutagenic component(s) seem to be heat stable and have hydrophobic chemical characteristics.[77,78] Doenjang showed a higher activity than Japanese miso to protect *Salmonella typhimurium* against the mutagenesis induced by aflatoxin B1 or MNNG in a comparative study.[40,74]

Mammalian cell culture and animal transplantation experiments have shown that in addition to providing protection against genotoxicity, doenjang also protects against the advancement of tumor transformation steps. Again, fermented foods showed much higher activities compared to raw soybeans or soy flour.[74] The relationship between fermentation and antimutagenic activity was clearly shown in an experiment with chongkukjang, by comparing soybeans at 0 and 48 hours of fermentation and 20 days of maturation.[76] It should be pointed that these experiments were again performed with organic solvent extracts, effectively eliminating salt from the test sample and also perhaps concentrating active components. Therefore, conclusions may not be directly applicable to whole foods as they are commonly eaten.

In an animal model, both organic solvent and boiling water extracts of doenjang showed an inhibition of solid tumor formation in BALB/c mice transplanted with Sarcorma-180. Results also showed the extension of survival time for those mice receiving the extract treatments compared to control mice.[77] In a similar study, longer aging time increased the anticancer and antimetastatic activities of methanol extract of

doenjang.[80] Tumor growth of transplanted sarcoma-180 were inhibited up to 38% by the treatment of 24-month old doenjang extract, while 3-month-old doenjang extract did not show inhibition. The metastasis of injected colon 26-M3 cells was blocked by 82% the extracts and the inhibition rate also increased with the ripening time. The result coincides with the higher antimutagenic activities of longer-ripen traditional doenjang compared to commercial one with shorter production time.

Modification of enzyme activities involved in the metabolism of xenobiotics in liver has been suggested as a biochemical mechanism for the beneficial effect of doenjang extracts. During a mouse tumor transformation assay, reduced activities of glutathione *S*-transferase and glutathione reductase and decreased glutathione content in the liver observed after the tumor cell implantation were restored in the animals treated with doenjang extracts. Lowered lipid peroxide levels indicated that antioxidation could be involved in the mechanism as well.[78,81] These authors identified the active components as linoleic acid and genistein, both of which were shown to block the passage of the cancer cell lines from the G2 to the M phase.

12.3.2 Cardiovascular Disease

A meta-analysis of 38 clinical studies showed that soy protein consumption reduced the level of serum cholesterol, LDL cholesterol, and serum triglyceride.[82] The American Heart Association recently stated that the daily consumption of soyfoods containing phytoestrogen could benefit hypercholesterolemic subjects. The possible mechanisms for the beneficial effect suggested by various authors involve trypsin inhibitors and/or other peptides, phytic acids, saponins, isoflavones, or any combination of these constituents of soy.[83] The protein component of soy seems to play an integral part of this lipid altering effect, based on the fact that isoflavones alone failed to show any effect.[84] Elimination of the phytoestrogen from soy reduced the effect on serum cholesterol levels.[83]

12.3.2.1 Inhibition of Angiotensin Converting Enzymes

Angiotensin converting enzyme (ACE) produces angiotensin II, a vasoconstrictor and inhibits the vasodilator bradykinin, leading to increased blood pressure. It has been shown that a number of food items including fermented foods have inhibitory activities on ACE.[85] Doenjang was tested both *in vitro* and *in vivo,* and shown to inhibit ACE with IC_{50} of 2.2 to 310 μg/ml. The inhibition of ACE may ultimately result in lower blood pressure.[86–89] Two ACE-inhibiting peptides found in doenjing have been isolated and characterized as Arg-Pro and His-His-Leu by two independent groups in Korea.[88,89] The tripeptide, which showed the higher ACE inhibitory activity, has been synthesized and shown to lower systolic blood pressure when administered to spontaneously hypertensive rats by intravenous injection at a level of 5 mg/kg body wt. The ACE activity in the aortic tissue was significantly lower in the treated rats, while the activity in serum was unchanged.[89] Since small peptides can be directly absorbed through the intestinal tract, the tripeptide can probably reach the circulatory system and exert its protective effect. However, it remains to be seen how much of the ingested food peptides survives the digestive system and get into the blood circulation.

12.3.2.2 Antithrombotic Peptides

Another cause of cardiovascular disease is thrombosis, which is the abnormal aggregation of blood platelets leading to atherosclerosis or hypertension. In his pioneering work, Shon searched for antithrombic peptides in doenjang extract and showed fractions containing basic peptides exhibited a higher activity against ADP-induced platelet aggregation than other known antithrombic peptides.[90] Recently, ADP receptor antagonists have been successfully developed and used to treat thrombosis.[91] Applying this same concept, it may be possible in the future to prevent the disease by daily consumption of doenjang and other antibiotic peptide containing foods.

As the antimutagenic/anticarcinogenic activities, the inhibitory activity of angiotensin converting enzyme and the fibrinolytic activity of doenjang extracts increase with ripening time up to six months, indicating slow proteolytic reaction during fermentation.[92, 93]

12.3.2.3 Isoflavones

Isoflavones, especially genistein from soy, have been implicated in the protection against a whole spectrum of chronic diseases including breast and prostate cancers, postmenopausal syndromes, osteoporosis, and cardiovascular disease. The protective role has been shown in many animal and *in vitro* studies, but only miniscule positive effects, if any, have been observed so far in human studies. For the details of the effects of isoflavones on various diseases, readers are referred to several excellent reviews.[94–97]

Isoflavones are known as weak estrogens; estrogen antagonists; antioxidants; inhibitors of topoisomerase II, HMG-CoA, angiogenesis and platelet aggregation, and as inducers of cell differentiation in animal and *in vitro* models.[94–98] It is likely that the association was not clearly observed in human studies because it requires long periods of time, perhaps decades, between the intake and the results. Furthermore, timing of isoflavone exposure seems important for the effect in humans. In one of the epidemiological studies, early exposure to soyfoods was necessary for the protection against breast cancer.[73] One hypothesis to explain this observation, based on an animal model, is that genistein, when administered to young subjects, promotes early cell differentiation during adolescence. This early differentiation in turn lowers epidermal growth factor signaling, which is frequently associated with breast cancer, in later mature years.[94]

Another likely explanation for the inconclusive human evidence is that while the experimental data were largely drawn from isoflavone exposure, epidemiological observations were focused only on the soy intake of the subjects. Most isoflavones in soy exist as glycosides and only small amounts are as free isoflavones (aglycon), which are in the form that can be absorbed into the body and exert physiological activities. Hydrolysis of the ingested glycosides is carried out by intestinal microbes, which are another variable in the human population. The ratio of free isoflavones to the glycosides has been shown to be much higher in fermented soyfoods than in their unfermented counterparts.[99,100] The urinary excretion of isoflavones was also higher in humans after the consumption of tempe (Indonesian fermented soyfood) than after the consumption of soybean pieces.[101] Doenjang is a promising source of

isoflavones, compared to other soyfoods because it has a higher proportion of free to glycoside-bound isoflavone.[102]

12.4 CONCLUSIONS

At this time there is no conclusive evidence that shows the human health benefits from the consumption of Korean fermented foods such as kimchi and doenjang. However, results from *in vitro* and animal studies as well as some human studies are promising for kimchi and fermented soyfoods. It appears that the fermentation process results in higher quality products. Both kimchi and doenjang show higher health promoting activities with the progress of fermentation. Well-ripened kimchi has a higher antimutagenicity than raw (unfermented) kimchi. Doenjang has greater amounts of peptides and free isoflavones than soybeans do. Both kimchi and doenjang have antihypertensive as well as anticarinogenic potential. One obstacle that needs to be overcome is the preference of Korean consumers for the salty taste of kimchi and doenjang, because the consumption of high salt levels can cause many health problems. It will be a challenge to investigate the effect of traditional fermented foods on human health and to develop improved versions of these foods, that have optimal health effects.

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13 *Lactobacillus plantarum*

The Role in Foods and in Human Health

Göran Molin

CONTENTS

13.1 HISTORY AND CULTURE

Consumption of live lactic acid bacteria (LAB) included in lactic acid fermented foods has been a regular part of the food intake of humans for a long time. In fact, archaeological evidence indicates that mankind has used this technique since prehistoric times.[1,2] This technique was presumably invented 1.5 million years ago by the early humanoids (Figure 13.1). Humans have in this way consumed large numbers of live LAB, and presumably those associated with plant material were consumed before those associated with milk-based foods. Lactic acid fermentation is the simplest and often the safest way of preserving food, and before the Industrial Revolution, lactic acid fermentation was applied just as much in Europe as it still is in Africa and parts of Asia. Thus, it could very well be that the human gastrointestinal (GI) tract evolved to adapt to a more or less daily supply of live LAB. This supply ceased in most industrialized countries during the 20th century, which might have led to GI problems, and even to immunologically dependant ones. (See Chapter 1 for more details on the history of fermented foods.)

Lactobacillus plantarum frequently occurs spontaneously in high numbers in most lactic acid fermented foods, especially when the food is based on plant material, for example, in brined olives,[6] capers (caper berries),[7] sauerkraut,[8] salted gherkins,[9] sourdough,[10] Nigerian ogi, (made from maize or sorghum),[11] Ethiopian kocho (made from starch from *Ensete ventricosum*),[12,13] Ethiopian sourdough made from tef (*Eragrostis tef*),[13,14] and cassava.[15,16] Thus, it is obvious that individuals consuming lactic acid fermented products of plant origin also consume large numbers of *Lb. plantarum*.

One example of the importance of lactic-acid-fermented plant material for indigenous living humans involves the Tschuktscer people living in Siberia on the Tschuktsch peninsula along the shore of the North Polar Sea. They were described by the explorer A. E. Nordenskiöld during his expedition around Asia in his voyages to discover the North-East Passage (1878 to 1880).[17] At that time, this population was a primordial society of hunters and fisherman, and a major component in their diet was lactic acid fermented plant material. During the summer months, the Tschuktscer collected different kinds of plant material, such as the leaves from Osier (*Salix*) and *Rhodiola*. After picking, the plant material was pressed into sealskin bags that were sealed and left to spontaneous ferment during the summer months. During the autumn, the contents froze in the shape of the outstretched bags. The frozen mass was cut in pieces and eaten as it was, or together with meat

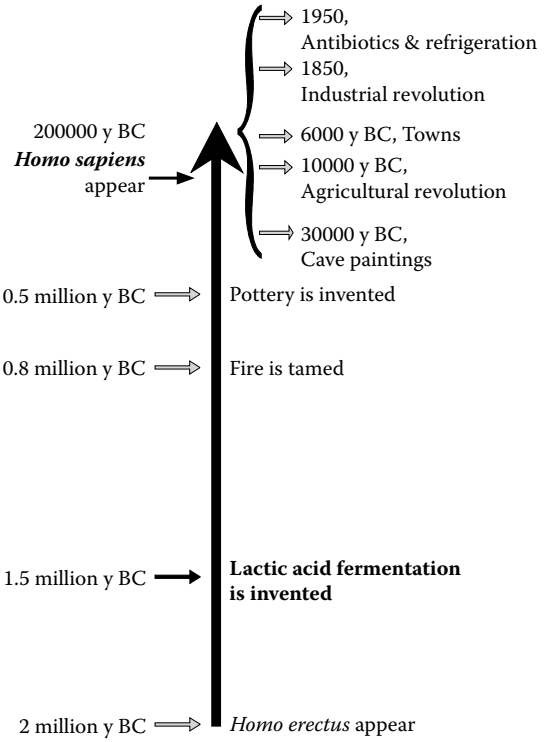

FIGURE 13.1 A suggested time scale for human development, showing how early the technique of lactic acid fermentation probably came into use. (Adapted from Leakey, R., *På spaning efter människans ursprung,* Natur och Kultur, Stockholm, 1993; Leakey R., *Hur människan blev till*, Natur och Kultur, Stockholm, 1995; Arrhenius, B., Grötfrukost på stenåldern, *Forskning och Framsteg*, 4-8, 1984; Lagerqvist, L.O. and Åberg, N., *Mat och dryck i forntid och medeltid*, Vincent Förlag and Statens Historiska Museum, Stockholm, Sweden, 1994; Larsson, L., *Ett fångstsamhälle för 7000 år sedan*, Signum, Lund, Sweden, 1988. With permission.)

or fish, or it could also be used in hot soups. Nordenskiöld speculated that the observed consumption of lactic acid fermented plant material could mimic the way hunters ate during the Stone Age.[17]

In Sweden and in the rest of North Europe, lactic acid fermented vegetables have been widely consumed up to modern times. In a Swedish cookbook from 1755, the procedures for making sauerkraut, fermented spinach, and sorrel is described.[18]

Sauerkraut has had a reputation of being healthy for a long time. Captain James Cook, during his sailing trips around the world (1768 to 1780), forced his crew to eat sauerkraut. James Cook became famous not only for his geographic discoveries, but also for the extraordinary record of survival of the seamen on board his ships.[19] *Lb. plantarum* are often spontaneously the dominating bacteria in sauerkraut, and may have been responsible for the good health of Cook's crews.[8] (See Chapter 14 for more details on sauerkraut.)

13.2 FOODS FERMENTED WITH *LACTOBACILLUS PLANTARUM*

13.2.1 Factors Affecting Fermentation

Lactic acid fermentation spontaneously occurs as soon as organic matter is enclosed in a limited space where access to oxygen is restricted. Thus, as the microorganisms grow, oxygen is consumed and carbon dioxide is produced. This change in gas atmosphere is the first environmental factor to control the microflora in favor of LAB. Production of organic acids and decreasing pH add to the altered gas atmosphere, and become critical for microbial control. In addition to these major environmental controlling mechanisms, antimicrobial compounds besides organic acids, such as hydrogen peroxide,[20,21] nitrogen oxide,[22,23] or antimicrobial proteins or peptides can be produced by the LAB.[24] The principle of a spontaneous lactic acid fermentation is shown in Figure 13.2.

In order to enhance the selection pressure of a spontaneous lactic acid fermentation, salt can be added or the water activity (a_w) can be decreased ($a_W = p/p_0$, where p = steam pressure over the product, and p_0 = vapor pressure over pure water). The water activity over pure water is 1.0, and the relative humidity (RH) in percent, in the gas phase, is the same as $a_W \times 100$.

A more sophisticated way to improve the control of the lactic acid fermentation is to add a starter culture, either as a pure culture, which is the modern industrial method, or by adding some material from a former produced product (*back slopping*), which is the more traditional method. The original purpose of performing a lactic acid fermentation was to increase the shelf life of the product. Empirically, humanity has learned that this is a safe way of preserving food, and also that the nutritional quality of the product persists for a considerable time and may even improve in some aspects. Lactic acid fermentation sometimes also has other advantages such as improving the taste and consistency of the product. In addition, it has also been recognized that beneficial health effects may result from consumption of the live LAB.

The method of pressing down plant material in a air tight bag of sealskin for spontaneous lactic acid fermentation as was done by the Tschuktscer people (see previous discussion) is a simple and efficient technique with the prime purpose of preserving

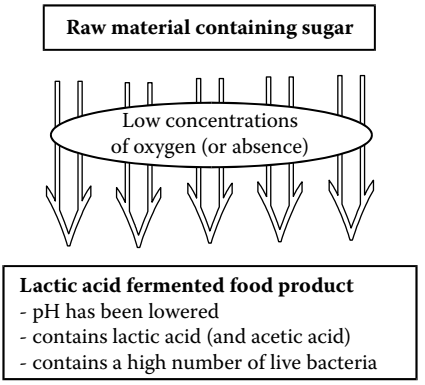

FIGURE 13.2 A schematic representation of lactic acid fermentation of food.

the material for later consumption. It has a modern equivalent in the silage technique, using big plastic bags, that is much favored by Swedish farmers. *Lb. plantarum* frequently occurs in high numbers in silage;[24–26] presumably the fermented product of the Tschuktscer people also contained high numbers of *Lb. plantarum*.

An even simpler technique than tight bags is to make a hole in the ground into which the fermentable material is pressed. Such an application was used in the ancient times in the north of Sweden for the preservation of salmon—the so-called *gravad lax* (buried salmon). During the summer, fresh-caught salmon were salted and buried on the sandy riverbanks at the mouth of rivers, and left to spontaneous ferment. The sour salmon could then be retrieved for consumption during the winter season. An example of the same technique of burying material for fermentation is still seen in Ethiopia today, where people make a lactic acid fermented product called *koho* containing high numbers of live *Lb. plantarum*. A little more advanced technique is traditionally used for the preservation of cucumbers. Instead of a hole in the ground, large open containers standing outdoors are used. The lactic acid fermentation is controlled with salt, and the result is salted gherkins. However, in a product such as brined olives, and even more so in products such as sourdough and ogi, the main purpose of the fermentation is not only to improve the shelf life, but to improve the eating and nutritional qualities of the food. In recent times, a new purpose has been added, and that is to make use of the health beneficial effects of live LAB, as is done in the product ProViva.®

13.2.2 Ethiopian Kocho

Ensete ventricosum (enset) is a perennial, banana-like, starchy root crop that grows in Ethiopia at altitudes of 1500 to 3000 m above sea level. The height of the plant can reach 6 to 8 m when the plant is harvested, 6 to 8 yr after planting. It is a leading staple crop for about 10 million inhabitants.[13] The plant is processed as follows: The pseudostem and corm are pulverized with a long wooden pestle and pounded into a pulp from which the fibers are removed. The remaining scrapings, the pulp, and the inner corm, are kneaded together, rolled into balls, and wrapped in fresh enset leaves. The leaf packages with fresh enset mash are packed into a pit in the ground that has been completely lined with leaves, and left for prefermentation for 2 to 5 d.[12,13] After this prefermentation, the packages are opened, and the mash is once again mixed and thoroughly kneaded, rolled into balls, and wrapped in new fresh enset leaves. The packages are then pressed by hands and feet into the pit. Some of the waste mash and celluloic material from the production are put on top to create a cover, and heavy stones are put on top of this cover. The aim is to limit the access of air into the pit. The major fermentation takes about 2 weeks at a temperature of 15 to 18°C (the same temperature range used for the fermentation of sauerkraut),[27] but the kocho can be left in the closed pit for periods of time from 6 months up to years (in the colder regions).

In the colder regions of Ethiopia, the general belief is that the quality of the kocho improves with storage time. In the warmer regions, the product becomes excessivley discolored if it is left too long.[12] Kocho is mainly baked into bread or cooked and eaten alone or in combination with various indigenous foods. High

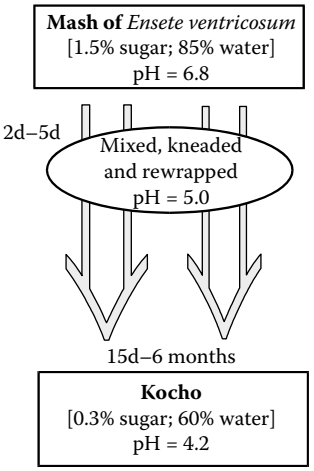

FIGURE 13.3 Flow scheme of the production of Ethiopian *kocho*.

quality kocho or variants of kocho are also eaten unheated and are considered by the public to posses beneficial health effects. The production of kocho is schematically shown in Figure 13.3.

In the beginning of the fermentation (first days), the fermentation is dominated by *Leuconostoc mesenteroides*, but after approximately a week *Lactobacillus* spp. reach similar numbers.[12] After 2 weeks, both *Lactobacillus* and *Leuconostoc* are present in large numbers (about 5×10^9 colony forming units (CFU) per gram). The viable count of *Lactobacillus* remains high for months, whereas the count of *Leuconostoc* rapidly declines. The same sequential pattern for *Leuconostoc* and *Lactobacillus* has been described for both sauerkraut and salted gherkins.[27] The pH may be a controlling factor. *Leuconostoc* is less resistant than *Lactobacillus* to low pH, and the species *Lb. plantarum* is especially hardy at low pH.[24,28]

The dominating LAB in Kocho bought in the market were *Lb. plantarum*, *Weissella minor*, and *Pediococcus pentosaceus*.[13] Gashe[12] also found that *Lb. plantarum* was one of the dominating species of lactobacilli.

13.2.3 Salted Gherkins

Cucumbers contain (on a percentage basis) a higher proportion of water than the Mediterranean Sea and, hence, are susceptible to microbial spoilage. They are harvested during a short season in the autumn and are used mainly for the production of different mixtures of pickles. Only a relatively small proportion is sold as salted gherkins, the traditional lactic acid fermented product. In Sweden, the production of salted gherkins to be used in the food industry as raw material for pickles is about 2000 tons per year, but can be up to 7000 tons depending on the harvest. Production in the United States is at least 100 times higher.

The type of cucumbers (*Cucumis sativis*) used for the lactic acid fermentation become heavily contaminated by microorganisms from the soil in the field during growth. A typical aerobic viable bacterial count can be 5×10^6 CFU/g; the count of

Enterobacteriaceae can be 1×10^6 CFU/g, while the count of lactobacilli is only 5×10^3 CFU/g. Thus, the odds of successful preservation by spontaneous lactic acid fermentation apparently seem low. But, with the use of salt (NaCl), it is feasible to control the fermentation and obtain a product with a long shelf life. The level of salt used is higher than for the fermentation of cabbage, but otherwise the process is similar. The steps of a traditional process are depicted in Figure 13.4. In Sweden, the cucumber is put into a brine to achieve a final concentration of 5% NaCl. Traditionally, this occurs in open containers of wood holding 25 tons of cucumber each, and situated outdoors.

After fermentation, the container is covered by a tarpaulin. In warmer countries, the salt concentration is usually higher during fermentation (up to 8% NaCl), and after the completed fermentation, the salt concentration is increased from 8 to 16% NaCl to ensure a long shelf life of the product. In this way, salted gherkins can be stored for at least 1 yr without problems.[27] In commercial processing plants, the containers have been modernized, and the cucumbers are purged with nitrogen (or air, which is less expensive) to displace accumulating carbon dioxide. The carbon dioxide can form pockets inside the cucumbers (so-called bloater formation). Control over the fermentation can also be achieved by acidifying the cucumbers with acetic acid (pH = 2.8) for a few days before starting the fermentation by increasing the pH to 4.6. In such a process, it can also be favorable to use a starter culture.

Under conditions where the salt concentration in the brine is not too high, the first LAB to increase to a dominating position are leuconostocs, and as the fermentation proceeds, they will be succeeded by lactobacilli and pediococci; this is the same succession as has been seen in Ethiopian kocho[12] and in sauerkraut.[27] A typical bacterial species found in high numbers at the end of fermentation is *Lb. plantarum*, both in salted gherkins and in sauerkraut.[27] However, when used as a starter culture, *Lb. plantarum* can cause gas pockets of carbon dioxide to form in the cucumbers.[29] Another problem is that carbon dioxide and lactic acid can be produced by malic acid fermentation.[30] A mutant strain of *Lb. plantarum* lacking the ability to ferment malic acid has been developed[31,32] and has been made commercially available for cucumber fermentation.[24]

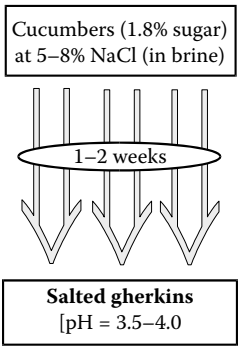

FIGURE 13.4 Flow scheme of the traditional production of salted gherkins. The sugar in the cucumbers consists mainly of glucose and fructose.

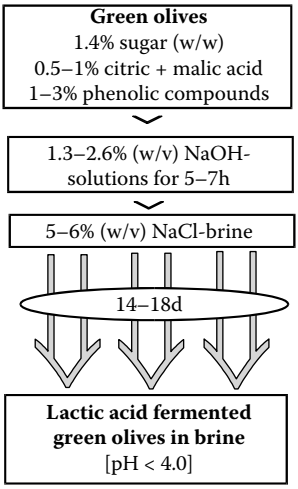

FIGURE 13.5 Flow scheme of the traditional production of green olives in brine.

13.2.4 Green Olives in Brine

The lactic acid fermentation of green olives (Spanish-style green olives) has major similarities to cucumber fermentation. After pretreatment with lye (1.3 to 2.6 [w/w] of NaOH) for 5 to 7 h to hydrolyze and remove some of the bitter tasting phenolic compounds (mostly ortho-diphenols and their glucosides as oleuropein),[27] the olives are put in a brine solution and subjected to a spontaneous lactic acid fermentation. The production procedure is schematically shown in Figure 13.5. After the fermentation is finished, the salt in the brine is increased to 8% (w/w) to ensure the keeping qualities of the olives for extended storage periods.

Lb. plantarum are normally found in high numbers at the end of the fermentation.[33] These bacteria are thought to be coexisting with a yeast flora.[10] The yeasts, are believed to release B vitamins that are utilized by the lactobacilli.[34,35] (See Chapter 15 for more details about olive fermentation.)

13.2.5 Sourdough

A sourdough contains a mixed population of LAB and yeasts. Originally, the use of sourdough was a way of providing yeast for bread making. Baker's yeast was not generally available before the 19th century. In the early 19th century, the beer making process was changed from top yeast to bottom yeast, and the production of baker's yeast became significant. A sourdough normally contains around 10^9 CFU LAB/g and 10^6 to 5×10^7 CFU yeasts per gram,[10,36] where *Lactobacillus* normally is the most frequently occurring genus of LAB[37] and *Lb. plantarum* often are isolated in large numbers.[10,38]

The lactic and acetic acid added to the bread by the lactobacilli improve flavor, and can also provide beneficial health effects to the consumer by reducing the glycaemic index by reducing the gastric emptying rate,[39,40] or by reducing the rate of starch digestion.[41] Furthermore, in rye breads, the acidification by the sourdough

is essential for the technological requirements of the bread (comprising > 20% rye flour), because the water-soluble proteins of rye flour do not form gluten. The dough structure relies on pentosans and mucilage, the contributions of which are enhanced in an acid environment.[10] Thus, the sourdough improves the water holding capacity of the starch and of the pentose containing polymers of the bread. It can also improve the shelf life[10] and increase the availability of minerals by enhancing the breakdown of phytic acid during the bread making process.[10,38] Phytic acids reduce the bioavailability of minerals by forming strong complexes.[42] The phytic acids can be broken down by the microflora (especially by yeasts) and by indigenous phytases of the cereals, which can be activated by pH lowering.[43]

A typical procedure for the production of rye sourdough production is illustrated in Figure 13.6. There are different options for inoculating a sourdough to be made for mixing into the bread dough (Figure 13.6). A new sourdough can be up-started by mixing rye flour with water; and after 2 d at 30°C a primary sourdough (Figure 13.6). However, the quality of this sourdough will not be optimal due to high numbers of *Enterobacteriaceae* that are present already in the flour.[38] To improve the quality of the sourdough, it has to be reused; part of the sourdough is mixed with new flour and water. As this procedure is repeated the quality gradually improves. Not all types of cereals that can be used for starting a sourdough. Wheat flour will not have enough selective power to control the lactic acid fermentation; it will allow bacterial groups other than LAB to flourish, which will disturb the sourdough development.[10] It can be speculated that the high content of phytic acid in rye provides a selection pressure. Phytic acid binds iron, but this is not a problem for organisms such as *Lb. plantarum* that have no requirement for iron.[44,45] Thus, when sourdough is used in wheat bread making, the rye sourdough must gradually be replaced by wheat flour by recycling the sourdough.

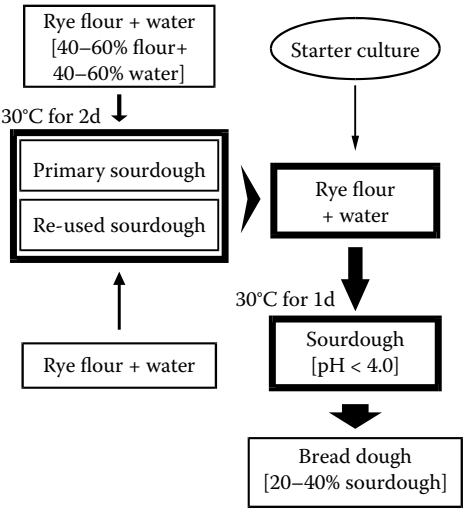

FIGURE 13.6 Flow scheme of the preparation of rye sourdough and rye bread dough.

An alternative to inoculating with old sourdough, is to add a starter culture containing, for example, a suitable *Lb. plantarum* strain, directly to the mixture of flour and water (Figure 13.6).

The sourdough technique is used in many parts of the world. One example is the tef-based *injera*-bread from Ethiopia. Injera, one of the major foods in Ethiopia, is normally prepared from cereals such as tef, wheat, barely, sorghum, maize, millet, or combinations.[13] However, injera from tef (*Eragrositis tef* of the grass family *Poaceae*) is the most popular. Tef flour is mixed with water (1 kg flour per 1.8 L of water), inoculated with a part of an old sourdough, and left in a jar at 18 to 21°C for 2 to 4 d, where the pH drops from 6.7 to below 4.0.[13,14] It has been shown that antinutrients such as phytate and tannins are reduced during the fermentation period.[45] From the microbial point of view, the lactic acid fermentations of rye and tef sourdough have much in common; *Lb. plantarum* frequently occur in high numbers in tef sourdough.[13]

13.2.6 Nigerian Ogi

Ogi is a traditional lactic-acid-fermented cereal-based product from Nigeria. It is used as a weaning food for children (pap) but is also widely consumed by adults as porridge at, for example, breakfast, or as a cooked stiff gel (*agidi*), eaten together with stews, soups, or fried bean cakes. Ogi can be made from maize, sorghum, or millet, but the most popular type is made from maize (*Zea maize*) and sorghum (*Sorghum bicolor* or *Sorghum dabar*). Maize is frequently used for the ogi-gel (agidi), whereas the food based on red sorghum (*Sorghum bicolor*) is preferred for weaning food. Red sorghum generally gives a pap of lower viscosity than maize does, which is advantageous when the consumers are young children, as the bulking effect of maize will reduce intake and increase the risk for malnutrition. Paradoxically, the content of tannins is high in red sorghum, and tannins can react with proteins to make them indigestible in the gut.[47–49] However, the protein digestibility of high-tannin sorghum was significantly improved by lactic acid fermentation.[43] Interestingly, *Lb. plantarum* are able to degrade tannins.[50,51] *Lactobacillus plantarum* is frequently a dominate part of the bacterial flora in ogi.[52] Furthermore, it was shown that *Lb. plantarum* could be used as a single strain starter for producing high quality ogi.[11,53]

Ogi is traditionally produced by a labor-intensive procedure (Figure 13.7). The cereal grains are cleaned and steeped in water for 1 to 3 d, and the first spontaneous fermentation occurs. After the water is poured off, the grains are wet-milled and wet-sieved through a muslin cloth or a fine wire mesh.[11,53] The pomace, mostly consisting of hulls, is discarded and usually used for animal feed. The remaining flour suspension is left for sedimentation for 1 to 3 d. During this step, a spontaneous lactic acid fermentation occurs. When the ogi is sour enough, the supernatant is decanted, and the flour cake is stirred with boiling water or with the decanted supernatant to form a gruel or a porridge. The ogi can also be cooked in water into a thick gel (agidi) that is put in leaf packages.

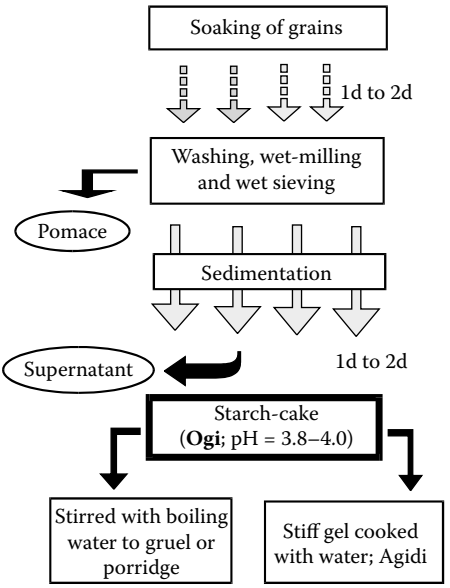

FIGURE 13.7 Flow scheme of the traditional wet-milling procedure for *ogi* production.

13.2.7 Tanzanian Togwa

Togwa is a cereal-based, lactic-acid-fermented beverage, frequently consumed in Tanzania by all age groups as a refreshment and by infants as a weaning food.[54,55] As part of the daily diet, children can be fed togwa two to three times a day, and sometimes children as young as 3 months old are given togwa. The consumption of togwa as a beverage depends on the age of the consumer. It can be taken by adults 2 to 3 times a day (0.3 to 0.7 l per occasion), especially during the dry and hot season. Children are given 0.2 to 0.5 l of togwa per feeding.[55] The togwa is mostly made from maize flour (*Zea mays*), sorghum (*Sorghum bicolour*) or finger millet (*Eleusine coracana*). Rice (*Oryza sativa*) and cassava (*Manihot esculenta*) flour or mixtures of cassava and cereals are also used in some areas.[55] It seems that sorghum-based togwa is preferred by many consumers. The different steps in the preparation of togwa are summarized in Figure 13.8. The flour is mixed with water (1 part flour and 9 parts of water) and cooked for 10 to 20 min. The gruel is then cooled down to about 35°C, and 10% (v/v) old togwa (back slopping) is mixed into the gruel together with 5% (w/v) malt flour.[54,55]

Malt flour is prepared from sorghum or millet. The malt flour is prepared by soaking the cereal grains of choice for 12 h; then, they are drain-dried, spread on broad leaves, and covered with new leaves.[54] A more modern alternative is to use winnowing trays, jute mats, or aluminum trays, and cover the grains with wet cloths. The grains are allowed to germinate at about 30°C for 3 to 6 d until the roots and plumule become pinkish. After sun drying and milling of the germinated grains, the

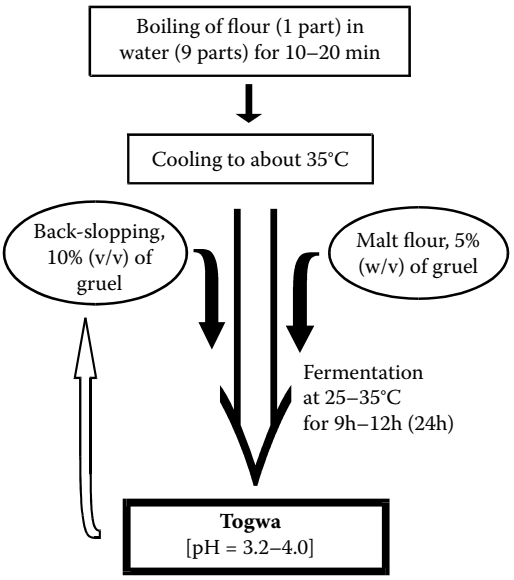

FIGURE 13.8 Flow scheme of the production of Tanzanian *togwa*.

malt flour is ready for use.[54] The malting procedure reduces up to 99% of the tannins in the sorghum.[56] Presumably, the supplementation of malt flour also leads to a decrease in tannins during the fermentation (Figure 13.8).

After mixing the heat-treated gruel with malt flour and old togwa (as a starter culture), the mixture is left for 9 to 24 h (usually 10 to 12 h) for lactic acid fermentation to produce togwa (Figure 13.8). The fermentation can be performed in gourd bottles, covered earthenware pots, aluminum pots or plastic containers.[55] Togwa has a pH of 3.2 to 4.0, mainly due to lactic acid formed during the fermentation, and contains high levels of *Lb. plantarum*.[55] It has been shown that togwa made by using *Lb. plantarum* as single-strain starter culture equals spontaneously fermented togwa in quality.[55]

In spite of the fact that togwa is often produced under poor hygienic conditions, where inferior water quality and the malting procedures expose the product to considerable hygienic obstacles, there has been no documented outbreaks of foodborne disease connected to togwa.[55] Furthermore, Mugula,[55] after interviewing mothers, didn't find any indication of togwa being incriminated in food poisoning. It has also been demonstrated that enteropathogenes (*Bacillus cereus*, *Campylobacter jejuni*, enterotoxigenic *Escherichia coli*, *Salmonella typhimurium,* and *Shigella flexneri*) inoculated before fermentation, disappear after 24 h in the fermenting gruel, provided that the pH during this 24 h period has fallen to ≤4.0, which is the normal pH fall in togwa.[57,58] The fermenting gruel also inhibited enterotoxin production by *Campylobacter jejuni* and *E. coli*, and even inactivated pure cholera toxin when they were added to the fermenting gruel.[58] It is thus obvious that the traditional lactic acid fermentation is a very safe process from a food hygiene point of view.

Togwa can lower the incidence of enteropathogens in feces of children,[59] but it has also been shown to improve the condition of the intestinal mucosa in children

with acute diarrhea as shown by measurements of intestinal permeability to lactulose and mannitol.[60] Under pathological conditions, intestinal permeability to larger sugars increases whereas permeability to smaller ones stays the same or decreases.

13.2.8 Swedish ProViva®

The probiotic strain *Lb. plantarum* 299v (DSM 9843) is included in a European "functional food" product with the brand name ProViva.®[61–63] The strain *Lb. plantarum* 299v found in this product has been isolated from healthy human intestinal mucosa[64] and is patented. ProViva is a fruit beverage that today is primarily marketed in Sweden. It s produced and marketed in Scandinavia by the company Skåne Dairies (Malmö, Sweden), whereas the holder of the rights to the strain *Lb. plantarum* 299v is the company Probi AB (Lund, Sweden). Probi AB licenses the rights to use *Lb. plantarum* 299v to Skåne Dairies. ProViva was launched in the Swedish market in 1992 and since then sales have steadily increased.

The lactic acid fermented component in the drink ProViva is an oatmeal pap that has been fermented with *Lb. plantarum* 299v. The lactic acid fermentation produces about 1×10^9 CFU of *Lb. plantarum* 299v per ml of oatmeal pap. This fermented oatmeal formula was originally developed as a new concept for enteral feeding (nasogastric feeding).[64] The lactic acid fermented oatmeal pap is an integral part of ProViva, where 5% fermented oatmeal pap has been mixed with different types of fruit drinks, including rosehip, strawberry, blueberry, black currant, rasberry, or mango. In the final product, there is about 5×10^7 CFU of *Lb. plantarum* 299v per ml of fruit drink.

The patented process to produce the lactic acid fermented oatmeal pap is schematically shown in Figure 13.9. It is interesting to note that the general procedure of the industrially adopted Swedish process of producing lactic acid fermented oatmeal pap has some important features in common with the traditionally produced Tanzanian

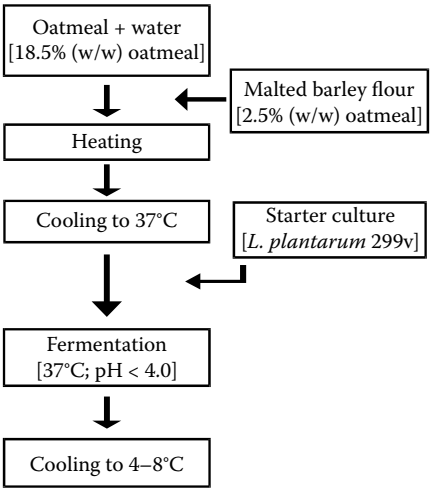

FIGURE 13.9 Flow scheme of the production of Swedish lactic acid fermented oatmeal gruel to be used in probiotic formulas.

togwa. Both are a cereal-based, lactic-acid-fermented pap of relatively low viscosity, providing the consumer with high levels of live *Lb. plantarum* (Figure 13.8). The viscosity of the products is lowered by the supplemented malt flour (malt flour of barely in Sweden and malt of sorghum or millet in Tanzania) in combination with a heat treatment, followed by the decreased pH in the lactic acid fermentation. In the Swedish product that originally was intended as a base for a nutritional formula for enteral feeding, the low viscosity and high energy content of the liquid were prerequisites.[65] Without added malt flour, oat meal pap of the stated concentration of flour (18.5%; w/w) will form a thick porridge impossible to administer through a thin tube.[65–67] The decrease in viscosity is presumably in large part due to degradation of starch. Malt is rich in amylases. There is also an increased solubility of β-glucans, and if large amounts of malt are used, or extra malt flour is added after the heat treatment, the total amount of β-glucan is substantially reduced.[66,67] However, the β-glucans are considered valuable, as they are believed to delay intestinal absorption and beneficially affect cholesterol and glucose metabolism. The process shown in Figure 13.9 causes a relatively small, if any, reduction of the total content of β-glucans, even if the viscosity is significantly affected.

The lactic acid fermented oatmeal pap (Figure 13.9) provides about 76% of the energy, and 70% and 99% of the protein and carbohydrate content, respectively, compared to the average nutrient content in commercial nutritive solutions intended for enteral feeding.[66] The pap is also relatively rich in β-glucans, thiamine, phosphorus, iron, copper, and manganese.[66] The amount of free amino acids in the lactic acid oatmeal pap is affected by the malt and by the activity of *Lb. plantarum* 299v. It has been shown for *Lb. plantarum* strains genetically closely related to *Lb. plantarum* 299v that the concentration of aspartic acid, asparagines, and alanine decrease during fermentation, whereas the concentrations of glutamic acid, proline, glycine, and arginine increase.[67]

A drawback of oats from a nutrition point of view is that oats contain large amounts of phytate (myoinositol hexaphosphate), which is one of the main inhibitors of absorption of iron and zinc in humans.[68] Even small amounts of phytate in the foods have a strong negative effect on the absorption of iron.[69] However, degradation of phytate during food processing can be accomplished by activation of intrinsic phytases in cereals. In fact, phytate can be completely degraded in wheat and rye, but to a lesser extent in oats by soaking at low pH (pH 5) and increased temperature (55°C).[70] In the oatmeal pap fermented with *L. plantarum* 299v, the phytate concentration is somewhat reduced with the help of the supplemented phytases from the malt flour, and the decrease in pH during fermentation at a fermentation temperature considered near optimum for the oat intrinsic phytases.[67,71] Due to the high stability of the oat phytate, substantial amounts remain in the fermented pap. The level of inositol hexaphosphate is around 12 μmol/g.[67] There is no data to indicate that *Lb. plantarum* 299v possess phytase activity.

13.3 THE SPECIES *LACTOBACILLUS PLANTARUM*

13.3.1 Systematics

13.3.1.1 Lactic Acid Bacteria

The organisms performing the conversion of carbohydrates to carboxylic acids—mainly lactic acid—are by tradition called LAB. The term was used early by food

microbiologists, and by 1919 the Danish bacteriologist Orla Jensen tried to define key features of LAB as follows: "The true lactic acid bacteria form a large natural group of nonmotile, nonspore-formers, Gram-positive cocci and rods that at fermentation of sugar mainly produce lactic acid." Based on definitions such as this, different systematically defined taxa have been included in the group LAB. However, LAB is not a systematically defined group based on evolutionary relationships. It is a functional characteristic that food microbiologists apply to bacteria, harmless to both food quality and human health, that occurs spontaneously in traditional lactic acid fermented foods. From the systematic point of view, this means a relatively wide variety of taxa. How many genera and species should be included depends as much on how many different types of foods that are included, and how strict the quality definitions are set for these food products. For example, the higher the eating quality of a lactic acid fermented food product is, the fewer types of bacteria generally involved in the final fermentation. In a product of poorer quality, all types of unwanted organisms can be present in high numbers in the final product. The only absolute condition for the organisms involved in lactic acid fermentation must be that they produce lactic acid as the major end-product in carbohydrate catabolism and that they are harmless to consume in high numbers, even for consumers with underlying illnesses that weaken their immunological defense. The taxa frequently occurring in high numbers in traditional and spontaneously fermenting lactic acid fermented foods are *Lactobacillus*, *Pediococcus*, *Weissella*, *Leuconostoc*, *Oenococcus, Lactococcus*, and *Streptococcus thermophilus* (and some closely related *Streptococcus* species). The genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella,* and *Oenococcus* have a relatively close phylogenetic relationship, and might all be included in the trivial expression "lactobacilli." However, *Lactococcus* and *S. thermophilus* have from a phylogenic point of view, nothing in common with the lactobacilli other than being members of the same general branch of evolution, i.e., the phylum (or division) *Firmicutes* (Gram-positive bacteria having a relatively low ratio of guanine and cytosine in their genome).

13.3.1.2 Phylogenetic Relationships

Lactobacillus plantarum is a bacterial species in the huge and relatively diverse genus of *Lactobacillus*, which comprises more than 90 validly named species or subspecies. The DNA guanine plus cytosine (G+C) content of the different species ranges from 32 to 54 mol %, which is about twice as large a range as that normally accepted for a well-defined genus.[72–74] By tradition, the *Lactobacillus* spp. have been divided into three groups depending on their fermentation abilities; the obligately homofermentatives (Group I), the facultatively heterofermentatives (Group II), and the obligately heterofermentatives (Group III).[75] Group I species ferment hexoses exclusively to lactic acid by the fructose-1,6-biphosphate pathway, but can not ferment gluconate or pentoses, whereas Group II species can ferment pentoses and/or gluconate. Group III species ferment hexoses to lactic acid, acetic acid, and/or ethanol, and carbon dioxide. *Lactobacillus plantarum* are facultatively heterofermentative, whereas the type species of *Lactobacillus*, *Lb. delbrueckii*, is obligately homofermentative. The mol % G+C of *Lb. plantarum* is 44 to 46%, whereas it is 49 to 51 for *Lb. delbruecki*.[75]

Paradoxically, the mol % G+C for some of the other well known obligately homofermatives species such as *Lb. acidophilus*, *Lb. crispatus*, *Lb. jensenii*, and *Lb. gasseri* are 32 to 37%, 35 to 38%, 35 to 37%, and 33 to 35%, respectively.[75] It is obvious that there are major genomic differences between *Lb. plantarum* and many of the obligately homofermentative species. *Lb. plantarum* has also a significantly large genome, which may indicate an ability to adopt to many different conditions.[76]

More recent taxonomic efforts to understand the phylogenetic relationships have been directed towards comparative analysis of 16S (and 23S) ribosomal ribonucleic acid (rRNA) gene sequences. These analyses divide *Lactobacillus* into several subgroups, but also point out that several of the facultatively heterofermentative *Lactobacillus* spp. are related to *Pediococcus* spp.[74,77–79] The *Lactobacillus* spp. were divided phylogenetically into three groups that were not altogether in agreement with the traditionally, phenotypically based, subgroups (fermentation groups). Thus, many of the obligately homofoermentatives (for example, *Lb. delbrueckii*, *Lb. acidophilus*, *Lb. crispatus*, *Lb. jensenii*, and *Lb. gasseri*) formed one subgroup. This rRNA group was called the "*Lb. delbrueckii* group." The second group was formed by more than 30 *Lactobacillus* spp., including *Lactobacillus* spp. of all three fermentation groups and also some *Pediococcus* spp., and called the "*Lb. casei* group." And the third group, the so-called *Leuconostoc* group, included *Leuconostoc* spp., some obligately heterofermentative *Lactobacillus* spp. and *Weissella* spp.[74,77–79] *Lb. plantarum* were included in the *Lb. casei* group.

The *Lactobacillus* have been further subdivided[77] and are now in five phylogenetical rRNA groups:

1. The *Lb. acidophilus* group (*Lb. delbrueckii* was regarded atypical for the obligately homofermentatives due to its high mol % G+C)
2. The *Lb. salivarius* group
3. The *Lb. reuteri* group
4. The *Lb. buchneri* group
5. The *Lb. plantarum* group

Surprisingly, the *Lb. plantarum* group included the obligately homofermentative *Lb. farciminis*, the facultativly heterofermentative *Lb. alimentarius*, and the obligately heterofermentative *Lb. collinoides*.[80] None of these species have in the past been regarded as *Lb. plantarum*-like. However, more closely related to *Lb. planarum*, and definitively most *Lb. plantarum*-like, are *Lb. pentosus*[77,81] and *Lb. paraplantarum*.[82] These two species and *Lb. plantarum* have not only high similarities in the 16S rRNA gene, they have also phenotypical similarities. All have also a cell wall peptidoglycan of the *m*-diaminopalemic-direct type[82] which is not the most common type among *Lactobacillus*. *Lb. agilis* also has this cell wall type and shows phenotypic similarities to *Lb. plantarum*.[83] The majority of the *Lactobacillus* spp. have the peptidoglycan type, L-Lys-D-Asp.

13.3.1.3 Diagnostic Features

Key features of *Lb. plantarum*, according to *Bergey's Manual*[75] are rod-shaped cells, growth at 15° C but not at 45°C, cell walls containing teichoic acid, cell wall peptidoglycan of the *m*-diaminopalemic-direct type, production of both isomers of lactic acid (DL lactic acid), inability to produce NH_3 from arginine, utilization of pentoses by the induction of phosphoketolase, and the mol % G+C = 44 to 46%. The type strain of *Lb. plantarum* is ATCC 14917.[75] The ability of different *Lb. plantarum* strains to ferment different carbohydrates at 37°C in the API 50CH test kit is shown in Table 13.1. *Lb. plantarum* possess a striking ability to ferment many different carbohydrates. In view of their use for fermentation of cereals, it is also interesting to note that some strains are able to ferment starch.[52]

TABLE 13.1
The Percentage of *Lb. plantarum* Strains Able to Ferment Different Carbohydrates in the API 50CH Test Kit[a] at 37°C

Carbohydrate	% Positive *L. plantarum* strains[b]
Ribose, mannose, galactose, glucose, fructose, mannitol, N-acetyl-glucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, gentiobiose	100
L-arabinose	75
D-xylose	15
Rhamnose	5
Inositol	5
Sorbitol	80
α-Methyl-D-mannoside	80
α-Methyl-D-glucoside	5
Trehalose	95
Melezitose	70
Raffinose	50
Starch	30
Glycogen	15
D-turanose	75
Gluconate	85
Glycerol, erythriol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, dulcitol, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2-keto-gluconate, 5-keto-gluconate	0

[a] API systems, S.A., Montalieu Versieu, France.

[b] 20 tested *Lb. plantarum* strains isolated from human intestinal sites and traditional lactic acid fermented foods.

Source: Data from Johansson, M.-L., Molin, G., Pettersson, B., Uhlén, M., and Ahrné, S., *J. Appl. Bacteriol.,* 79, 536-541, 1995. With permission.

The phenotype of *Lb. plantarum* can be heterogeneous, which may hamper a phenotypic identification.[52,84] However, ribotyping patterns of the 16S rRNA genes, i.e., restriction fragment length polymorphism (RFLP) of the 16S rRNA gene, is extremely homogeneous within the species of *Lb. plantarum*, irrespective of the phenotype and source of isolation.[85] Thus, a genomic identification is much more precise, and must strongly be recommended.

Subgrouping of strains/isolates of *Lb. plantarum* below the hierarchical level of the species can conveniently be performed with the polymerase chain reaction (PCR)-based technique of randomly amplified polymorphic DNA (RAPD).[86] However, if individual strains are to be identified, restriction endonuclease analysis (REA) of total chromosomal DNA is recommended. By use of relatively frequently cutting restriction enzymes, such as *Eco*RI and *Cla*I, and traditional agarose gel electrophoresis, a reproducible identification with high resolution capacity can be obtained.[87] This method was successfully used for identification at re-isolation of *Lb. plantarum* strains 299 and 299v from mucosal biopsies obtained in administration studies in humans.[88] The genomic position of these two strains were confirmed when the genome was compared with 19 other *Lb. plantarum* strains by microarrays containing a subset of small genomic fragments of the strain *Lb. plantarum* WCFS1.[89] *Lb. plantraum* 299v was shown to be genomically different from all the tested strains, and was most closely related to the strain, *Lb. plantarum* 299 (DSM6595).[89]

13.3.2 Physiology and Ecology

13.3.2.1 Ecological Niches

Lactobacillus plantarum is often the dominating *Lactobacillus* spp. in traditional lactic acid fermented foods based on plant material, as has been described previously in this chapter. The fermented foods can be inoculated with *Lb. plantarum* directly from the plant, as *Lb. plantarum* can be present in low numbers (less than 10 CFU/g of plant material on living plants).[90] However, the plant material can also be inoculated with lactobacilli originating from animals and from the humans preparing the product for fermentation. *Lb. plantarum* are often present on the human mucosa, from the mouth to the rectum,[64,91] and are also present in the GI tracts of several domestic animals such as dogs, pigs, and horses. *Lb. plantarum* may even be present in insects, spiders, and snails. Thus, *Lb. plantarum* may cyclically change its environment from the human or animal intestinal tract, via plants and lactic-acid-fermented foods, back to the mouth and the intestinal tract of humans and animals. This behavior puts high demands on the adaptability and competitiveness of the organism and may be one reason for the relatively large genome of *Lb. plantarum* and the high ability to utilize different carbohydrates.

One factor of importance contributing to the ability of *Lb. plantarum* to go from food to the GI tract is that the organisms have the ability to survive in the GI-environment and to adhere to the mucosa in order to avoid immediate washout. Short duration establishment in high numbers of two *Lb. plantarum* strains on the intestinal mucosa after oral administration in lactic-acid-fermented oatmeal pap (freeze dried) has been shown for two genetically closely related strains of *Lb. plantarum* (strain 299v [DSM 9843] and strain 299 [DSM 6595]).[88] *Lb. plantarum* 299v,

when administrated in the probiotic fruit drink ProViva, has theability to survive the passage through the human GI tract and to establish itself for a short period of time in the intestine after consumption has been confirmed.[92,93]

13.3.2.2 Adhesion

The *Lb. plantarum* strains 299 and 299v are included in a genetic subgroup within the species *Lb. plantarum*[87] where the members mostly originate from the intestinal mucosa, but also can be found in traditional lactic acid fermented foods.[64,91] The strains of this subgroup have been shown to have a pronounced ability to attach to human mucosa cells in vitro; the adhesion is dependant on a mannose-binding adherence mechanism.[91,94] Moreover, *Lb. plantarum* strains of this particular genomic subtype frequently dominate the total *Lactobacillus* flora of healthy individuals, both on rectal and on oral mucosa.[91,94]

Yeasts also have mannose receptors on their surface, and *Lb. plantarum* strains with mannose-sensitive capacity for adhesion can bind to the surface of the yeast cell.[94] *Lb. plantarum* often occurs together with yeasts in traditional lactic acid fermented foods. Presumably, *Lb. plantarum* benefits from attaching to the surface of eucaryotic cells, perhaps by utilizing growth factors leaking out from the eucaryotes. *Lb. plantarum* requires calcium, pantothenate, and niacin.[75] The yeasts may also have a protective effect against the noxious effects of oxygen.

Lb. plantarum 299v have been shown to inhibit enteropathogenic and enterohemorrhagic *Escherichia coli* adhesion in vitro to intestinal epithelial cells in culture by inducing mucin expression, i.e., intestinal epithelial cells produce more mucin that limits access of pathogens to their surface.[95,96] The ability of *Lb. plantarum* 299v to reduce the secretory response of intestinal epithelial cells to enteropathogenic *E. coli* (EPEC) has also been shown in vitro.[97] The observed effect was due to reduced attachment of EPEC to epithelial cells in presence of *Lb. plantarum*.[97]

13.3.2.3 Oxidative Reactions

Lactobacillus plantarum possess enzymatic systems of its own to handle oxygen radicals. However, this bacterium should be regarded as a microaerobe; it has less efficient systems for handling oxygen radicals than fully aerobic organisms do. Oxidative reactions that can occur with *Lb. plantarum* are:

1. With pyruvate oxidase, produces H_2O_2, CO_2, and acetyl phosphate from pyruvate, O_2, and phosphate
2. With L-lactate oxidase, or NAD-independent D-lactate dehydrogenase, produces pyruvate and H_2O_2 from lactate and O_2
3. With NADH oxidase, produces NAD^+ and H_2O_2 from NADH, H^+, and O_2
4. With nonenzymatic superoxide reduction by manganese, produces H_2O_2 from O_2^- and hydrogen[24]

Lactobacillus plantarum has a high growth requirement for manganese and can also accumulate high intercellular levels of manganese.[98] Interestingly, plants are rich in manganese, and manganese provides a defense for *Lb. plantarum* against

oxygen toxicity by the reduction of oxygen radicals to H_2O_2.[99] The produced H_2O_2 can be converted to O_2 and water by manganese cofactored pseudocatalase in *Lb. plantarum*.[100,101]

13.3.2.4 Carbohydrate Fermentation

Although lactic acid always is the major end product of glucose under anaerobic conditions (2 moles lactic acid per mole hexose), considerable amounts of acetic acid has been shown to be produced by *Lb. plantarum* under aerobic conditions.[102] About one third acetic acid and two thirds lactic acid were produced by *Lb. plantarum* ATCC 8014 under aerobic conditions.[103]

Lactobacillus plantarum are not only able to ferment hexoses and pentoses (produce 1 mole each of lactate, acetate, and CO_2 per mole of pentose), but they can also utilize many organic acids such as malic, tartaric, and citric acids to produce CO_2 and lactic or acetic acid and other byproducts. The breakdown of malic acid to lactic acid and CO_2 (malolactic fermentation) is important in winemaking.[83] The metabolic options of *Lb. plantarum* have been reviewed by Vescovo et al.[83] and Daeschel and Nes.[24] *Lb. plantarum* can also produce smaller amounts of diacetyl or acetoin, which have antimicrobial properties and may also affect the taste of foods.[24,83,103]

13.3.2.5 Resistance to Low pH

The fact that *Lb. plantarum* frequently predominate in spontaneously lactic acid fermented foods, where the pH usually is below 4.0 (see previous discussion), and also survive the passage through the acid conditions of the human stomach,[85] point to its high resistance to acid conditions. This organism also has a high tolerance to low pH compared to other lactic acid bacteria.[24] For example, a comparison between *Lb. plantarum* and *Leuconostoc mesenteroides* showed that the growth of *Lc. mesenterodes* ceased when internal cellular pH reached 5.4 to 5.7 and growth of *Lb. plantarum* stopped when their internal pH dropped to 4.6 to 4.8.[104] *Lb. plantarum* maintained its pH gradient down to an external pH of 3.0.

13.3.2.6 Breakdown of Tannins

Besides pH, a controlling factor in the fermentation of plant material may be the presence of tannins and other phenolics. Tannins are naturally occurring water soluble polyphenols of varying molecular weight, which differ from most other natural phenolic compounds because of their ability to precipitate proteins from solutions.[105] Tannins inhibit the growth of a number of microorganisms and are resistant to microbial attack.[48,106] So-called condensed (nonhydrolysable) tannins are more resistant to microbial degradation than hydrolysable tannins. Tannins are commonly found in fruit and seeds such as grapes, apples, olives, beans, grains of sorghum and finger millets, coca, tea, and coffee. Fungi and yeasts and some aerobic bacteria are usually able to degrade tannins but also anaerobic degradation also occurs, for example in the intestinal tract.[48,107] Strains of *Lb. plantarum*, *Lb. pentosus,* and *Lb. paraplantarum* can possess tannase activity,[50] and are also able to metabolise phenolic acids.[108,109] *Lb. plantarum*, which grows in environments where high concentrations of tannins

are often present, have the unusual ability to break up tannins and to metabolize the phenolic acids. *Lb. plantarum* may modify the tannins and produce breakdown products from them, for example, substituted phenyl-propionic acids.[109] It has also been shown that *Lb. plantarum* is able to produce small amounts of phenyl-acids such as benzoic acid[110] and phenyl-lactic acid,[111] with strong antifungal properties.

Phenolics, as flavonoids, have strong antioxidative capacity which can have positive physiological effects. The combined effect of rosehips, i.e., fruits of roses, which are extreamly rich in biologically active polyphenols, and *Lb. plantarum* were tested in a ischaemia/reperfusion (I/R) model in mouse.[112] I/R of the colon is an inflammatory condition leading to tissue injury where reactive oxygen species play a central role. *Lb. plantarum* 299v possesses enzymatic activity toward polyphenols (tannins), which can split up the tannis to flavonoids and thus increase the antioxidative capacity of rosehip. It was shown that administration of rosehip and *Lb. plantarum* 299v, together, significantly decreased lipid peroxidation (the content of malondialdehyde [MDA] was taken as an index of lipid peroxidation) in caecum tissue. The results support an additive role of rosehip and *Lb. plantarum* in reducing lipid peroxidation.[112]

The degradation of tannins by *Lb. plantarum* will positively affect the nutritional value of tannin-rich fermented food products, and this may have physiological effects in the gastointestinal (GI) tract of the host. It also seems likely that a tannin-rich environment will give *Lb. plantarum* a selective advantage compared with other microorganisms that are unable to degrade tannins and that even may be inhibited by them.

13.4 HEALTH EFFECTS

13.4.1 The Intestinal Microflora

13.4.1.1 Probiotics and the Bacterial Balance

It is well established that high numbers of lactobacilli counteract many pathogenic and potentially pathogenic bacteria, regardless of whether the system is a lactic acid fermented food or the human intestine.[113,114] The original concept of probiotics implies that the balance between beneficial and harmful bacteria in the microflora of the GI tract can be positively affected by eating the right type of living microorganisms.[115,116] Even if the term "probiotics" today is used in a broader sense—to refer to live microorganisms with beneficial health effects when administrated to animals and humans—the original concept of counteracting deleterious bacteria in the GI-tract still remains interesting.

After oral administration *Lb. plantarum* 299v, *Lb. plantarum* was found in high numbers on the rectal mucosa,[93] and in feces.[92,93,117–120] *Lb. plantarum* 299v already adhere to the tonsillar mucosa directly after oral intake.[121] Furthermore, *Lb. plantarum* 299v increases the total viable count of lactobacilli in feces.[118,119,122,123]

The actual presence of live and metabolic active *Lb. plantarum* 299v on human intestinal mucosa after ingestion of the bacteria in a drink has been verified by hybridization to a DNA microarray comprising clones covering the *Lb. plantarum* genome.[124] It was shown that about 10% of the genes were expressed and genes were

detected for all functional classes. The expression differed between individuals, and to a lower degree, between the small and large intestine.[124]

In any case, one key question is, what components of the intestinal flora should be suppressed? That the probiotics should inhibit pathogens is self-evident, but the normal intestinal flora is much more than pathogens. Unfortunately, the human bacterial flora (and animal flora as well) is poorly defined, and many components have not been systematically described, not even on the hierarchical level of genus.[125] Examples of frequently occurring components of the human intestinal flora that presumably can have negative health implications and therefore should be counteracted are *Bacteroides fragilis* and species of the family *Enterobacteriacieae* (for example, *Escherichia coli* and *Klebsiella pneumoniae*). These groups found in the normal bacterial flora frequently are involved in abdominal infections and sepsis.

Lactobacillus spp. are usually present in varying numbers in the human GI tract, but are normally present in lower numbers than many other components of the normal flora such as, for example, *Bacteroides*, clostridia/eubacteria, and *Ruminococcus*.[126,127] An ingested probiotic will not only work in the colon, but will come in contact with the mucosa of the mouth, and then the intestinal mucosa and its microbial inhabitants all along the small intestine. This means the probiotic have exposure to a huge interface that is harboring a smaller population of resident bacteria than that found in the colon. The effects and actions in the small intestine will probably also have an influence on the colonic environment.

Lactobacillus plantarum frequently occur on the human GI mucosa.[64,91] The two strains of *Lb. plantarum*, 299 (DSM 6595), and 299v (DSM 9843) that have been shown to survive the passage through the human GI tract,[89] have also been shown in vitro to possess antimicrobial activity against potentially pathogenic species such as *Listeria monocytogenes*, *Bacillus cereus*, *E. coli*, *Yersinia enterocolitica*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Enterococcus* faecalis,[128] *Lb. plantarum* 299v showed a relatively strong antagonistic properties against *Salmonella enterica* subsp. *enterica*,[129] and a more intermediate antagonistic activity against *Helicobacter pylori*.[129] Furthermore, when healthy volunteers consumed a mixture of lactobacilli strains, including *Lb. plantarum* 299 and *Lb. plantarum* 299v, the level of lactobacilli in the intestine increased, and levels of Gram-negative anaerobes, *Enterobacteriaceae*, and sulfite-reducing clostridia decreased.[89] The inhibitory effect of *Lb. plantarum* 299v against *Enterobacteriaceae* and Gram-negative anaerobes has also been demonstrated in rat models simulating severe clinical conditions.[130,131]

Gram-negative anaerobes are often are involved in secondary infections after abdominal surgery.[132–134] Furthermore, Gram-negative bacteria always contain endotoxins and they initiate, even when present in small numbers, violent inflammatory reactions. Gram-negative anaerobes are also suggested to be producers of carcinogenic substances in the intestine.[135,136] Rats pretreated with the Gram-negative anaerobe *Bacteroides fragilis* before the onset of an acute liver injury developed a significantly poorer liver status than control rats with the liver injury but without bacterial pretreatment.[137] Some strains of *B. fragilis* can also secrete a toxin that has shown to activate T-cell-factor-dependant β-catenin nuclear signalling in intestinal epithelial cells, and it has been suggested that this event may contribute to oncogenic transformation in the

colon.[138] The inhibitory effect of *Lb. plantarum* 299v against *Bacteroides* was shown in a placebo-controlled study in patients with inactive ulcerative colitis.[139]

The group of sulfite-reducing clostridia can contain subgroups that produce toxins. Sulfite-reducing clostridia also produce hydrogen sulfide which, has a general toxicity. Furthermore, clostridia can produce carcinogenic substances in the intestine.[135] *Enterobacteriaceae* is a genomically close family including many pathogenic taxa, and even normally nonpathogenic taxa have a pathogenic potential in situations where the immunological defense of the host is failing. *Lb. plantarum* 299v have been shown to inhibit enteropathogenic *E. coli* adherence in vitro to HT-29 intestinal epithelial cells by inducing intestinal mucin gene expression. When this gene is expressed, epithelial cells produce more mucin, and the slime protects the cells from the enteropathogenic *E. coli*.[140] It has also been shown that the colonization of *Lb. plantarum* 299v competes with that of *E. coli* in gnotobiotic rats.[141]

In a study in Tanzania, *Lb. plantarum* 299v was used as a starter culture for producing the cereal-based, lactic-acid-fermented beverage togwa. *Lb. plantarum* 299v was used for producing 50% of the test togwa, while the other 50% was made by traditional back sloping.[59] Spontaneously fermented togwa is frequently dominated by *Lb. plantarum*.[55] The product was given to children (< 5 yr) once a day for 13 consecutive days, and the presence of fecal enteropathogens such as *Campylobacter*, entero-pathogenic *E. coli*, *Salmonella,* and *Shigella* was evaluated. The proportion of children with isolated fecal enteropathogens decreased significantly ($P < 0.001$) during the study period.[59]

The ingestion of probiotics can positively alter the GI microflora, as has been seen by the decreased plate counts of *Enterobacteriaceae* and sulfite-reducing clostridia after ingestion of lactobacilli.[88] In a randomized, placebo-controlled, double-blind study in healthy volunteers that consumed *Lb. plantarum* 299v in a fruit drink (2×10^{10} CFU/d for 3 weeks), the total level of carboxylic acids in feces increased;[92] this increase was due to increases in the concentration of acetic acid and propionic acid.[92] The carboxyl acids are produced by the GI microflora, and this change in acid composition points to significant changes in the flora. *Lb. plantarum* 299v are not known to be able to produce propionic acid. The increased concentration of acetic acid and propionic acid must be regarded as beneficial from a health perspective. Both types of short-chain fatty acids are utilized as an energy sources by the mucosa cells of the intestine. Short-chain fatty acids are, in fact, the major energy source of the colonic mucosa cells. An increased level of short-chain fatty acids in the lumen is therefore beneficial for the condition of the mucosa. Moreover, absorbed propionic acid comes via the portal blood to the liver, and there it can have positive effects on both lipid metabolism and inflammatory responses in the liver.

Healthy subjects receiving *Lb. plantarum* 299v also experienced a decrease in flatulence during the treatment period,[92] which might indicate that the concentration of gas-producing microorganisms in the GI tract decreased.

13.4.1.2 Intestinal Mucosal Status and Reduced Translocation

The effect of *Lb. plantarum* on the mucosal status and barrier function has been extensively studied in rat models. When the status of the intestinal mucosa was evaluated

using the content of protein or content of rRNA and DNA as an indicator, an improvement in status was shown in rats with acute liver injury that had been pretreated with *Lb. plantarum* 299v.[142,143] An improved mucosal status was also seen in rats with enterocolitis that had been treated with *Lb. plantarum* 299v.[130] In this study the permeability of ethylenediaminetetra-acetic acid (EDTA) through the mucosa was measured and found to decrease in animals receiving *Lb. plantarum* 299v.[130]

Translocation (the passage of viable bacteria through the epithelial mucosa into the *lamina propria* and then to the mesenteric lymph nodes and possibly other tissues)[144] can be reduced due to the improved status of the intestinal mucosa. Translocation can be studied in rats with an acute liver injury induced by an injection with D-galactose-amine, which causes a severe liver inflammation.[145,146] Twenty-four hours after the onset of the liver injury, translocating bacteria can be found in organs such as the liver and spleen and in the portal and arterial blood. The liver injury does not directly affect the intestinal mucosa, but the immunological defense of the animal is severely weakened, which allows the translocating bacteria to travel beyond the mesenteric lymph nodes and the liver. However, by pretreating the animals with *Lb. plantarum* 299v, translocation can be significantly decreased.[131,137,142,147] Another strain of *Lb. plantarum* (DSM 6595) has been shown to have an effect in the liver failure model,[137] as have some strains of *Lactobacillus* spp. other than *Lb. plantarum*.[131] However, *Lb. plantarum* 299v seems to be an especially effective strain in this respect (see Figure 13.10).

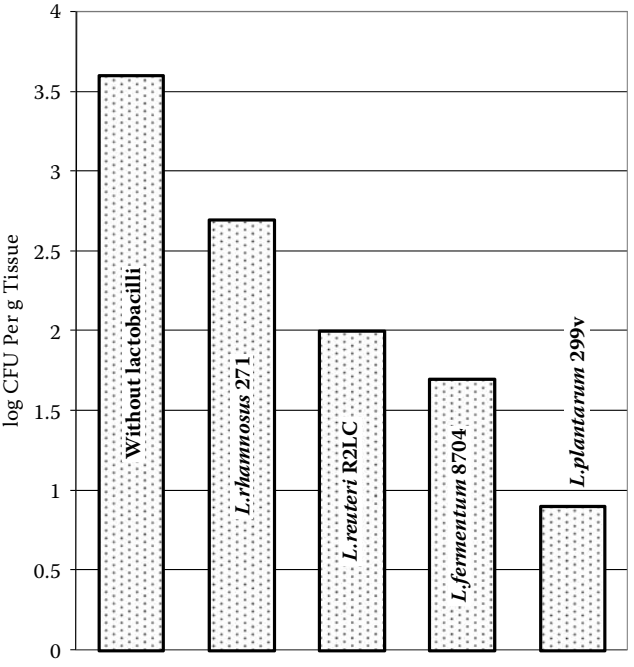

FIGURE 13.10 Bacterial translocation to the liver 24 h after onset of the liver injury of rats pre-treated with different *Lactobacillus* strains (From Adawi, D., Molin, G., Ahrné, S., and Jeppsson, B., *Microb. Ecol. Health Dis.,* 11, 47–54, 1999. With permission.)

It is interesting to identify what type of bacteria are translocating in the rats with liver failure.[147] In rats that had not received any lactobacilli treatment, the majority of the bacteria found in the liver originated from the dominating population of the intestinal mucosal flora, i.e., *Lb. animalis*, *Lb. reuteri* and *Lb. acidophilus* (*Lactobacillus* species are much more dominant in rats than in humans), but *Proteus vulgaris*, *Bacteroides distasonis*, *Enterococcus faecalis*, and *Staphylococcus aureus* were also found in the liver. *P. vulgaris* and *S. aureus* were also found in the arterial blood.[128] However, pretreatment for 8 d with *Lb. plantarum* 299v before the liver injury not only decreased the rate of translocation to the liver, but no bacteria translocated to the blood and only *Lb. animalis*, *Lb. reuteri*, and *Lb. acidophilus* were found in the liver.[147] The *Lb. plantarum* treatment not only decreased the rate of translocation, it obviously had a controlling impact on the intestinal microflora and enhanced the domination of *Lactobacillus*. It can also be noted that *Lb. plantarum* was never found in extra-intestinal sites in spite of the large pretreatment dose.[147] It has also been shown that pretreatment of rats with *Lb. plantarum* 299v in drinking water for a week inhibited *E. coli*-induced permeability of the intestine.[129] This was shown in intestinal segments mounted in Using chambers, where the permeability of mannitol was measured. Exposure to *E. coli* in the Using chamber normally increases the permeability, but the pretreatment of the living rats with *Lb. plantarum* abolished this increase in permeability.[148]

Many of the intestinal bacteria that translocate in the rats with liver failure end up in the liver, which enhances the inflammation of the liver, causing the condition of the liver to worsen. This deterioration can be measured by the concentration of liver enzymes in the blood. In the liver failure model, it was shown that pretreatment with *Lb. plantarum* 299v decreased the concentration of the liver enzymes aspirate transaminase and alanine transaminase in the blood, indicating that the liver status was improved by the treatment.[131,142,143] This was also true for another, genetically similar *Lb. plantarum* strain, DSM 6595.[137]

The preventive effect of *Lb. plantarum* 299v on translocation has also been seen in other experimental models in rats. *Lb. plantarum* 299v significantly reduced translocation in rats with enterocolitis induced by Methotrexate.[130] In this model, the mucosa is inflamed and damaged, in contrast to the liver failure model, where the mucosa is unaffected. The administration of lactobacilli to the rats with the enterocolitis mitigated the mucosal injuries induced by the chemotherapy.[130] Also, *Lb. plantarum* strain DSM 6595 possessed this effect. In a comparison with the type strain of *Lb. acidophilus* (strain DSM 6595), the effect was shown to be more pronounced for the *Lb. plantarum* strain than that for the *Lb. acidophilus* DSM 6595.[149] Furthermore, a decreased translocation has been observed after treatment with *Lb. plantarum* 299v in an experimental rat model with pancreatitis,[150] in a DSS-induced colitis model in rat,[151] and in a septic rat model.[152]

There can be several explanations as to how *Lb. plantarum* can improve the mucous status and decrease the translocation rate. One is the traditional probiotic effect, i.e., that the administrated probiotic strain counteracts adverse bacteria. These aggressive, adverse bacteria can induce and maintain an inflammation; they may be especially suited for translocation, and are capable of fighting off the host's immunological defense. It is also possible that the probiotic strain not only counteracts adverse

components of the flora, but it might also stimulate beneficial components that are part of the resident flora. In fact, the domination of resident intestinal lactobacilli of rats increased after treatment with *Lb. plantarum* 299v.[147] This was also indicated in humans when the amount of propionic acid in feces increased after consumption of *Lb. plantarum* 299v, because propionic acid is not produced by 299v.[92] However, the improved barrier effect of the mucosa could also be due to an immunomodulation (see the following section) and to a stimulation of the mucin production of the human mucosa cells.[95,96]

13.4.2 Risk Factors for Coronary Artery Disease

Lactobacillus plantarum 299v have surprisingly been shown to be able to decrease different risk factors for coronary artery diseases in individuals at risk. In a small randomized, placebo controlled and double-blind study on men with slightly elevated cholesterol levels, it was shown that the concentrations of total cholesterol and of low-denisty lipoprotein (LDL) cholesterol were decreased after consumption of *Lb. plantarum* 299v in a fruit drink.[153] The study included 30 individuals divided into two groups, where the treatment group consumed 200 ml fruit drink (rose hip), containing 5×10^7 CFU/ml, for 6 weeks, and the placebo group consumed the fruit drink without lactobacilli. The fall in cholesterol level was small but statistically significant.[153] However, even more surprising, it was shown in the same study that the fibrinogen level of the serum also was decreased significantly ($P < 0.001$), representing a reduction of 13.5%.[153] Fibrinogen is an acute phase protein that reflects the inflammatory status of the individual and also is an independent risk factor for coronary artery disease.[154] In a subsequent placebo controlled randomized double blind study with 38 healthy smokers, it was shown that the consumption of 400 ml ProViva daily for 6 weeks, not only significantly decreased the level of fibrinogen, but also F_2-isoprostans and interleukin (IL)-6, which are other inflammatory markers.[155] Moreover, *Lb. plantarum* 299v in the fruit drink also positively affected the systolic blood pressure and the insulin and leptin response.[155]

13.4.3 Decreased Systemic Inflammatory Response in Critically Ill Patients

One hundred and three critically ill patients were randomized to receive an oral preparation containing *Lb. plantarum* 299v (ProViva, Strawberry) in addition to conventional therapy (treatment group, $n = 52$) or conventional therapy alone (control group, $n = 51$).[156] On day 15, serum IL-6 levels were significantly lower in the treatment group compared to controls.[156] IL-6 is a cytokine produced by many cell types, including lympocytes, fibroblasts, and monocytes. It has a variety of systemic effects including activation of B and T lymphocytes, and induction of acute phase protein production in the liver. IL-6 appears to be a good indicator of activation of the cytokine cascade and predicts subsequent organ dysfunction and mortality.[157] Thus, the enteral administration of *Lb. plantarum* 299v to critically ill patients was associated with a late attenuation of the systemic inflammatory response.[156] This was associated with a change in EndoCAB levels in the patients administered *Lb. plantarum* 299v, indicating a decreased endotoxin exposure.[156]

13.4.4 Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is common, but its cause is unknown. It is not a single condition, but rather a collection of disorders causing similar symptoms of abdominal pain, diarrhea, constipation, or variability of bowel habit. The absence of strict pathogenic features has made IBS a disease without a proper diagnosis. Attempts have been made to develop criteria for a positive diagnosis of IBS.[158,159] Among patients coming to gastroenterology clinics, 20 to 50% are suffering from IBS, even if most patients with IBS do not seek medical care.[160] IBS is a chronic relapsing condition that perhaps occurs in most adults at some point in their lives. Symptoms begin before age 35 in 50% of patients, and 40% of patients are aged 35 to 50.[160] IBS was found in 18% of the adult population in the Bristol area in the United Kingdom.[161]

Lb. plantarum 299v in the fruit drink ProViva (rose hip) was administrated to patients with IBS in two double-blind, placebo-controlled studies, one in Poland[162] and one in Sweden.[93] In both studies, the patients were divided into two groups: one was given *Lb. plantarum* 299v and the other a similar rose hip drink without *Lb. plantarum* 299v (placebo). In the Polish study, that the magnitude of several of the IBS symptoms decreased in the *Lb. plantarum* group, and a higher proportion of the patients were free from symptoms in the treatment group than in the placebo group.[162] In the Swedish study, *Lb. plantarum* 299v significantly decreased the subjective bloating experienced during the treatment period.[93] Pain was also significantly reduced in both the treatment group and in the placebo group, but the decrease was more rapid and more pronounced in the *Lb. plantarum* group. Twelve months after the treatment, the patients given *Lb. plantarum* 299v in the study still experienced a better overall gastrointestinal function than the patients who had received the placebo.[93]

The bloating and pain experienced by IBS patient might be due to abnormal colonic fermentation giving rise to an excess of gas production, especially of hydrogen.[163] Presumably, *Lb. plantarum* 299v suppresses the components of the intestinal microflora that are responsible for this gas production. Further support for this suggestion is found in a small, randomized, placebo-controlled study on *Lb. plantarum* 299v, where the gas production and composition of the gas was objectively and carefully collected and recorded in a plastic tent for each subject after 4 weeks consumption. No direct differences were seen between the placebo and the treatment group.[164] However, if the volunteers were provoked by consuming 20 g lactulose, the hydrogen in the breath was significantly decreased in the group treated with *Lb. plantarum* 299v. Thus, the intestinal microflora seemed to have been changed in some way.

13.4.5 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a chronic inflammation along the GI tract. It can be limited to the large bowel (ulcerative colitis) or it can be situated anywhere along the GI tract (Crohn's disease). Ulcerative colitis is a relatively superficial ulcerative inflammation, while Crohn's disease is a transmural, granulomatous inflammation. IBD is thought to be due to an abnormal and uncontrolled immune response to normally occurring constituents of the intestine. The etiology of IBD is unknown. Microbial agents appear to be involved in the pathogenesis of IBD, and intestinal

bacteria seem to be an important factor in its development and chronicity.[165–167] In these conditions, the bacteria, mucosa, and immune system interact in complex ways, and this interaction is far from clear.[166]

Inflammation and the potential of *Lb. plantarum* 299v to counteract the inflammation has been studied in different animal models. In rats with enterocolitis induced by treatment with methotrexate, administration with *Lb. plantarum* 299v mitigated the mucosal injuries induced by the chemotherapy.[130] Furthermore, inflammation in the intestinal mucosa of rats after radiation was decreased by administration of *Lb. plantarum* 299v in fermented oatmeal pap.[168]

In a study using interleukin-10 deficient mice in germ-free and specific pathogen-free (SPF) environments, *Lb. plantarum* 299v was able to attenuate the established colitis where the bacterium had colonized the gastrointestinal tract of the mouse before the mouse was transferred to the SPF environment.[167,169] It was also demonstrated that a mono-association with *Lb. plantarum* 299v (i.e., *Lb. plantarum* 299v was the only bacterium in the animal) did not induce colitis, but only initiated a very mild immune response.

A frequently used animal model to mimic ulcerative colitis is rat given dextan sulphate sodium (DSS) in the drinking water; after some days the animals develop colitis. The DSS-induced lesions and the location of the lesions (mainly the left colon) have resemblances to ulcerative colitis in humans. Treatment with *Lb. plantarum* 299v decreased the severity of the colitis measured on a disease activity index (DAI; based on weight loss, diarrhea, and blood coming from rectum).[170]

There are today few clinical data on attempts to treat IBD in humans with probiotcs. Oral administration of a mixture of probiotic strains of different genera and species, including one strain of *Lb. plantarum*, in an open label trial was performed during a 6-week treatment period in ambulatory patients with active ulcerative colitis.[171] Intent to treat analysis demonstrated remission in 53% and response in 24%. The treatment resulted in a combined induction of remission–response rate of 77%.[171] The same probiotic mixture was earlier shown to prevent flare-ups of chronic pouchitis.[172] Pouchitis is a nonspecific inflammation of the ileal reservoir made after surgery for ulcerative colitis.

13.4.6 Immune Modulation

13.4.6.1 Expression of Cytokines in Cells *in vitro*

The cytokine response of human peripheral blood mononuclear cells differs between different *Lactobacillus* spp. It has been shown that different strains of *Lb. plantarum* of intestinal origin are able to induce the production of the cytokines IL-12 and IL-10 from blood mononuclear cells.[173] Compared to *E. coli*, less IL-10 was produced but considerably more IL-12 was produced. In the same study, *Lb. paracasei* induced the production of a higher proportion of IL-12, and *Lb. rhamnosus* induced a higher proportion of IL-10. The response of the mononuclear cells was more balanced in respect to IL-10 and IL-12 production when they were exposed to *Lb. plantarum*, than to the other two *Lactobacillus* spp.[173]

The cytokine response of bone marrow-derived murine dendritic cells, when exposed to different probiotic strains of lactobacillus, has also been shown to vary.[174]

Substantial differences could be seen between strains in their capacity to induce IL-12 and TNF-α production in dendritic cells. The ranking among the tested strains was as follows: *Lb. casei* subsp. *alactus* CHCC3137 >> *Lb. plantarum* Lb1 > *Lb. fermentum* Lb20 > *Lb. johnsonii* La1 > *Lb. plantarum* 299v >> *Lb. reuteri* DSM 12246.[174] Similar but less pronounced differences were observed among the test strains in the induction of IL-6 and IL-10.

The ability of the proinflammatory cytokine tumor necrosis factor, TNF-α to influence epithelial IL-8 responses to *Lb. plantarum* 299v has been analyzed in the HT-29 colonic epithelial cell line.[175] The results showed that TNF-α sensitises HT-29 cells to *Lb. plantarum* 299v and the IL-8 mRNA expression was increased above levels induced by TNF-α alone. However, even if the expression had been increased, the IL-8 secretion was most unexpectively decreased in the HT-29 cells that had been exposed to *Lb. plantarum* 299v. This means that even if *Ll. plantarum* 299v sensitizes the HT-29 cells, the bacteria exert a protective effect by down-regulating IL-8 secretion (IL-8 is a strongly proinflammatory cytokine).[175] In a way, this gives an explanation to the paradox that probiotics is able to both up-regulate the immunological response and exercise an anti-inflammatory effect.

13.4.6.2 Experimental Models in Rat

The subnormal levels of secretory IgA antibodies in the intestines of rats with enterocolitis were increased, and approached a more normal level, after the administration of *Lb. plantarum* 299v. Also the levels of CD4 and CD8 lymphocytes in the intestinal lamina propria increased to more normal levels after treatment with *Lb. plantarum* 299v.[176]

The levels of total serum IgA antibodies increased, and the IgA and IgM antibody levels against *Escherichia coli* were marginally higher in gnotobiotic rats colonized with *E. coli* together with *Lb. plantarum* 299v, compared with rats that only were colonized with *E. coli*.[122] The group treated with *Lb. plantarum* 299v also showed a significantly increased density of CD25-positive cells in lamina propria, and displayed a decreased proliferative spleen cell response after stimulation with ConA, one week after colonization. The results indicated that *Lb. plantarum* 299v can modulate a response to antigens presented via the gut.

Multiple sclerosis (MS) is a Th1 cell-mediated chronic inflammatory disease of the central nervous system. Treatment of experimental autoimmune encephalomyelitis (EAE) in a mouse model with *Lb. plantarum* 299v suppressed EAE development.[177] Treatment with *Lactobacillus paracasei* PCC 101 or *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 had no such effects.[177]

13.4.6.3 Immune Response in HIV Positive Children

Children congenitally exposed to human immunodeficiency virus (HIV) have received *Lb. plantarum* 299v in a fermented oatmeal pap (freeze dried), in a pilot study. The results suggested that *Lb. plantarum* 299v elicits specific systemic immune responses after oral supplementation.[178,179]

13.4 SAFETY ASPECTS

The safety of consuming high numbers of live bacteria has been questioned, and there are reports that *Lactobacillus* spp., including *Lb. plantarum* strains, have been isolated from diseased sites in patients.[180] However, the potential of *Lactobacillus* spp. to cause serious infections has been assessed by studying the prevalence of bactermia due to *Lactobacillus* spp. during a 4-yr period. This study indicated that the pathogenic potential of *Lactobacillus* spp. is low.[181]

The fact that many traditional lactic acid fermented foods spontaneously contain high numbers of *Lb.* plantarum,[6–10,12,14–16,52] and that these products all over the world have a reputation of being safe and wholesome, strongly indicates that live *Lb. plantarum* can safely be consumed. This becomes especially obvious if the long historical tradition of the lactic acid fermented foods is taken into account (Figure 13.1). However, in the case of the *Lb. plantarum* strain 299v, the safety has been more directly confirmed in a series of different studies.

Lactobacillus plantarum 299v has been given in high doses to immune-compromised children with HIV for extended time periods without any adverse effects.[178,179] *Lb. plantarum* 299v was given in a daily dose of 10^{10} CFU to two patients with small bowel bacterial overgrowth in short bowel syndrome (with D-lactic acidosis).[182] No negative effects of the *Lb. plantarum* 299v administration were noted. Instead, it was concluded for the whole case study, including six patients, that "Preliminary experience with probiotics to change the flora to nonpathogenic organisms is promising and may demonstrate greater effectiveness and results in fewer long-term complications."[182]

Lb. plantarum 299v has been given in doses of 2×10^{10} CFU per day to 64 patients undergoing elective major abdominal surgery for at least a week preoperatively, and in the postoperative period, without any negative signs, e.g., increased translocation due to the increased bacterial load.[183]

Lb. plantarum 299v has been given, to a critically ill patient in the intensive care without any adverse effects.[156,184] *Lb. plantarum* 299v was never found in the blood.

The risk of endocarditis has been tested in an experimental rat model.[185] A catheter was passed down the right common carotid artery into the lumen of the left ventricle. The catheter was tied in place, and the neck incision was closed. After 48 h, 10^8 CFU of *Lb. plantarum* 299v were injected (0.5 ml of bacterial suspension) through the tail vein. Four days after the injection of the *Lb. plantarum* strain, the rats were sacrificed and the blood, heart tissue, and catheter were sampled for bacteria. No *Lb. plantarum* 299v could be found in any of the sample sites.[185] Thus, even with this animal model, using a very unusual and challenging situation where a high dose of the bacteria is injected directly into the blood stream of an animal with an implant of artificial material in the artery and heart, *Lb. plantarum* strain was removed from the system before causing any damage.

This strain of *Lb. plantarum* appears to be perfectly safe, and so presumably are other strains of the species *Lb. plantarum*.

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14 Sauerkraut

Wilhelm Holzapfel, Ulrich Schillinger, and Herbert Buckenhüskes

CONTENTS

14.1 HISTORY AND CULTURE RELATED TO SAUERKRAUT

Present-day sauerkraut is a product resulting from lactic acid fermentation of shredded, salted white cabbage (*Brassica oleracea var. capitata* for. *alba L.*). There is no doubt that the preservation of plant material by fermentation dates back to prehistoric times. Plinius the Elder, in the first century A.D., is said to have been the first who described the production of sauerkraut by preservation of so-called salt cabbage in earthen vessels. It can be assumed that under the conditions described, the cabbage was fermented by microorganisms, some of which were typically associated with the plant phylloplane, but most of which were located in the pores of the fermentation vessels or originated from a former fermentation. Heads of white cabbage, the raw material of today's sauerkraut, seem to have been known as early as the eighth century A.D.[1] Lind[2] was the first to describe sauerkraut manufacture comparable to contemporary processing. An important date in the history of sauerkraut was the year 1775, when Captain James Cook was awarded the Great Copley Medal for his observations and conclusions about sauerkraut as an effective food for the prevention

of scurvy which, up to that time, was feared as the plague of the sea.[3] (See Chapter 1 for more details on the history of sauerkraut.)

14.2 PRODUCTION AND REGIONS OF IMPORTANCE

The results of the European Research Program "COST 91 bis"[4] were published in 1990, and indicated that in Europe, 21 different vegetables are commercially preserved by lactic acid fermentation. The economical importance of the different products is not quite clear, as fermented vegetables are not separately listed in the national statistics. However, based on the European production figures of 1985, fermented olives represent the most important fermented product, with a total amount of more than 510,000 tons per annum The second place was held by sauerkraut with a total of 220,000 tons, followed by 45,000 tons of fermented cucumbers. Of this total, 122,000 tons, of sauerkraut were produced in Germany.[4] During the 1990s the consumption of sauerkraut decreased in Germany from 1.7 kg per capita per year in 1989 to 1.2 kg in 1999. As a result of this development, the production also decreased to approximately 104,000 tons in 2004.[5]

Outside Europe there are two other regions with a significant production of sauerkraut: Korea, China, and other Far Eastern countries on the one side, and the United States on the other. Whereas American sauerkraut is very similar to the European products, the Asian *kimchi* is produced from Chinese cabbage. (See Chapter 12 for more details on the production and health properties of kimchi.)

14.3 FERMENTATION

14.3.1 The Manufacturing Process

At the present day, sauerkraut is manufactured in all European countries by small, medium, and large companies. Consequently, the production procedures differ within a wide range. Nevertheless, the basic principles are rather similar in all cases, and therefore it is possible to summarize them as is shown in the simplified flow sheet given in Figure 14.1. Following quality control of the delivered cabbage (estimation of external quality criteria such as color, spoiled leaves, damages including insect damage, and internal tip burn, as well as the determination of the dry matter, sugar, vitamin C, and nitrate content, and residues of plant protection agents), the outer green and dirty leaves are removed, and the core of the heads is bored or partly removed. Subsequently, the cabbage is shredded into 0.7- to 2-mm wide strips which, in most cases, are salted on a conveyor belt between the shredding station and the fermenter with 0.7 to 2.5% sodium chloride. Under commercial conditions, the shredded cabbage is placed into fermentation containers of volumes with capacity between 100 kg and 40 tons of cabbage.

Apart from salting, filling is one of the critical control points in sauerkraut manufacture. It is of major importance that the air between the particles be removed as much as possible. Filling and pressing the cabbage into the fermentors together with added salt leads to an osmotic withdrawal of water out of tissue cells; the water replaces the air between the cabbage shreds. It is of utmost importance to exclude air in order to support the subsequent lactic acid fermentation, and to prevent mold and

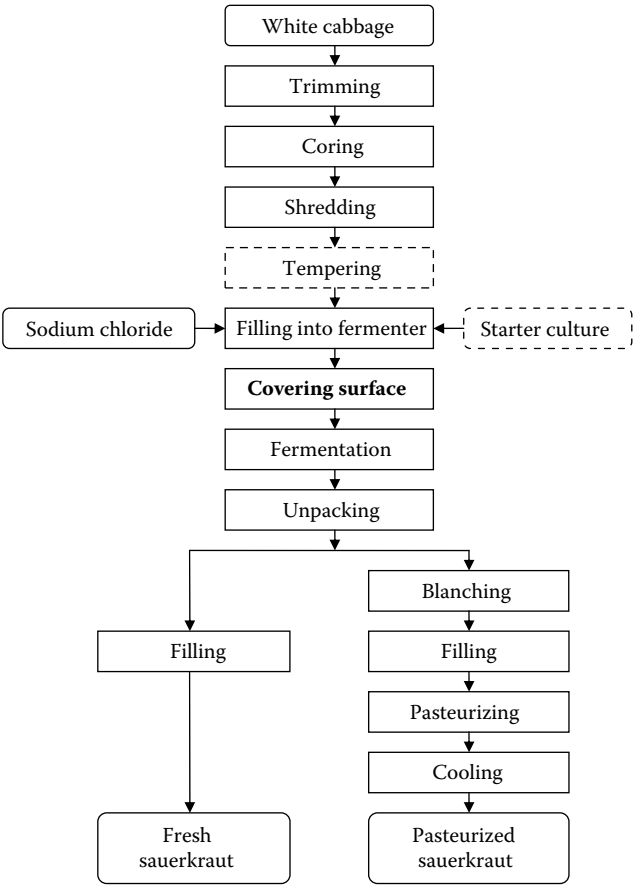

FIGURE 14.1 Flowsheet of the production of fresh and pasteurized sauerkraut.

yeast growth. Finally, the surface of the filled containers must be covered carefully in order to exclude oxygen and microbial contamination. Depending on the temperature, a "spontaneous" fermentation will start within a few hours to 1 to 2 d, and will continue between 7 d and several weeks.

If the sauerkraut is distributed as a fresh and unheated product, the fermentation has to be continued until all fermentable carbohydrates have been metabolized. Otherwise, a secondary fermentation by yeasts may occur, resulting in an alcoholic fermentation. If the sauerkraut is to be distributed as a pasteurized product, the fermentation can be stopped any time after the pH falls below 4.1. After the kraut is taken out of the fermentors, it is blanched, canned, pasteurized, and finally cooled in order to prevent heat induced quality losses.

14.3.2 Factors Affecting Sauerkraut Fermentation

The fermentation of cabbage to sauerkraut represents a very complex system of microbial, biochemical, enzymatic, chemical, and physical processes. The complexity of the

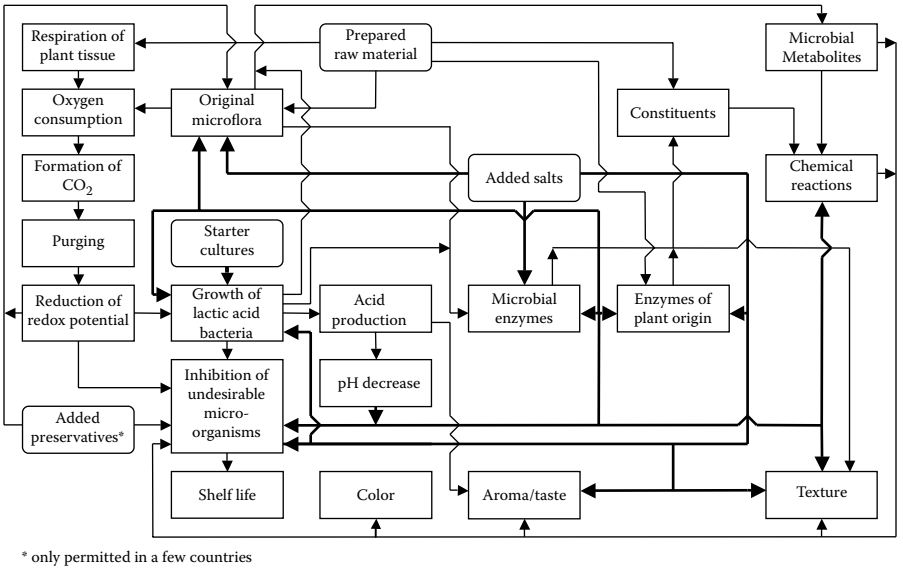

FIGURE 14.2 Interaction of various factors with processing steps involved in sauerkraut fermentation.

system is schematically demonstrated in Figure 14.2. Only the few major reactions and interrelations of the sauerkraut fermentation that influence the sensory properties and the self life of the final product are depicted. To complicate matters, fermentation can be influenced by a multitude of technological, microbiological, and raw material inherent factors, which are summarized in Table 14.1. The most important factors are described in more detail below.

14.3.2.1 Addition of Sodium Chloride

The addition of salt (sodium chloride) as well as its even distribution throughout the cabbage fibers, is one of the critical control points in sauerkraut production.[6] Not only the development of anaerobic conditions and the type and extent of microbial growth, but also the sensory properties of the final product are affected by the amount of salt used.

Directly after its addition, as well as during compression of the shredded cabbage into the fermentation vessels, the sodium chloride fulfills its first function, namely causing the osmotic withdrawal of water from the cabbage cells. The emerging liquid fills up the space between the pieces of shredded cabbage, and thereby supports the development of anaerobic conditions, which comprise the selective basis for the lactic acid fermentation.

From the onset of fermentation, the amount of salt added affects the microbial population, as it selectively favors growth of desired groups of bacteria. An increased salt content limits growth of undesirable microorganisms such as pseudomonads, flavobacteria, *achromobacter,* or fungi, whereas growth of particular lactic acid bacteria (LAB) is promoted.[7] Heterofermentative LAB are more sensitive to high

TABLE 14.1
Important Factors Influencing the Sauerkraut Fermentation Process

Raw material inherent factors
Variety of cabbage
General quality (fresh harvested or stored heads)
Dry matter content
Content of fermentable carbohydrates
Vitamin C content
pH-value
Buffering capacity
Microbial factors
Spontaneous microorganisms or starter cultures
Home flora
Bacteriophages
Residues of plant protective substances
Technological factors
Amount of added sodium chloride
Degree of shredding
Size and material of fermentor
Exclusion of oxygen
Temperature of the raw material

salt concentrations than homofermentative lactobacilli, with some species tolerating levels > 3%. Therefore, high salt levels favor the growth of homofermentative LAB, resulting in an accelerated production of lactic acid. However, fermentations are more than acidification, and it has been shown that the flavor of sauerkraut produced under these conditions is unbalanced because of the lack of acetic acid and other metabolic products normally produced by the heterofermentative species. On the other hand, too low salt concentrations (< 0.8%) may result in undesirable fermentation as well as in a poor-quality product such as soft sauerkraut.

Sodium chloride is a major flavor and modifying ingredient. Sauerkraut usually contains between 0.6 and 2% of sodium chloride (Table 14.2). The amount used depends on consumer demands and on traditional considerations in the producing countries. For instance, the salt content of canned sauerkraut produced in Germany tends to be lower (average content: 11.3 g/kg) than in the United States (average salt content: 16.7 g/kg). Following the general trend in industrialized countries to reduce the salt content of such products, the average amount in German sauerkraut decreased between the 1960s and 1980s from an average value of 12.9 g/kg to 11.3 g/kg.[8] The vegetable fermentation industry is also interested in low-salt fermentations as a means to reduce the chloride waste from sauerkraut fermentations.[9] Finally, it should be mentioned that the amount of salt used, to some extent, depends on the desired degree of acidity, because practical experience has shown that both the total acid and salt content should be in a certain ratio to ensure a balanced taste of the resulting sauerkraut.

TABLE 14.2
Initial Ecological Conditions within the Substrate

The substrate consists of solids in a liquid environment. In sauerkraut production it is not common to circulate the brine in order to distribute the microorganisms as well as released nutrients, sodium chloride or metabolites throughout the fermenter	
Microorganisms originating from the raw material or proceeding from the equipment (home flora) are distributed throughout the fermentation stock during shredding and filling	
Salmonella, clostridia, listeriae, and other undesirable microorganisms are present in all probability	
The currently used cabbage varieties are rich in nutrients, growth factors, and minerals	
Water activity	a_w = 0,95–0,99
PH	5.9–6.5
Temperature	5–20°C
Sugar content	20–50 g/kg
Vitamin C content	300–700 mg/kg
Buffering capacity	0.45–0.65 g of lactic acid /100 g cabbage
Sodium chloride	0.6–2.0% (sometimes up to 2.5%)

Source: Buckenhüskes, H.J., in *Food Microbiology—Fundamentals and Frontiers,* 2nd ed., Doyle, M.P., Beuchat, L.R., and Montville, T.J., Eds., ASM Press, Washington, 2001, pp. 665–679. With permission.

14.3.2.2 Carbohydrates

The amount of fermentable sugars available is a major factor affecting the development of the LAB that convert the carbohydrates into lactic and acetic acids. The resulting pH decrease depends on the amount and kind of produced acids as well as the buffering capacity of the fermenting cabbage, from which it is possible to estimate the minimum amount of fermentable carbohydrates required to reduce the pH below 4.1.[10] The modern cabbage varieties normally consist of enough sugar for sufficient fermentation, and sufficient remaining sugar (after fermentation) to give an acceptable flavor.

14.3.2.3 Temperature

As in other biological processes, sauerkraut fermentation is influenced by the temperature. From a sensory point of view, the best results are obtained at temperatures between 15 and 20°C.[11] Higher temperatures will cause an accelerated acid production, which leads to products with a so-called green and immature flavor.[7] Temperatures below 10°C hamper the start of fermentation and favor the spoilage of the cabbage. Although some experiments have been conducted to control the temperature of the fermenting cabbage, this has not been very successful. The only method industrially used to affect the temperature is the warming up of cold cabbage by steam injection, resulting in a temperature increase of approximately 5°C.

14.3.2.4 Microbiology

As shown in Figure 14.3, sauerkraut fermentation is a complex microbiological process resulting from the metabolic activity of a definite sequence of different microorganisms, predominantly of heterofermentative and homofermentative LAB. This succession is a

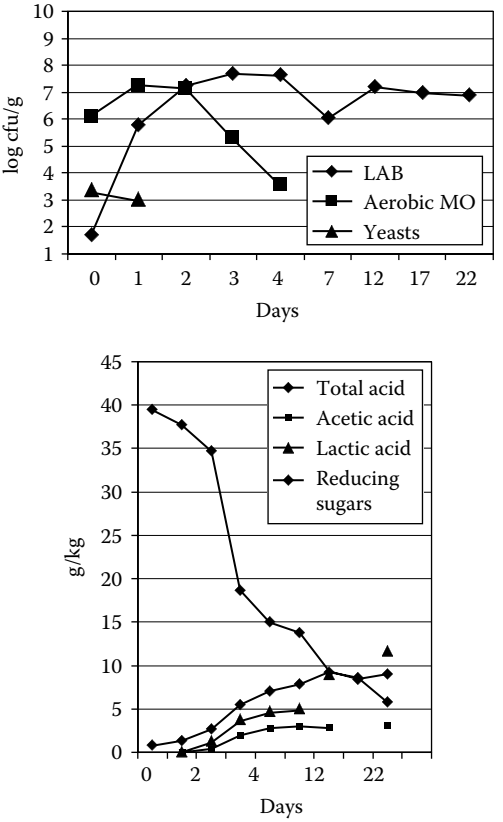

FIGURE 14.3 Sauerkraut fermentation from early harvested cabbage, conducted at 19°C in laminated plastic poches. (a) Changes in major microbial groups. (b) Changes in some analytical parameters. (From Schneider, M., Microbiology of Sauerkraut Fermented in Small Ready-to-Sell Containers (in German), dissertation, Hohenheim University, Stuttgart, Germany, 1988. With permission.)

consequence of the changing environmental conditions within the fermenting substrate. The initial ecological conditions are listed in Table 14.2. The growth sequence of spontaneously fermenting cabbage is invariably initiated by *Leuconostoc mesenteroides*, which comprise > 90% of the LAB population at this stage, followed by heterofermentative lactobacilli and finally by homofermentative lactobacilli.[13,14]

Fresh plant material harbors numerous and various types of microorganisms. The initial microflora is dominated by aerobic bacteria like pseudomonads, Enterobacteriaceae, and coryneforms.[14] On cabbage leaves LAB are only present in extremely small numbers, representing 0.15 to 1.5% of the total bacterial population. The identification of the LAB initially present on 30 different batches of cabbage has shown that nearly all belong to *Leuconostoc mesenteroides* ssp. *mesenteroides*, whereas only in a few cases some "streptococci" (probably enterococci or lactococci) were found. According to older literature, yeasts should play an important role in flavor formation, however, their share in the total microbial count is normally less than 0.1%.[11]

The traditional process of the spontaneous fermentation of cabbage can be divided into four steps.[12,15]

1. Fermentation starts as soon as the cabbage is filled into the containers. When the cabbage fibers are tightly packed, the number of strictly aerobic bacteria such as *Pseudomonas*, *Flavobacterium*, and *Acinetobacter* species initially present, decreases immediately. Anaerobiosis is rapidly attained as a result of the respiration of the plant material and the consumption of oxygen by facultative anaerobic enterobacteria which multiply for the first 2 or 3 d. Oxygen deprivation is accompanied by a change in pH resulting from the organic acids (lactic, acetic, formic, and succinic acids) formed. It is not clear whether or not the microorganisms present at this stage of fermentation have a significant influence on flavor development.
2. The more anaerobic atmosphere/lower redox potential, the added salt, and the reduced pH favor the facultatively anaerobic LAB, which soon become the predominant organisms. Although *Leuconostoc mesenteroides* is not as acid tolerant as other LAB species, it generally initiates the fermentation, as it is present at sufficiently high numbers and is well adapted to the substrate. Under ideal conditions, e.g., as in cabbage juice, this species may achieve a maximum cell population of more than 10^8 colony forming units (CFU)/ml after 12 to 14 h incubation.[16] *Lc. mesenteroides* produces lactic and acetic acids that quickly lower the pH. As a heterofermentative LAB, it produces carbon dioxide supporting the replacement of air and providing an anaerobic atmosphere favorable for the stabilization of vitamin C (ascorbic and dehydro-ascorbic acid) and the natural color of the cabbage. It has recently been shown that not only *Lc. mesenteroides*, but also strains of *Lc. fallax* are involved in this stage of fermentation.[17] Growth and fermentation patterns of these strains have been found highly similar to those of *Lc. mesenteroides*. Growth of *Lc. mesenteroides* and other leuconostocs is followed by growth of the heterofermentative species *Lactobacillus brevis,* which is more acid- and salt-tolerant than *Leuconostoc*. Depending on the temperature, the first two stages of sauerkraut fermentation are completed after 3 to 6 d. During that time, the concentration of lactic acid will increase up to approximately 1%.[15]
3. The third stage of fermentation starts with another shift in the lactic population; homofermentative lactobacilli become the predominant organisms mainly due to the combined effect of anaerobiosis, lowered pH, and elevated levels of salt. Among these, streptococci (most probably enterococci) and pediococci represent a minor component usually less than 10% of the total LAB population. The dominating homofermentative component consists of members of the former "subgenus" *Streptobacterium*. In the older literature, these are usually ascribed to a single species, namely *Lactobacillus plantarum* (formerly referred to as *Lactobacillus cucumeris*.)[18] Investigations in the 1980s revealed that *Lb. plantarum* comprises only 30 to 80% of the "streptobacteria," and that *Lb. sakei* and *Lb. curvatus* are also present in large numbers during this stage of sauerkraut fermentation.[19] The LAB convert the largest part of the available carbohydrates (glucose, fructose, and sucrose) to

organic acids, predominantly lactic acid. During the third stage of fermentation, the total acid content (calculated as lactic acid) will increase to 1.5 to 2.0%. Presently, most of the sauerkraut in Europe is unpacked and pasteurized when it reaches a pH of 3.8 to 4.1, because consumers increasingly prefer mild products, in terms of both acid and salt content.

4. Only fresh distributed, unpasteurized sauerkraut will undergo the final stage of fermentation, when *Lb. brevis,* and some other heterofermentative species able to metabolize pentoses such as arabinose and xylose, become dominant. Living plant material normally does not possess free pentoses. However, they are liberated after harvest as a result of acid-induced hydrolysis of hemicellulose.[20] Due to the increase of the total acid content up to 2.5%, the pH of the sauerkraut will decrease as low as pH 3.4.

14.3.2.5 Starter Cultures

Up to now, the majority of the sauerkraut produced in Europe and North America is still prepared by spontaneous fermentation. Due to current thinking about food security and product quality, modern approaches include the use of defined

TABLE 14.3
Traits Considered Relevant to Starter Cultures for Sauerkraut Fermentation

Criteria	Relevance
Technologically relevant criteria	
Rapid and predominant growth	Important
Salt tolerance	Advantageous
Acid production and tolerance	Important
Inability to metabolize organic acids	Important
Growth at low temperatures	Important
Formation of dextrans	Detrimental
Pectinolytic activities	Unacceptable
Formation of bacteriocins	Useful
Bacteriophage resistance	Not relevant
Sensorially relevant criteria	
Heterofermentative metabolism	Important
Formation of flavor precursors	Important
Nutritionally relevant criteria	
Reduction of nitrate and nitrite	Useful
Formation of L(+) lactic acid	Advantageous
Formation of biogenic amines	Unacceptable

Sources: Buckenhüskes, H.J., in *Food Microbiology—Fundamentals and Frontiers, 2nd ed.,* Doyle, M.P., Beuchat, L.R., and Montville, T.J., Eds., ASM Press, Washington, 2001, pp. 665–679; Lücke, F.-K., Brümmer, J.-M., Buckenhüskes, H., Garrido Fernandez, A., Rodrigo, M., and Smith, J.E., in *Processing and Quality of Foods, Vol. 2 Food Biotechnology: Avenues to Healthy and Nutritious Products*, Zeuthen, P., Cheftel, J.C., Eriksson, C., Gormley, T.R., Linko, P., and Paulus, K., Eds., Elsevier Applied Science, London, 1990, p. 11. With permission.

starter cultures for sauerkraut fermentation. Because it is not possible to eliminate the natural microflora of the shredded cabbage by appropriate methods, suitable strains should be highly competitive and should lead to a product of consistent quality. Traits considered relevant in starter cultures for sauerkraut fermentation are listed in Table 14.3. Experiments using strains of *Lb. plantarum* as a starter culture showed that this starter caused a rapid decrease of the pH; however, the resulting products showed a poor, unbalanced and less complex flavor due to the lack of different metabolites normally produced by other microorganisms. On the other hand, sauerkraut with excellent flavor characteristics was obtained using selected strains of *Lc. mesenteroides*.[21]

Use of selected starter cultures, and especially strains of *Lb. plantarum*, resulted in a significant reduction in the formation of biogenic amines, particularly tyramine, putrescine, and cadaverine.[22,23] In addition, Kalač et al.[23] also observed a significant reduction in the formation of acetic acid, ammonia, and alpha-amino groups during the fermentation.

14.4 COMPOSITION AND CHANGES AS A RESULT OF FERMENTATION

Cabbage consists of crude fibers, carbohydrates, proteins, lipids, and ash in relatively high proportions (see Table 14.4). The major change that takes place during fermentation is the conversion of the carbohydrates to lactic and acetic acids, ethyl alcohol, carbon dioxide, mannitol, and dextrans. The proteins, lipids, glucosides, and other constituents of cabbage are also affected by the fermentation process, resulting in alterations of the chemical and physical properties of the product.

The major changes during fermentation are indicated in Table 14.5 where some analytical data from the salted cabbage and from the product after 23 d of fermentation at 19°C are listed.

A completely fermented sauerkraut contains from 1.8 to 2.25% acid and, occasionally, total acidities of about 2.5% are attained. Lactic and acetic acids are the predominating acids and are normally formed in a ratio of 4:1. Other organic acids such as succinic, malic, and propionic acids may also be formed in much smaller quantities.[26] Ethanol and CO_2 are produced in variable amounts as a result of the metabolism of heterofermentative LAB.

Marked changes also occur in the lipid components of the cabbage including waxes, fats, and phospholipids, although they are present in minor amounts.[27,28] For instance, a high proportion of the acetone-soluble true fats are hydrolyzed to glycerol and free fatty acids, and phospholipids may be fermented to yield glycerol, free fatty acids, phosphates, and free choline.[29]

Volatile sulfur compounds such as hydrogen sulfide, methanethiol (methyl mercaptan), dimethyl sulfide, and allyl isothiocyanate can be detected in the headspace of sauerkraut and sauerkraut juice, and have a great impact on the flavor of

TABLE 14.4
Constituents of White Cabbage and Sauerkraut (average values)

Constituents	Dim	White cabbage	Sauerkraut
Main ingredients			
Water	g	90.4	90.7
Total nitrogen	g	0.22	0.24
Protein (N × 6.25)	g	1.37	1.52
Fat	g	0.20	0.31
Available carbohydrates	g	4.18	0.77
Total dietary fiber	g	2.96	2.14
Available organic acids	g	0.23	1.60
Minerals	g	0.66	2.35
Minerals and trace elements			
Sodium	mg	12	355
Potassium	mg	255	288
Magnesium	mg	14	14
Calcium	mg	45	48
Manganese	µg	200	140
Iron	µg	412	600
Copper	µg	33	130
Zinc	µg	224	320
Phosphorus	mg	36	43
Vitamins			
Retinol equivalent	µg	12	3
Total carotenoids	µg	69	18
β-carotene	µg	69	18
Vitamin K	µg	70	62
Vitamin B1	µg	43	27
Vitamin B2	µg	45	50
Nicotinamide	µg	320	170
Pantothenic acid	µg	260	230
Vitamin B6	µg	190	210
Folic acid	µg	31	31
Vitamin C	mg	48	20
Special carbohydrates			
Glucose	mg	2039	420
Fructose	mg	1762	210
Sucrose	mg	347	140

Source: Souci, S.W., Fachmann, W., and Kraut, H., *Food Composition and Nutrition Tables*, Medpharm Scientific Publishers, Stuttgart, 2000. With permission.

TABLE 14.5
Selected Analytical Data from Fermenting Sauerkraut on Days 1 and 23 of Fermentation at 19°C

Attribute	First day	23rd day
pH	6.18	3.41
Total acidity (titrated to pH 8.4; calculated as lactic acid)	0.38 g/kg	12.45 /kg
Lactic acid	0.09 g/kg	11.67 g/kg
D(–) Lactic acid	0.02 g/kg	8.77 g/kg
L(+) Lactic acid	0.07 g/kg	2.90 g/kg
Acetic acid	0.03 g/kg	3.07 g/kg
Reducing sugars	38.0 g/kg	5.8 g/kg
Dry matter	9.1%	8.3%
Sodium chloride	17.4 g/kg	17.5 g/kg

Source: Gail-Eller, R., Beitrag zur Kenntnis der Inhaltssoffe von Sauerkraut in Kleinbehältern, Hergestellt Nach Einem Neuen Verfahren, Diieratation, Hohenheim University, Stuttgart, Germany, 1984. With permission.

the sauerkraut.[30] A variety of additional volatile compounds, including carbonyls such as diacetyl and acetaldehyde, are produced by the bacteria, or by autochemical reactions or by the intrinsic enzymes of the fermenting cabbage itself.[31] Acetal, isoamylalcohol, *n*-hexanol, ethyl lactate, and *cis*-hex-3-ene-1-ol were among the substances identified as major volatiles in sauerkraut.[32]

White cabbage contains variable amounts of different glucosinolates.[33] After wounding the vegetable tissue, the glucosinolates are hydrolyzed by the enzyme myrosinase (E.C. Number 3.2.3.1), resulting, after some chemical reactions, in the formation of thiocyanates, which are responsible for the typical cabbage-like taste. During sauerkraut fermentation, the goal is to remove this taste, otherwise the sauerkraut is said to be green or immature. Gail-Eller and Gierschner.[30] have found that the hydrolysis of the glucosinolates is temperature dependent. At a fermentation temperature of 19°C, hydrolysis is completed within 3 d, whereas at 5°C it takes up to 8 d.

During the last centuries, sauerkraut was one of the major sources for vitamin C in the diet. From a technological point of view, the vitamin C content is a typical indicator for good manufacturing practice of sauerkraut, and it is important to stabilize the taste and the color of the final product as well.[34] Bohrer[34] investigated the influence of the different steps in sauerkraut processing on the vitamin C content. It could be demonstrated that all steps cause different losses in total vitamin C (sum of ascorbic and dehydro-ascorbic acid). Depending on the quality of the raw material and the processing parameters, the losses of vitamin C from the fresh cabbage to canned and pasteurized sauerkraut ranged from 13.2 to 52.7%.

Boskov Hansen et al.[35] investigated the effect of lactic acid fermentation of cabbage on the content and solubility of dietary fibers. In the fermented product, the content of total dietary fiber was higher than in the original cabbage, which can be

explained by the concentration increase due to the removal of cell liquid. The percentage composition of soluble and insoluble fibers was only slightly changed. However, it could be shown that changes in the fibers occur during fermentation. The solubility of the dietary fiber decreased within 7 d of fermentation. During further fermentation for 3 weeks, the solubility increased to the same percentage as in raw vegetables.

14.5 PRODUCT QUALITY AND FORMATION OF METABOLITES

14.5.1 Biogenic Amines

Some microorganisms associated with food fermentations may cause the formation of biogenic amines (BA) from free amino acids by the activity of amino acid decarboxylase enzymes. BA are low-molecular-weight organic bases; they are biologically active and may cause intoxications. The "classic" BA intoxication is caused by histamine, which at levels of ≥ 50 mg/100g may cause toxic effects or allergy-like symptoms such as sneezing, headache/migraine, shortness of breath, etc. Therefore, the presence of BA products of microbial metabolism in fermented and spoiled foods is important. Histamine production is more typically associated with Gram-negative bacteria such as the *Enterobacteriaceae*. However, these bacteria may only occur in significant numbers during the early stages of sauerkraut fermentation and are soon inhibited by the competing LAB and the concomitant reduction of pH (see Figure 14.4).

Taylor et al.[36] suggested that histamine production mainly occurs during the first fermentation phase. This explains why histamine is not generally considered as a typical major BA in sauerkraut. Still, its presence, together with that of putrescine, has been noted. The histamine concentration in sauerkraut has been reported to fall within the range of 9 to 200 mg/kg,[37,38] although typical amounts seem to range between 12 and 78 mg/kg.[39] Average values of 174, 146, and 50 mg/kg have been reported for tyramine, putrescine, and cadaverine, respectively, in household and

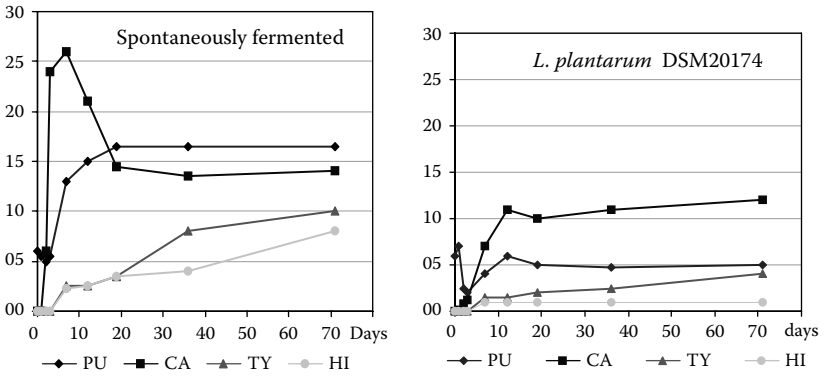

FIGURE 14.4 Biogenic amine formation (mg/100 g dry matter) during sauerkraut fermentation without starter culture and with *Lactobacillus plantarum* DSM20174. (From Halásaz, A., Baráth, ă ., and Holzapfel, W.H., The influence of starter culture selection on sauerkraut fermentation, *Z. Lebensm. Unters. Forsch.*, 208, 434–438, 1999. With permission.)

commercial sauerkraut from the Czech Republic and Austria, with the lowest concentrations in the household product.[40] Generally, BA concentrations are much higher in the brine than in the fermented cabbage,[22] although Kalač et al.[41] observed no differences. They also reported the highest levels for tyramine during sauerkraut storage, followed by putrescine and cadaverine.

It has clearly been shown that the concentration and type of BA in sauerkraut are influenced by fermentation conditions. Well-controlled fermentations followed by storage at 4°C resulted in reduced levels of cadaverine, putrescine, and spermidine,[22] whereas an increase in histamine appears to be associated with reduction of the pH below 3.8 to 3.6.[39] Moreover, it is known that only a small proportion of the food-associated bacteria may be able to produce toxicologically significant amounts of BA, and even within a species, strains may have different levels of decarboxylase activities. In an attempt to reduce BA production during sauerkraut fermentation, selected strains of *Lb. plantarum* with low amino acid decarboxylase activities, have been applied as starter cultures, and the generation of BA during fermentation was compared to that of spontaneously fermented cabbage.[22] In this way, the formation of all BA, and especially of cadaverine and putrescine, could be significantly reduced (see Figure 14.4). Starter culture concentrations necessary for this effect appear to amount to at least 5×10^6 CFU/100 g of cabbage.[22]

14.5.2 Bacteriophages

Bacteriophages do not appear to cause problems in sauerkraut fermentation. Pure-culture fermentations are not common, and if one or several LAB strains are infected by phages, other naturally occurring strains will become dominant and carry out the fermentation. Dissemination of bacteriophages within a fermentor is very limited, because the brine is normally not circulated in sauerkraut fermentations. However, dissemination may occur during the early stages of fermentation as long as carbon dioxide is produced by tissue respiration or microbial metabolism. The gas bubbles force their way to the surface and therefore enable transport through the fermenting substrate.

In a recent study by Yoon et al.,[9] nine different phages were isolated from commercial sauerkraut fermentations. They were characterized as members of the *Siphoviridae* and *Myoviridae* families and were found to be active against *Lc. mesenteroides* and *Lb. plantarum* strains isolated from the same product. Phages were also detected against a *Lc. mesenteroides* used as a starter culture in brine samples from the first day of inoculation. *Leuconostoc* phages reported in the literature are of the *Siphoviridae* family and were mainly of dairy origin. The diversity of phages indicates that they should have the potential to play a significant role in the ecology of "natural" sauerkraut fermentation.[9]

14.6 HEALTH PROPERTIES OF SAUERKRAUT

Sauerkraut is considered to be a healthy product as it is an important source of vitamins (especially vitamin C), mineral salts, and dietary fibers (see Table 14.4). In our modern Western diet, however, sauerkraut no longer plays an essential role as source of vitamin C.

The lactic acid produced during sauerkraut fermentation normally consists of both isomers, the L-(+) and D-(-) forms. There is still discussion about the importance of L-(+)- versus D-(-)-lactic acid. Because the majority of the lactate produced by the metabolism of mammals is of the L-(+) form, this isomer is called the physiological form. Whereas L-(+)- lactic acid is metabolized by a specific lactate dehydrogenase, the D-(-) isomer can only be used by a nonspecific D-2-hydroxy carbonic acid dehydrogenase. The oxidation rate of the non-specific enzyme is much lower than that of the lactate dehydrogenase, and it can be inhibited by L-(+)-lactate. This inhibition functions at all steps, which means that the intake to the liver, the transport within the kidneys, as well as the oxidation by D-2-hydroxy carbonic acid dehydrogenase will be inhibited. For a long time, it was assumed that large amounts of D-(-)-lactic acid ingested with foods would lead to a lactate acidosis. Today, it is agreed that the intake of D-(-)-lactic acid is not be a problem for healthy people. Only for babies in their first year it is recommended to exclude foods containing the D-(-) isomer from the diet.[42]

Despite these scientific opinions, there is still a need for products containing predominantly L-(+)-lactic acid, and therefore such products are offered by the industry. The investigation of facultatively heterofermentative lactobacilli in sauerkraut fermentation has led to the isolation of strains characterized by the exclusive formation of L-(+)-lactic acid. Originally, such strains were designated as a new species named *Lactobacillus bavaricus.*[43] However, this "species" was renamed *Lb. sakei* after a high DNA similarity of these strains was found with *Lb. sakei.*[44] The so-called L-(+) sauerkraut, which is distributed in health food stores, is produced by the application of such a racemase-defective strain of *Lb. sakei.* This starter culture is highly competitive and well adapted, and is able to suppress *Lc. mesenteroides*, which otherwise initiates the fermentation. *Lb. plantarum* strains are also outnumbered. Therefore, in sauerkraut freshly fermented by such L-(+)-lactate producing strains, the L-(+) isomer represents more than 90% of the total lactic acid. However, the flavor is poorly developed because of the suppression of the heterofermentative LAB.

A health-promoting effect of sauerkraut may be linked to the high content of glucosinolates (up to 1% of dry weight) of the white cabbage.[45] Glucosinolates undergo hydrolysis during fermentation[46] by the enzyme myrosinase. Some of the resulting metabolic products, including indoles and isothiocyanates are highly reactive compounds and were shown to be powerful inhibitors of carcinogenesis in laboratory animals.[47] Isothiocyanates are able to inhibit mitosis and stimulate apoptosis in human tumor cells,[48] and influence phase I and phase II biotransformation enzyme activities, thereby possibly influencing several processes related to chemical carcinogenesis e.g., the metabolism, DNA-binding, and mutagenic activity of promutagens.[49] One of the major underlying mechanisms appears to be the selective inhibition of cytochrome P450 enzymes involved in carcinogenic metabolic activation.[47]

Another health-promoting property of sauerkraut may result from the LAB involved in sauerkraut fermentation. As with other fermented products, unpasteurized sauerkraut contains high numbers of viable LAB, which may include organisms showing a beneficial effect on the intestinal ecosystem of the consumer. Up to now, however, there are no reports on the probiotic efficacy of typical sauerkraut LAB, and the health effects of these organisms still have to be demonstrated.

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15 New Trends of Table Olive Processing for Quality Control and Functional Proprieties

Moktar Hamdi

CONTENTS

15.1 INTRODUCTION

The olive tree, *Olea europea L.*, is the only species of the Oleacea with edible fruit. Cultivation began in the Mediterranean countries more than 6000 yr ago, was developed in Andalucia by Arabs, and then came to be introduced to the America continent. In the last decades, cultivations were promoted in Asia, Australia, and South Africa.

Among the 1500 olive cultivars catalogued in the world, only approximately 100 cultivars are classified as main producing varieties and according to use of their fruits: oil extraction, table olive processing, and dual-use cultivars. A number of olive cultivars are being cultivated in Mediterranean countries for processing as table olives (see Table 15.1).[1] Some Spanish and Italian cultivars such as Gordal

TABLE 15.1
Main Cultivars Used in Table Olive Processing

Country	Cultivar
Spain	Alorena,[a] Gordal Sevillana,[a] Hojiblanca, Mazanilla Cacerena, Manzanilla de Sevilla,[a] Morisca, Villalonga[a]
Italia	Ascolana,[a] Nocellara del Belice,[a] Nocellara Etnea, Sant' Agostino[a]
Greece	Chalkidiki,[a] Kalamon, Konservolia
Turkish	Ayvalik, Domat,[a] Memicik, Memeli
Tunisia	Meski,[a] Chetoui
Potugal	Carraquenha, Galega Vulgar
Morroco	Meslala,[a] Picholine Marocaine

[a] Used only for table olive. Others are dual-use cultivars.

Source: IOOC, *Catalogo Mundial de Variedades de Olivo*, Madrid: International Olive Oil Council, 2000.

Sevillana, Manzanilla de Sevilla, and Ascolana have been exported to the other countries (including Argentina, Australia, United States, and Israel) to produce table olives.

The practice of olive fermentation was started on a small scale and has developed into large-scale production processes. Table olives are among the most important fermented vegetable food produced in the world. Fermentation, which is a widely practiced, ensures the increased shelf life and microbiological safety of many foods. Lactic acid bacteria (LAB) because of their unique metabolic characteristics are involved in many fermentation processes of milk, meats, cereals, and vegetables. The fermentation process was developed to preserve foods at a low cost, instead of canning and freezing. Olives are consumed only after fermentation because it makes the olive more digestible, and reduces the bitterness and toxicity of phenols. Fermented olives contain vast quantities of food compounds in a wide diversity of flavors, aromas, and textures that enrich the human diet.

The world production of table olives is estimated to surpass 1.5 million tons per year, with the Mediterranean countries being the main producers. There has been an increased demand for fermented green and black table olives in recent years in all regions of the world because their nutritional and functional foods proprieties. The International Olive Oil Council (IOOC) statistical data for 1989–1990 and 2000–2001 show that the production of table olives increased in the majority of countries during the last decade (see Figure 15.1). [2] Spain and Turkey are the main producers of green olives and naturally black olives, respectively.

15.2 OLIVE FRUIT QUALITY AND STORAGE

The quality (nutritional, functional and technological potential) of the olive fruit is influenced by the cultivar, the region of production, the degree of drupe maturation, and postharvest conditions.

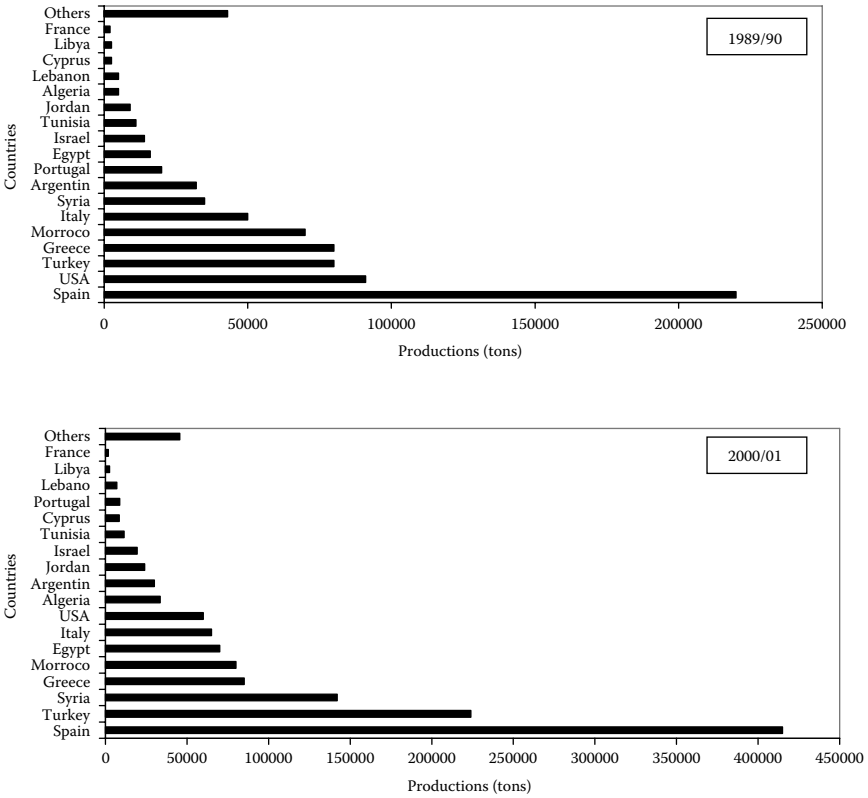

FIGURE 15.1 World production of table olives evolution from season 1989/90 to season 2000/01. (From IOOC, (http://www.fao.org/docrep/006/y4890e/y4890e0h.htm), 2001.)

15.2.1 Olive Fruit Composition

The olive tree produces an oval fruit that is a fleshy green drupe. The olive fruit consists of a pulp and a stone representing 70 to 90% of the olive weight, and the pit another 10 to 30% (see Table 15.2).[3,4] The pulp consists mainly of oil (10 to 25%) and water (60 to 75%). The oil fraction includes mainly triglycerides, diglycerides, monoglycerides, free fatty acids, sterol esters, terpenes alcohols, and phospholipids.

The ash in olives is relatively low, between 0.6 and 1%, and includes minerals especially potassium, calcium, phosphorus, sodium, and magnesium. The olive fruit texture is attributed to the presence of the fiber fraction (1 to 4%)[5] and the pectic substances (0.3 to 0.6%).[6] Sugars and polyols represent 20% of the fresh pulp weight. Free sugars including mainly glucose, fructose and sucrose range from 3 to 4%, and they are very important for olive fermentation. The main organic acids present in the pulp (0.5 to 1%) are oxalic acid, malic acid, and citric acids. The protein fraction

TABLE 15.2
Major Components of Olive Fruit and Table Olives

Major components (%)	Fruit olive	Spanish-style green olives	Californian-style black olives	Greek-style black olives
Moisture	60–75	69	69	60
Lipids	10–25	21	21	23
Fiber	1–4	1.4	2.4	2.2
Proteins	1–3	1.0	1.1	1.2
Ash	0.6–1	4.6	2.0	6.7
Sodium	0.008	1.435	0.634	3.740
Cacium	0.051	0.054	0.068	0.028
Potassium	0.283	0.044	0.012	0.029

Source: Fernández Dıéz, M.J., de Castro Ramos, R., Garrido Fernández, A., González Cancho, F., González Pellissó, F., Nosti Vega, M., Heredia Moreno, A., Mínguez Mosquera, M.I., Rejano Navarro, L., Durán Quintana, M.C., Sánchez Roldán, F., García García, P., Castro Gómez-Millán, A., in Biotecnología de la Aceituna de Mesa, Consejo Superior de Investigaciones Científica-Instituto de la Grasa, Madrid-Sevilla, 1985; Rejano, L., El aderezo de lasaceitunas, in *El Cultivo Delolivio,* Barranco, D., Fernandez-Escolar, R., Rallo, L., Eds., Junta de Andaluciaand Ediciones Mundi-Prensa, Madrid, 1977, pp. 565–586.

is low and can vary between 1 and 3%. Hassapidou et al.[7] reported the presence of vitamins typically found in edible olives and olive oil. The green color of olives is attributed to chlorophylls (1.8 to 13.5 mg/100g fresh pulp) and carotenoid pigments (0.6 to 2.4 mg/100g fresh pulp).[8,9] The variations in concentrations of most nutrients are influenced by the type of cultivar, the growing conditions, and the degree of ripeness. During the ripening process, the ratio between chlorophylls and carotenoids changes because the chlorophylls decrease. In the development of the olive fruit, three phases are usually distinguished:[10] a growth phase, during which accumulation of oleuropein occurs, a green maturation phase, coinciding with a reduction in the levels of chlorophyll and oleuropein, and a black maturation phase, characterized by the appearance of anthocyanins, and during which the oleuropein levels continue to fall.

The phenolic compound content in olive fruit can vary between 1 and 2% and is represented mainly by the oleuropein. The bitter taste of olives is largely ascribed to the content of oleuropein.[11,12] During maturation, oleuropein is partially converted into demethyloleuropein which becomes the major phenol in black olives.[13] Saija and Ucelle[14] summarized the phenolic compounds structures, which range from quite simple compounds to highly polymerized substances such as tannins.

A pulp-to-stone ratio of olive fruit of approximately 6, and the maturation degree of drupe, are the two main technological requirements for table olive processing.

15.2.2 Postharvest Alterations of Olive and Storage Control

Olive fruit are harvested mature-green or black, depending on the cultivar and the processing method. The harvested olives need to be stored and transported by methods that minimize physical damage, and chemical and microbiological contamination. A

great deal of variation on storability between cultivars has been observed in the same growing area. For example, fresh Chondrolia green olives were very sensitive to chilling injury. They lost their capacity to develop skin color and ripen after 2 to 4 weeks of cold storage with excessive internal browning, resulting in pitting and external discoloration.[15] Mature green olives are chilling sensitive when kept long enough at temperatures below 5°C, whereas the fruit of some cultivars can be damaged at temperatures as high as 10°C.[16,17] The main chilling injury symptoms of olives include internal browning of the flesh around the pit, pitting appearing as dull skin color and, progressively, external browning.[18] In the altered zone of the olive, the surrounding components of pigments were affected, with the degeneration of proteins and phospholipids forming pigment-lipoprotein complexes.[19]

Microbial invasion of fruit tissue by bacteria and fungi can occur when storage conditions are favorable. The postharvest handling can produce breaks in the tissue allowing microorganisms to enter and affect the fruit quality. Specific species of microorganisms are able to grow in the olive fruit and may cause spoilage and poisoning. El Adlouni et al.[20] reported that *Aspergillus* and/or *Penicillium* are able to develop on olive and produce ochratoxin A and/or citrinin and/or aflatoxin B after harvest, and during drying and storage of olives. Aflatoxigenic strains inoculated onto fresh damaged black olives and fresh black olive paste grew extensively, but on fresh whole black olives, they grew less well. Strong aflatoxigenic strains produced low levels of aflatoxins on fresh whole black olives, fresh damaged black olives, and fresh black olive paste.[21]

The quality of the fermented olives depends on the skin color and flesh firmness of the raw product at the time of processing. Several studies of the effects of controlled atmosphere during low temperature storage on olive fruit have been done to improve olive storage, and allow for an extension of the processing period using the Spanish method for fresh green olives.

Reduced O_2, increased CO_2 levels, or their combinations in the storage room atmosphere can inhibit chlorophyll degradation, color development, and loss of flesh firmness.[18]

Green olives (Olea europaea L. cvs. Conservolea and Chondrolia), destined for Spanish-style processing, were harvested at the beginning and the end of commercial harvest period, and stored at 5°C and 7.5°C in air or various controlled atmospheres. Experiments showed that Conservolea green olives can be stored up to 37 days at 5°C in air or for up to 22 d at 7.5 °C and 2 kPa O_2 plus 5 kPa CO_2.[15] Panagou[22] reported that packing the fruits in a high carbon dioxide atmosphere or dipping then in a potassium sorbate solution prior to packing, in combination with low storage temperature (4°C), can effectively inhibit yeast and fungal growth during long-term storage.

Dourtouglou et al.[23] showed that postharvest storage of olives under a CO_2 atmosphere for a period of 12 d resulted in color and flavor development and reduced bitterness. The gradual loss of bitterness observed during storage under CO_2 may be due to oleuropein decomposition. The antioxidant characteristics were lower in olives stored under air than under CO_2 atmosphere, which is an indication that the functional properties of olives may be enhanced after CO_2 storage.

15.3 TABLE OLIVE PROCESSING AND FERMENTATION TECHNOLOGY IMPROVEMENT

The fermentation process design should take into account specific biological and technological constraints, and minimize the cost during production and downstream operations. All fermented foods are made in fundamentally the same way with three steps: pretreatment of raw material, fermentation, and product recovery. Table olive are prepared by three processing methods: the Greek, the Spanish, or the Californian style (see Figure 15.2). The olive fruits are harvested at different degrees of ripeness,

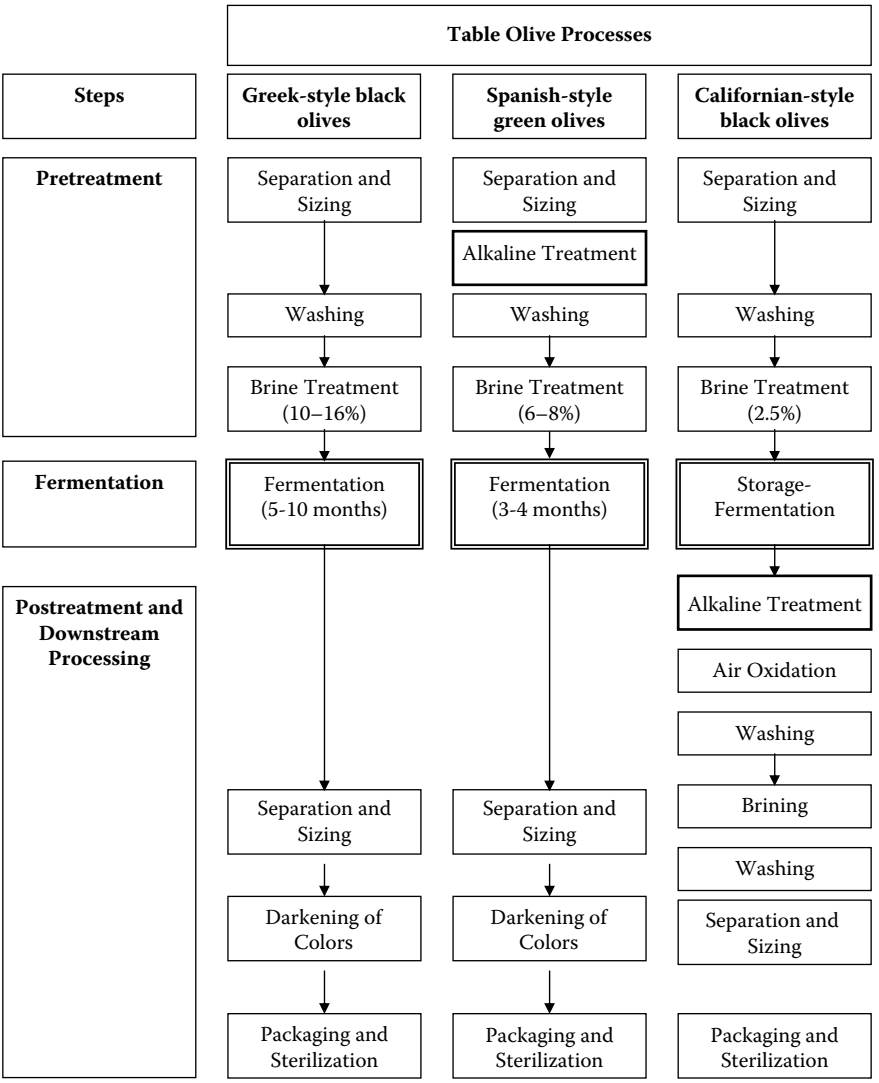

FIGURE 15.2 Flow sheet of table olives preparation according the Greek, the Spanish and the Californian styles.

yellowish-green, turning color, and black for Spanish style, Californian style and Greek style, respectively. The harvesting period of green olives is short, followed by storage in brine before final processing. Olives destined for Californian and Greek style processing are harvested mature, when skin color is not important.

The Greek style method of treatment is mild, and includes washing, natural fermentation in brine, air oxidation for color improvement, sizing, and packing. The Spanish processing method includes treatment with sodium hydroxide solution, for the total removal of the bitter compound oleuropein, washing, brining and fermentation, sorting and size grading, and packaging. The Californian method of treatment includes lye treatment, washing, iron salt treatment and air oxidation, washing, sizing, canning, and sterilization. All these procedures result in a decrease of the total amount of phenols. The typical traditional method known as "naturally black dry salted olives" is based on the fermentation of olives under high osmotic pressure (40 g/100 g) for 40 to 60 d.[22]

The use of a suitable pretreatment of the olive fruit and the control of the fermentation microbial flora (LAB and yeast) in the olive fermentation is the modern way to produce fermented olives on an industrial scale.

15.3.1 Pretreatment of Olives

The choice of suitable operating conditions for the pretreatment of table olives must take into account the microbial flora used, the quality of the olive fruit, and the desired final table olive composition. The majority of studies of pretreatment of the green olives have been carried out to reduce the olive fermentation duration, and to improve the final quality of the fermented olives.

The Spanish style and Californian style processes include pretreatment with lye, which hydrolyzes the bitter glycoside oleuropein and increases the permeability of the olive skin, resulting in the efflux of flesh nutrients into the surrounding liquid.[24]

Rejano[4] reported the characteristics of the alkaline treatment (2 to 3.8% NaOH during 6 to 11 h), the first washing (2 to 5h), and the second washing (12 to 16h), for the main cultivars used in Spain. The degree of penetration of NaOH through the olive fruits—which varies from two-thirds to three-quarters of the distance between the skin and the stone of the fruit—depends on the variety.

The study of the effect of pretreatment with lye at different concentrations (1.0, 2.0, and 2.5%) on some physico-chemical changes of green table olive Azizi cultivar during the fermentation process indicated that lye treatment at 1% and 2% using sodium hydroxide were the more suitable concentrations for processing the green table olive Azizi cultivar under Egyptian conditions, where the quality in texture, color, flavor, and appearance were recorded. A positive correlation was found between the physico-chemical properties and the sensorial properties of pickled olives.[25]

Alkaline aerobic oxidation, during ripe olive processing of the Intosso cultivar (Italy), induced significant changes in the total phenolic concentration. Oleuropein aglycones diminished considerably, whereas tyrosol and hydroxytyrosol increased markedly.[26]

Green olives of the Tunisian variety Meski were treated according to a Spanish-style green olive process by using an alkaline treatment (1.5, 2, and 2.5% [w/v] NaOH) to eliminate bitterness, combined with different brine concentrations (6, 9,

and 12% [w/v] NaCl). Results showed that 2% NaOH solution and 9% sodium chloride brine was an optimal combination inducing the best growth of *Lactobacillus* species (10^8 colony-forming units [cfu]/ml), and acidity of 0.726 g lactic acid/100 ml brine.[27]

New types of pretreatment of olives designed to improve the fermentation step and the product's shelf life are under investigation. Thermal treatment eliminates the background and competitive flora, and make olives more fermentable. However, sterilization of the brine resulted in the decrease of volatile compounds, whereas pasteurization did not lead to significant changes in the volatiles.[28]

Green table olives (Greek cultivar Conservolea), unheated and pasteurized, were supplemented with glucose or sucrose in various amounts, and inoculated with *Lactobacillus plantarum*.[29] This fermentation study showed that sugar supplements did not affect the LAB growth rate, but they increased the rate of pH drop and the production of acids. Adequate supplements of sugars (0.5%, 1.0% w / v), of both types, in unheated olive fermentation, resulted in a fast pH drop, causing a satisfactory halt of Enterobacteriaceae growth in the first days of fermentation, and a subsequent population decline occurred in the following days, eliminating the danger of early stage spoilage, and ensuring the safety of the final product. In pasteurized olives where *Lb. plantarum* starter was the only microbial flora, it was considered worth evaluating the fermentation progress without any competition.

The hydrolysis of the bitter compound oleuropein, either by miroorganisms or acids needs further study. The selected strain of *Lb. pentosus* 1MO allowed the reduction of the debittering phase period to 8 d.[30] Postharvest storage of olives under a CO_2 atmosphere for a period of 12 d resulted in color and flavor development, and reduced bitterness to provide a natural debittering without use of chemicals (e.g., alkaline solutions, brine). Further investigation for the development of table olive processing that will enable fast olive debittering with minimal environmental impact is required.[23]

15.3.2 Brining of Olives and Fermentation Control

The knowledge of microorganisms involved in olive fermentation is crucial in to order define a procedure to control the fermentation and the safety of final product. In the green table olive process, the removal of bitterness by alkaline treatment, and the resulting residual sodium hydroxide occurs before fermentation. Although in the Greek-style black olive, the fruits are directly placed in brine for fermentation, which contributes to a partial removal of bitterness. LAB dominate in the brine of green olives, whereas mainly fermentative yeasts are found in the brines of black ones. *Saccharomyces cerevisiae* and *Candida boidinii* were the most frequently identified species in green seasoned olives and processed black olives, respectively.[31]

15.3.2.1 Green Table Olive Fermentation (Spanish style)

The washed olives are placed in a sodium chloride solution (8 to 10%), which decreases in concentration to 5 to 6% as the salt penetrates in the fruit. The three first days of brining corresponds to the first step, which starts at pH 10 because the residual lye comes out of the pulp. The soluble compounds of the fruit pass by osmosis into the brine and serve as substrates for growth of the main active bacteria

(enterobacteriaceae, cocaceae LAB, and sporulated gram positives) and are responsible for the decrease of the pH to 6. Concentration of soluble nutrients, mainly the sugars, in the brine controls the fermentation rate, because it is dependent on the rate of diffusion of the sugars from the olive flesh into the brine. The second step begins after 3 d, and lasts 15 to 20 d. Lactic acid fermentation commences spontaneously in the brine because of the suitable environment (NaCl presence and low oxygen tension) for the selection and subsequent dominance of cocaceae lactic acid bacteria, mainly *Pedicoccus* and *Leuconostoc*. The pH drops to 4.5 and produces a decrease in the Gram-negative bacteria counts. The third step lasts 30 to 60 d when the pH becomes less than 4. The *Lb. plantarum* population generally coexists with a yeast population until the end of the fermentation process and during storage.[32,33,34]

Among the LAB of the natural flora, *Lb. plantarum* is the predominant microorganism for successful green olive fermentation.[35,24,27] LAB consume the sugars of the fruit, which are present in brine and produce mainly lactic acid, and to a lesser extent other acids, causing the pH drop to 3.9. The developed acidity and pH drop is the determining factor for the success of fermentation, and safety of the final product as suppression of both spoilage and pathogenic microorganisms takes place.[36]

In some cases, lactic acid is not produced in the amounts needed for the adequate fermentation of olives, and spoilage occurs through subsequent contamination by other microorganisms.[37,38] Some alterations of green table olives caused by undesirable bacteria are summarized in Table 15.3.

15.3.2.2 Natural Black Table Olive Fermentation (Greek style)

High salt fermentation (HSF) is usually employed in Turkey and Greece, where the salt concentration of the brine is between 10 and 14%.[39] Growth of spoilage bacteria is controlled mainly with the high salt concentration, which favors the growth of yeasts rather than LAB. However, when the concentration of sodium chloride is too high, the olives become wrinkled; this phenomenon may be irreversible and detrimental to the final product quality.

TABLE 15.3
Main Microbial Alterations during Green Table Olive Processing

Alterations	Microbial agents/products	Remedies
Fish-eye and gas-pocket	Gram-negative bacteria/CO_2-H_2	Acidification by acetic acid
Butyric alteration	*Clostridium*/butyric acid	Good practice of hygiene and raising NaCl higher than 5%
Zapateria (unplesant smell)	*Clostridium* and *Propionibacterium*	Raising NaCl and keeping pH below 4.2
Softening (pocked)	Bacillus, yeasts, and molds	Maintaining good anaerobic
Sedimentand gas (pocked)	Bacteria and yeasts	Heat treatment as pasteurization

Source: Fernández Díez, M.J., Olives, in *Biotechnology: Food and Feed Production with Microorganisms*, Rehm, H.-J., and Reed, G., Eds., Verlag, FL, 1983, pp. 379–397; Garrido Fernández, A., García García, P., and Brenes Balbuena, M., Olive fermentations, in *Biotechnology: Enzymes, Biomass, Food and Feed*, Rehm, H.-J., and Reed, G., Eds., VCH, New York, 1995, pp. 593–627.

HSF takes 9 to 10 months due to the slow leaching out of soluble components such as sugars and oleuropein into the brine. The final pH is stabilized between 4.5 and 5 depending on the variety. Citric, malic, and tartaric acids were the major organic acids accumulating in the brine during fermentation.[40]

During fermentation of naturally black olives, the numbers of Enterobacteriaceae and *Pseudomonas* spp. in the brine decreased, whereas LAB and yeast populations increased. The pH is not reduced to the desired level because of the inhibition of LAB growth by the salt and polyphenols, and by the low sugar content of the brine. Polyphenols have a more inhibitory effect on the LAB than yeasts.[39]

A molecular approach used for the study of yeasts associated with table olives allowed the identification of three yeast species *(Issatchenkia occidentalis*, *Geotrichum candidum,* and *Hanseniaspora guilliermondii*) that had not been described previously.[31]

The acid hydrolysis of oleuropein and the polymerization of anthocyanins pigments are the main changes observed in the Greek-style black olives.[41,42]

After 4 months of preservation in brine, the oleuropein content decreased markedly, because of bacterial metabolism of the fermenting brine. Vanillic acid and flavonoids, contents (which were low in fresh olives), disappeared completely, whereas tyrosol increased, showing the same trend as hydroxytyrosol.[26]

15.3.2.3 Improvement of Table Olive Fermentation

The production of the fermented foods is based on microbial activity that induces beneficial changes and produces flavor ingredients that give the final product its distinctive taste. The approach to improve a fermented olive is based on the control of the olive fruit composition, and the fermentation conditions to produce the required quality of the final table olive (see Figure 15.3).

Some research has been conducted to improve fermentation kinetics and to control the quality of table olive product. To overcome all of these problems, several solutions have been proposed, such as the use of a starter (*Lb. plantarum, Lb. pentosus,*

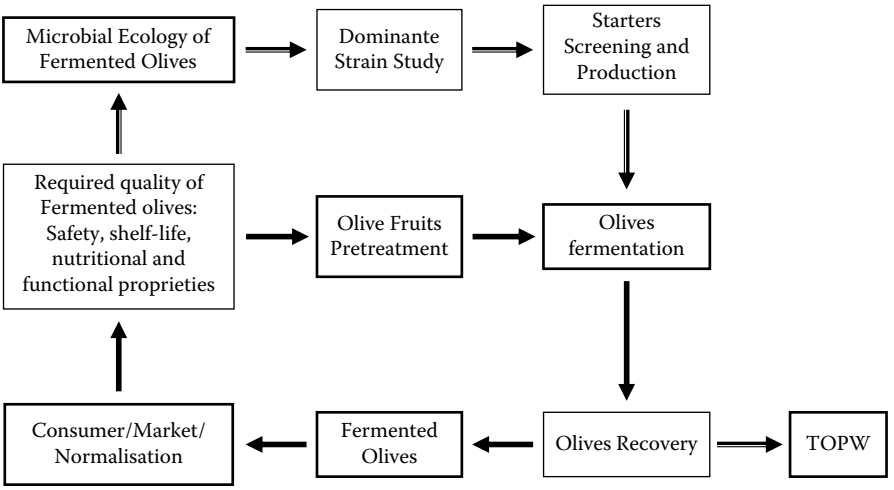

FIGURE 15.3 Cycle development of fermented olives.

Enterococcus casseliflavous and bacteriocin–producing strains), addition of sugars, an extra salt supplement, and the acidification of the brine.[43,34,44,4]

Inoculation is strongly advisable to control all stages of the fermentation, and reduce the risks of microbial alterations. Chammem et al.[27] reported the decrease in the cell count of the *Lb. plantarum* starter culture just after inoculation. The total number of the lactobacilli in the inoculated fermentors was similar to that in the spontaneous process, and similar to those found in previous studies.[34,43–45]

Inoculation with the *Lb. plantarum* starter culture leads to a faster pH decrease in green table olive processing compared to the spontaneous one, and this may help to reduce the risk of spoilage during the first days of fermentation.[24]

Leal-Sánchez et al.[47] indicated that Spanish green table olives could be improved by using *Lb. plantarum* LPCO10 as a starter culture at 10^7 cfu/ml of brine, and well defined starting brine parameters, i.e., 4% w/v of NaCl, and initial pH correction with acetic acid, with inoculation to take place 1 to 4 d after brining.

Green olives were fermented with starter cultures of *Enterococcus casseliflavus* cc45 and *Lb. pentosus* CECT 5138. This is the first report dealing with the presence of *E. casseliflavus* in table olive brines and their utilization as a starter culture.[45]

A strain of *Lb. pentosus* was also selected and used as a starter to ferment, on a pilot plant scale, black olives (Itrana and Leccino cv.) at 28°C. The temperature-controlled fermentation of Leccino cv. olives resulted in obtaining ready-to-eat, high quality table olives in a reduced-time process. However, these fermented olives showed a decrease of oleuropein and an increase of the hydroxytyrosol concentration.[30]

Different initial brining conditions on the spontaneous fermentation of cv. Conservolea green olives including (a) brine acidification with 2% (v/v) lactic acid (serving as a control), (b) addition of 25% (v/v) 1 N HCl (c) substitution of the initial brine by 20% (v/v) with a brine from a previous fermentation (brine reuse) were investigated.[48] The brine reuse process was the most effective in minimizing the likelihood of spoilage, as it decreased the survival period of enterobacteria (24 d), followed by the HCl treatment (28 d) and the control (35 d). All supplementary treatments were effective in reducing the pH to a final value of 4.3 to 4.5. However, glucose increased the final concentration of lactic acid in the brine reuse and HCl-treated processes (73.4 and 67.8 mM, respectively) compared with the control that was lacking in acidity (44.7 mM), denoting a clear advantage of glucose over lactic acid as a supplement.[48]

15.3.3 Table Olives Recovery and Storage

The fermented olives are sized graded after separation of defective fruits. Olive fermentation increases the palatability of the fruit. Typical compositions of the different styles of table olives were summarized by Garrido-Fernández et al.[24] (see Table 15.2). The main changes that occurred after olive processing are the increase of ash caused by the alkaline treatment and the brining, and the decrease of carbohydrates and phenolic compounds that have diffused into the brine. Average values of water activity were 0.976, 0.977, and 0.990 for green, directly brined, and ripe (by alkaline oxidation) olives, respectively. Mean values of the pHs were 3.69, 3.92, and 6.52, whereas salt levels were 5.53, 4.98, and 2.55 for the same commercial products.[49]

Moisture content of the black processed olives was in the range 49.58 to 50.79 g/100 g. The water activity (a_w) value of olives fermented with the high salt brine was found to be 0.947, whereas a_w values of olives fermented with the low salt brine were 0.962 to 0.964. Protein content of the olives ranged between 1.76 to 1.95 g/100 g and the ash contents were found to be 2.41 to 2.81 g/100 g in olives with low salt brine and 4.70 g/100 g in olives with high salt brine.[39]

The established quality regulations for Spanish-style green olives require that table olives must be packed in solution with the characteristics: pH 3.2 to 4.1, free acidity 0.4 to 0.6, NaCl 5 to 7% and residual lye 0.02 to 0.07N.

Some spoilage microorganisms occur during storage, increasing the microbial and chemical contamination. Caggia et al.[50] showed that *L. monocytogenes* can survive and grow in green table olives prepared by both the Spanish-style and biological methods, and fermented with starter cultures of LAB (*Lb. plantarum, Lb. casei,* and *Lb. pentosus*). Panagou et al.[51] reported that fungus *Monascus ruber van Tieghem* (the main spoilage microorganism during storage of table olives) is salt- and acid-tolerant, being able to grow at NaCl concentrations of up to 9% (wt/vol) and pH values of as low as 2.2, depending on the incubation temperature.

Contents of biogenic amines in Spanish-style green or stored olives increased throughout the brining period. Putrescine and cadaverine concentrations in directly brined olives were markedly lower than contents in Spanish-style olives.[52]

Garcia et al.[53] reported that the formation of biogenic amines (cadaverine and tyramine), which only occurs during storage and might be related to zapatera spoilage, is affected by temperature and the type and time span of the debittering process.

Packaging that makes table olives more attractive and convenient for use ensures greater safety of the food against biological and chemical changes, and can result in longer shelf life. Montano et al.[43] studied that the kinetics of ascorbic acid (AA) loss during storage of both pasteurized and unpasteurized packed table olives with two different levels of added AA. The effect of pasteurization treatment alone on added AA was not significant in the unpasteurized product; AA was not detected after 20 d in samples stored at room temperature.

Research about possible contamination of table olives with components or degradation products of plastic packaging materials have not been reported.

15.3.4 Treatment of Table Olive Processing Waste Waters (TOPW)

The table olive processing wastewaters (TOPW) constitute a major environmental problem due to their high organic load and the large volumes generated. TOPW are unsuitable for disposal at municipal or industrial wastewater treatment plants due to its high organic and phenol content. Typical flow rates of TOPW are 3.9 to 7.5 m^3 and 0.9 to 1.9 m^3 per ton of processed green olives and black olives, respectively. The TOPW have a pH 3.6 to 13.2, suspended solids 0.03 to 0.4 g/l, dissolved solids 0.2 to 80 g/l, a biological oxygen demand in 5 days (BOD_5) 0.1 to 6.6 g/l, a chemical oxygen demand (COD) 0.3 to 18.2 g/l and a sodium chloride content 0.17 to 80 g/l. The large fluctuations in these characteristics are caused by variations in water consumption, the factory capacity, the clarity and ripeness of the olive preparation conditions.[54] In most cases, these wastewaters are dumped into the environment untreated. In other

cases, the most common treatment is to retain them in evaporation ponds, which cause undesirable smells and the possibility of polluting surface and ground waters. Garrido et al.[55] showed that the recycling of such brines, after treatment with both 0.6% (w/v) of activated carbon and ultrafiltration through a polysulphone membrane of 1000 daltons pore size with minor composition adjustments, can be added to the solution employed for packing the final product. This recycling could eliminate about 80% of the total pollution and facilitate treatment of the remaining wastewater.

Some research has reported innovative chemical and biological technologies to reduce the pollution caused by TOPW.

15.4 FUNCTIONAL PROPERTIES OF TABLE OLIVES

Table olives are a traditional fermented functional food, and one of the most important components of the Mediterranean diet. The benefit of olive oil has been under investigation for many years. However, table olives have not been investigated as extensively. Table olives are an essential source of linoleic acid and monounsaturated fatty acids, both of which have a high biological and nutritive value. The benefit of the olive products is the absence of the saturated fatty acids. In addition to monounsaturated fatty acids, polyphenols, chlorophylls, and carotenoids contribute to the nutritional benefits and biological functions of table olives.

Olives (*Olea europaea*) contribute to the daily intake of nutritional antioxidants because they contain an array of polyphenolic phytochemicals, including various hydroxytyrosol derivatives (e.g., oleuropein) and flavone glycosides.[14,13] The consumption of table olives in combination with the consumption of olive oil provide a large intake of natural antioxidants in countries where these products are common in the diet as compared to the 23 and 28 mg of flavones and flavanones intake per day in the Netherlands and Denmark, respectively, and of 115 mg per day for the United States, as reviewed by Ross and Kasum.[56] Recently, Bouskou et al.[57] mentioned that the consumption of table olives is considered to offer a high intake of antioxidants, mainly polyphenols, and so provide a health benefit for the prevention of many chronic diseases. The phenolic fraction of table olives is very complex (Table 15.4)[57] and can vary both in the quality and quantity of phenolic compounds.[58] It is dependent upon the processing method,[42] upon the cultivar,[59] upon irrigation regimes, and upon the degree of drupe maturation.[60] The most important changes in the phenolic fraction are due to the depletion of oleuropein during the olive fruit development and the increase in concentration of tyrosol and hydroxytysol.[61,62,63,30] The major phenolic compounds present in table olives are tyrosol, hydroxytyrosol and oleanolic acid and the concentration of these compounds is dependent upon the degree of maturation and the method of treatment of olive drupe until they become edible.[64,65,42,13] The concentration of phenolic compounds decreased slightly during the fermentation process, particularly hydroxytyrosol, which was found in high concentration in waste waters.[66] The sterilization step did not change the tyrosol and the hydroxytyrosol contents of the processed olives.[26]

The total phenolic content of table olives is made up of different polyphenols in different quantities that varies between types of olives. In general, about 5 to 10 table olives supply the daily intake of polyphenols. An analysis carried out on

TABLE 15.4
Major Phenols and Phenolic Acids of Greek Table Olives (Cultivars: Kalamon and Tsakistes)

	Kalamon		Tsakistes	
Polyphenol	**Kernel**	**Flesh**	**Kernel**	**Flesh**
Cinnamic acid	2	5	4	2
Tyrosol	14	22	21	14
p-Hydroxy-benzoic acid	0.4	0.6	0.9	0.4
p-Hydroxy-phenyl-acetic acid	0.9	0.9	6	3
p-Hydroxy-phenyl-propanoic acid	7	8	6	3
Vanillic acid	0.2	0.2	0.3	0.2
Hydroxy-tyrosol	39	81	114	61
Protocatechuic acid	1	1	2	0.5
3,4-Dihydroxy–phenyl-acetic acid	0.2	0.6	10	4
p-Coumaric acid	0.2	nd	0.7	0.2
Ferulic acid	nd	nd	0.4	nd
Caffeic acid	0.6	1	4	nd
Oleanolic acid	12	nd	25	nd

Note: Expressed in mg of each polyphenol per 100 g of flesh or kernel (mg/100 g).

Source: Boskou, G., Fotini, N., Chrysostomou, S.S., Mylona, A., Chiou, A., and Andrikopoulos, N.K., Antioxidant capacity and phenolic profile of table olives from the Greek market, *Food Chem.*, 94, 558–564, 2006.

48 samples showed that olives with changed color in brine had the highest concentration in polyphenols (approximately 1200 mg/kg), whereas oxidized olives had the lowest (approximately 200 mg/kg).[42]

Owen et al.[65] investigated the antioxidant capacity of two Italian brined olive drupe varieties, (one black and one green) and showed that black olives, which contain higher concentrations of phenolic compounds, present higher antioxidant activity compared to green olive extract.

As mentioned by Bouskou et al. [57] table olives are a good source of antioxidants, mainly polyphenols, which protect the body tissues against oxidative stress. Polyphenol intake is beneficial for human health because their antioxidant activity has been associated with a lower risk of coronary heart disease, preventing some types of cancer, reduce inflammation, and inhibiting platelet-activating factor activity.[67] Soni et al.[68] showed that the consumption of standardized aqueous olive pulp extract is considered safe at levels up to 20mg/kg/d. Among the polyphenols found in the extract, the major constituent of biological significance is hydroxytyrosol (50 to 70%).

The concentration of provitamin A carotenoids in table olives was determined for nutritional labelling or for use by nutritionists to estimate provitamin A intakes in diets that include table olives. Only β-carotene was found in all commercial products. Concentrations of β-carotene, ranged from an average of 197 to 1,387 mg in green olives, from 37 to 726 mg in directly brined olives, and from 39 to 333 in ripe (darkened by oxidation) olives per 100 g edible portion.[6]

With the aim of developing new functional foods, the table olive was used as a vehicle for incorporating probiotic bacterial species. Survival studies in table olives of *Lactobacillus rhamnosus*, *Lb. paracasei*, *Bifidobacterium bifidum*, and *B. longum*, demonstrated that bifidobacteria and one strain of *Lb. rhamnosus* showed a good survival rate at room temperature.[70]

The depolymerisation of phenolic compounds and their conversion by *Lb. plantarum*[71,72,73] should be an interesting way to improve the functional proprieties of olives and related products. In fact, it has been found that the higher the molar mass of tannin molecules, the stronger are the antinutritional effects and the lower the biological activities.[74]

Recently, several methods have been proposed for the improvement of the nutritional value of various plant food commodities by increasing their content of biologically active polyphenolic phytochemicals. The improvement of the functional properties of the table olive requires a new approach to control olive harvesting, processing, and storage. Saija and Ucelle[14] suggested that an understanding is important of olive growing and processing technologies, and the behavior and measurement of macro- and micro-, bio- and techno-components of extra virgin olive oil and table olives after the raw material has been subjected to appropriate harvesting, milling, malaxation, extraction and debittering treatments, in order to communicate the hedonic sensory quality and functional quality of olives to consumers.

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16 Traditional Chinese Fermented Foods

Y-H. Peggy Hsieh, Steven Pao, and Jiangrong Li

CONTENTS

16.1 INTRODUCTION

China, with its population of 1.3 billion, is one of the world's largest food producers and consumers. Long before the science of microbiology became established, the fermentation process was already being used in China for food manufacturing and preservation. It is believed that the region's people long ago discovered that some food materials under certain conditions of storage could develop special aromas, pleasing flavors, and different textural characteristics. These changes not only satisfied sensory desires, but also stabilized the shelf life of food or even offered some health benefits. Over time, these processes were recorded, repeatedly practiced, and refined to produce a variety of foods that we today call fermented foods.

Traditional or indigenous fermented foods and beverages have been popular since the earliest recorded history, and they form an integral part of the Chinese diet.[1] Fermented products have long been documented in Chinese history, marking

life events including celebrations, feasting, and ritual activities based on their perceived sensory and health benefits. Inscriptions from the late Shang Dynasty (about 1600 B.C. to 1046 B.C.) record at least three types of fermented beverages, and these fermented beverages and other foods were offered as sacrifices to royal ancestors in the bronze vessels that were used to store, serve, and ritually present fermented beverages during that period, likely accompanied by elite feasting.[2] Using a combined chemical, archaeobotanical, and archaeological approach, evidence of fermented beverage production in ancient China has been documented that extends back nearly nine thousand years.[2]

Fermented foods are traditionally prepared in the household using relatively simple techniques, offering an economical way to preserve food materials, and producing a variety of tasty products. However, research on the microbial activity associated with the fermentation process has been documented in China only since the mid-19th century.[3] Understanding the essential role and ecology of the microorganisms such as molds, yeasts, and bacteria used in food fermentation makes it possible to use more controlled and efficient processes. The rapid pace and complexity of modern life has resulted in the development of more specialized products, with the fermentation system gradually adopting a standardized approach to making traditional fermented foods using selected starter cultures. With the many alternative food preservation methods available today, fermentation is no longer the most pressing requirement for preserving food in most developed countries; fermented foods are manufactured in modern societies for their widely appreciated flavor, aroma, and textural attributes.[4] More recently, further advances in our understanding of nutrition and food science has led to an increased realization and appreciation of many traditional Chinese fermented foods for their inherent beneficial health effects.

16.2 CATEGORIZATION OF CHINESE FERMENTED FOODS

Traditional Chinese fermented foods have had a strong influence on products developed in other Asian countries. Consequently, Asian fermented products share many similarities, including the development of a special saccharification fermentation system in which fungi (mold) break down the polysaccharides into dextrin, maltotriose, maltose, and glucose in the food substrate.[5] Most of the early food substrates such as rice, legumes, and other grains used for making traditional fermented food in China lack the simple sugars needed for yeast or lactic bacteria's action, and therefore the exogeneous saccharifying enzymes that are supplied by mold growth are required. Traditionally, a thick mold mycelium is grown on a variety of steamed cereals, legumes, and other materials to make a saccharification-fermentation agent variously called *qu* or *koji*. Here, amylolysis fermentation, which exploits the fungi of the genera *Aspergillus, Rhizapus, Monascuc*, and others, depending on the environmental availability, is used to break down the carbohydrates of cereals and legumes into fermentable simple sugars. Yeast and bacteria then enter the process either opportunistically, in the traditional process, or as an added inoculants, in the controlled process. When amylolytic *koji* starters are used, they can be made by growing molds on rice, wheat, barley, or some combination of these in the form of a starchy cake containing simple mold strains or mixed cultures of molds and

yeasts or bacteria. These starters provide a wide variety of enzymes that hydrolyze starch, protein, and lipid components in the raw materials. These commercial *koji* starters can be stored at ambient temperatures without significant loss of viability for at least 6 months.[1]

The activities of fermenting organisms are dependent on their intrinsic and extrinsic parameters of growth. The same food materials processed under different environmental conditions and geographical areas using nonstandardized techniques can thus be fermented into different kinds of products yielding distinctively different flavors and aromas. The diversity of traditional Chinese fermented foods that has developed over thousands of years is therefore immense. Many of these products that are manufactured in a traditional localized "farmhouse" manner are now considered to be at a premium, because they retain unique flavor and aroma characteristics that many claim are simply not available in the equivalent factory-manufactured products.[4] In general, Chinese fermented foods are classified into four categories depending on the primary microorganisms involved in the production. Some types of foods only require a single starter culture containing mold, yeast, or bacteria to complete the entire fermentation process, whereas others may employ two or more different cultures to produce the desired end products. Selected examples are summarized in Table 16.1.

16.2.1 Foods Fermented by Mold

Foods that are fermented by mold alone are very common; examples include *tian jiu-niang* (fungal-fermented sweet rice), *douchi* (fermented whole soybean), *furu* (fermented soybean curd or *tofu*), and *hongqu* (red mold rice or red fermented rice). *Tian jiu-niang* is a fermented glutinous rice product that is a popular snack food in China (Figure 16.1). It is a mixture of rice grains and saccharified liquid, typically containing 1.5 to 2.0% alcohol with an acidity of 0.5 to 0.6% as lactic acid, and some glucose, maltose, and oligosaccharides.[6] The steamed sweet rice is fermented with *jiu-yao* (starter culture) that contains *Rhizopus, Mucor, Monilia,* and *Aspergillus*.[7] The acidity may increase to 1%, and the alcohol content may reach 5%. The product is consumed largely in the winter season by children and adults to keep the body warm by enhancing the blood circulation, and is also used as an ingredient in making special dishes.[6] *Furu, douchi,* and *hongqu* (Figure 16.2) will be discussed in detail later in this chapter.

16.2.2 Foods Fermented by Yeasts

Examples of foods fermented by yeasts include fruit wines and steamed yeast buns.[3] The fermentation processes for both types of products are similar to the fruit wines and yeast bread commonly found in Western countries. *Saccharomyces cerevisiae* is used as the starter culture to ferment sugars into CO_2 and alcohol. Fruits such as grapes, pears, and plums that have a high sugar content are suitable for making fruit wines, which usually have an alcohol content below 10%. Steamed yeast buns (Figure 16.3) are the Chinese equivalent of Western style yeast bread; the main difference is the method of cooking, namely steaming versus baking. There are many varieties of steamed yeast buns, including stuffed meat buns, stuffed vegetable buns,

TABLE 16.1
Popular Chinese Fermented Foods

Product name	Description	Main microorganisms involved	References
		Fermented by molds	
Douchi	Fermented whole soybeans	*Aspergillus oryzae* or *Mucor* spp.	86
Furu	Fermented soybean curd	*Actinomuco* spp., *Mucorwutungkino* spp., *Mhiemelis* spp., and *Rhizopus* spp.	20, 26, 19
Tian-jiu-niang	Fermented glutinous rice	*Rhizopus, Mucor, Monilia,* and *Aspergillus* spp.	7
Hongqu	Red fermented rice	*Monascus. purpureu, M. pilosus, M. ruber,* and *M. froridanus*	128
		Fermented by yeasts	
Fruit wine	Liquid from fermented fruits	*Saccharomyces cerevisiae*	3
Yeast buns	Steamed yeast buns, sweet, blend, or savory	*Saccharomyces cerevisiae*	3
		Fermented by bacteria	
Jiang tsai	Fermented salted dry vegetables	Lactic acid bacteria (*Streptococcus faecalis, Lactobacillus plantarum, Lactobacillus brevis, Pediococcus,* spp., and *Leuconostoc mesenteroides*)	9
Suan cai, pao cai	Fermented salted dry cabbage products	Lactic acid bacteria	8
Bacteria-fermented and enzymatically fermented *furu*	Bacteria-fermented and enzymatically fermented soybean curd	*Bacillus* spp. or *Micrococcus* spp.	20
		Fermented by mixed molds, yeasts and bacteria	
Jiang	Fermented bean paste or wheat flour paste	*Aspergillus oryzae* or *Aspergillus soyae, Pediococcus halophilus, Lactobacillus. delbrueckii, Zygosaccharomyces rouxii,* and *Torulopsis* spp.	14
Soy sauce	Liquid from fermented soy bean and wheat flour	*Aspergillus oryzae* or *Aspergillus soyae, Pediococcus halophilus, Lactobacillus. delbrueckii, Zygosaccharomyces rouxii,* and *Torulopsis* spp.	14
Rice vinegar	Fermented rice or rice wine	*Rhizopus, Mucor, Aspergillus,* and *Monascus* species, yeasts, and bacteria	167, 168
Gray *furu*	Mold and bacteria fermented soybean curd	Mold and bacteria species	20

FIGURE 16.1 *Tian-jiu-niang.*

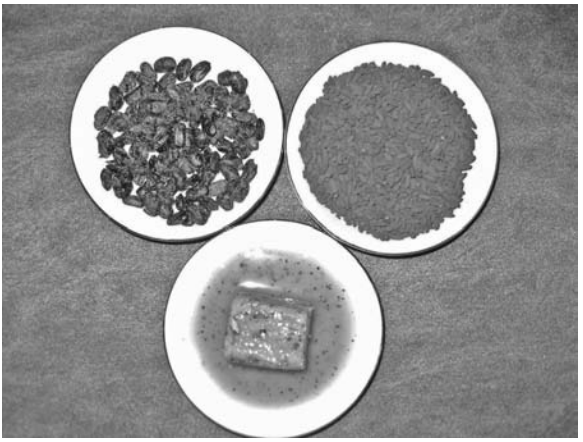


FIGURE 16.2 *Furu* (lower)*, douche* (upper left), and red mold rice (upper right).

FIGURE 16.3 Steamed yeast buns.

sweet buns stuffed with red bean paste or locus bean paste, and plain buns with different shapes and flavors, and they represent one of the most popular food choices for both a staple diet and as a snack food. Yeast fermented dough is also used to prepare a variety of bakery products.

16.2.3 Foods Fermented by Bacteria

Fermented vegetables, including cabbage, cucumber, radish, beets, ginger roots, and leafy greens, are mainly produced by mixed lactic bacteria fermentation. They are made and consumed in almost every Chinese household year-round. Fermented vegetables are commonly used as a side dish, or prepared with meats as part of a main dish for the family meal. After cleaning, the vegetables are partially air dried and then placed in a container, salted layer by layer, and sealed. Normally the NaCl concentration and the final pH of fermented cabbage products such as *Suan cai* are around 2% and from 3.1 to 3.7, respectively.[8] The acidity and simple sugar content of the vegetables permit the growth of natural lactic acid bacteria (LAB) including S*treptococcus faecalis, Lactobacillus plantarum, Lactobacillus brevis, Pediococcus,* spp., and *Leuconostoc mesenteroides*, producing a spectrum of small flavoring compounds such as lactic acid, acetic acid, ethanol, and glucose.[9] Some of these LAB are now known to be probiotics, hence consuming fermented vegetables containing live bacteria can be considered beneficial due to the multiple beneficial health effects associated with probiotics.[10] In recent years, lightly salted/fermented vegetables that are usually consumed within 2 d of preparation are becoming more popular because of the modern trend towards low-salt and rapid production.[11] However, food borne outbreaks associated with lightly fermented vegetables have been reported,[12,13] and control of pathogenic bacteria in lightly fermented (salted) vegetables is currently a subject of research.[11]

16.2.4 Foods Fermented by Mixed Mold, Yeast, and Bacteria

Jiang (fermented soybean and wheat flour paste) and soy sauce (liquid from *jiang*) are typical examples of foods fermented by mixed mold, yeast, and bacteria. The characteristic flavor and aroma of *jian* and soy sauce are the result of the combined enzyme activities of these microorganisms. Soybeans, which contain high protein and oligosaccharide levels, but no significant amounts of simple sugars, are normally stable to the activities of yeast and bacteria due to the lack of amylase in most of these organisms. Therefore, the first step in manufacturing *jiang* and soy sauce is to inoculate the steamed food substrate with *Aspergillus oryzae* or *A. soyae* to make *koji*. The blooming fungi produce saccharifying enzymes that break down the poly- and oligosaccharides into simple sugars for subsequent bacteria and yeast activities. These fungi also produce acid and alkaline proteases that hydrolyze proteins in the food substrate to peptides and amino acids. The bacteria and yeast involved in the secondary fermentation include *Pediococcus halophilus, Lactobacillus delbrueckii, Zygosaccharomyces rouxii,* and *Torulopsis* species.[14]

16.3 COMMON TRADITIONAL FERMENTED FUNCTIONAL FOODS IN CHINA

Functional food is defined as food that is thought to confer a health benefit beyond its basic nutrition. Recently, some fermented foods have been shown to have various beneficial health and physiological properties in addition to their taste and basic nutritional values, thus allowing them to be considered functional foods. Most Chinese fermented foods and beverages are derived from plant materials such as vegetables, grains, and legumes. Rice and soybeans are two major crops that are cultivated and used to produce a variety of fermented products. Five well-known traditional Chinese fermented foods, namely, *sufu, douchi*, red yeast rice, and rice wine and vinegar derived from rice or soybeans are introduced herein because the Chinese have long recognized the health values associated with consumption of these products.

16.3.1 Fermented Soy Products

In addition to their high protein content and other nutrients, soybeans contain polyphenols, amino acids and peptides, soluble fiber, and isoflavones, all of which exert a variety of biological activities both *in vivo* and *in vitro*, and thus confer properties of a functional food. Consumption of soy foods has been associated with reduced risks of cardiovascular diseases;[15] several cancers, including lung, colon, rectal, breast, stomach, and prostate cancers;[16,17] and improved bone health.[18] However, unprocessed soybeans also contain antinutritional factors and have high levels of oligosaccharides such as stachyose, raffinose, and melibiose, which are difficult to digest, and cause flatus in some people. To counteract this effect, the Chinese learned how to transform the soybean into more palatable and digestible forms through fermentation. Hydrolytic breakdown of undesirable components, such as allergens and phytates, takes place during fermentation, making fermented soybean products such as *jiang*, soy sauce, *furu*, and *douchi* nutritionally improved, easily digested, and widely accepted. They are used in daily cooking as spices, condiments, a main dish, or mixed with other food materials in prepared dishes, and have been an essential part of the Chinese diet for thousands of years.

16.3.1.1 *Furu*

16.3.1.1.1 Background and History

Furu or *sufu* is a fermented soybean curd (*tofu*) product originating in China. It is an inexpensive and popular side dish consumed mainly with breakfast rice congeal or as a spread on steamed bread. It can also be used as a spice ingredient in cooked vegetables and meats to enhance the flavor or color. Literally, *furu* means "molded milk." Microbial action on soy proteins results in a soft cheese-like product with a spreadable creamy consistency and a pronounced flavor.[19]

This product has appeared in English literature under many different names due to the phonetic transliteration from various dialects used in China, including *sufu*, *tousufu*, *dou-fu-ru, tou-fu-ru*, *jiang-dou-fu, fu-yu*, and *foo-yue*.[20] *Furu* is also known

as *tofuyo*, *hyu-fu* or *fu-nyu* in Japan,[21] *chao* in Vietnam, *ta-huri* in the Philippines, *taokaoan* in Indonesia and *tao-hu-yi* in Thailand.[22] These names have caused some confusion to Western people as well as Chinese, which indicates the popularity of *furu* in Asia. The first written records of *furu* consumption date back to the Wei Dynasty (220 to 265 A.D.),[23] and it became popular in the Ming Dynasty (1368 < to 1644).[24] The annual production of *furu* was 45,000 tons in 1997,[25] and today is estimated to be over 300,000 metric tons in China alone.[20] More detailed information on *furu* is available in the review by Han et al (2001).[20]

16.3.1.1.2 Starter Culture

Furu is made by fungal solid-state fermentation of *tofu* followed by aging in brine containing salt, spices, alcohol, and other ingredients. The fungal starters used to produce furu include *Actinomuco* spp., *Mucorwutungkino* spp., *Mhiemelis* spp., and *Rhizopus* spp.[20,26,19] The majority of furu fermentation starters used in commercial practice represent species of *Mucor* and *Actinomucor.* Among these, *Actinomucor elegans* and *Actinomucor taiwanesis* seem to be the preferred molds that are most widely used for the commercial production of *furu* in China and Taiwan, respectively.[27] Because most of these commercial starter species grow best at 25 to 30°C, they are not suitable for furu production during hot summers with indoor factory temperatures above 30°C. For this reason, *Rhizopus oligosporus,* which grows well at temperatures up to 40°C, has been explored as a potential alternative to *A. elegans* as the starter for furu production during the hot season.[26]

16.3.1.1.3 Manufacturing Process

The review by Han et al. describes how both traditional processes with natural fermentation and innovative commercial processes are used to manufacture furu.[20] In general, furu is produced in stages. The first stage is the preparation of *tofu,* which is made by soaking, grinding, and filtering the soybeans to obtain soymilk, followed by coagulating the milk by adding calcium salt to form soybean curd. The curd is then pressed into sheets and cut into cubes of tofu. The second stage is the fermentation or preparation of *pehtze*. Here, tofu is inoculated with a mold starter and incubated for between 2 and 7 d at 12 to 30°C, resulting in mycelium covered pieces of pehtze. The starter may either be inoculated with a pure culture applied as a sprayed suspension, or by natural house flora inoculated onto the tofu by direct contact with utensils. The purpose of the pehtze fermentation step is to encourage the formation of a white cover of mold mycelia surrounding the cubes of tofu. The final stage consists of the ripening of the pehtze in a dressing mixture or brine containing rice wine, salt, and other product-specific ingredients for color and flavor. A number of proteolytic, lipolytic, and other enzymes formed by the molds will act upon the pehtze during the ripening stage, resulting in softening and flavor development.[26]

The processing can be adjusted to produce mold-fermented *furu*, naturally fermented *furu*, bacterial-fermented furu, or enzymatically ripened furu. For mold-fermented furu and naturally fermented furu, the general preparation steps described above are followed, whereas for the bacterial fermented furu and enzymatically ripened furu products, a 2-d presalting step is required before the *pehtze* preparation. Here, the *tofu* absorbs the salt until the salt content reaches about 6.5%. The *pehtze* is then prepared by inoculating salted tofu with pure bacterial culture, *Bacillus* spp.,

or *Micrococcus* spp., at 30 to 38°C for about a week to produce bacterial fermented *furu*. There is no *pehtze* preparation step for the enzymatically fermented *furu*. Instead, some *koji* is added directly in the dressing mixture to ripen the salted *tofu*.

There are also varieties of *furu* produced using different dressing ingredients during the ripening stage. Based on its color and flavor, *furu* can be classified as red *furu*, white *furu*, and grey *furu*, among others. Of these, red *furu* is the most popular due to its attractive color, strong flavor, and perceived healthy ingredients, which are derived from the red fermented rice with which it is made. The dressing mixture for red *furu* consists of salt, red fermented rice, an alcoholic beverage, sugar, spices, and flour or soybean paste. White *furu* uses similar ingredients in the dressing mixture, omitting only the red fermented rice. Grey *furu* is ripened with a special dressing mixture that utilizes both bacteria and mold enzymes, resulting in a strong but offensive flavor. The production of grey *furu* has been a closely held secret, however, and as a result, may be becoming a lost art.[28] Other types of *furu,* including a drink *furu*, are prepared by adding higher concentrations of alcohol and a wide range of dressing ingredients, including vegetables, rice, and bacon.

Regardless of their differences in color and flavor, most types of *furu* contain approximately 58 to 70% moisture, 12 to 17% crude protein, 8 to 12% crude lipid, and 6 to 12% carbohydrates. Salt content is high, which contributes to the 4 to 9% ash content. The minerals found in *furu* (based on 100 g fresh weight) include Ca (100 to 230 mg), P (120 to 300 mg/g), and Fe (7 to 16 mg/g). The vitamins provided are thiamin (0.04 to 0.09 mg), riboflavin (0.13 to 0.36 mg), niacin (0.5 to 1.2 mg), and vitamin B_{12} (1.7 to 22 μg).[19,23] Overall, 18 amino acids have been identified in *furu*. Glutamic acid and aspartic acid are the most abundant amino acids found in red *furu* and grey *furu*. The ratio of the combined glutamic acid and aspartic acid to the total amino acid content is approximately 30%, which provides furu with a pronounced savory taste.[29] The complicated flavor is attributed to the presence of 22 esters, 18 alcohols, 7 ketones, 3 aldehydes, 2 pyrazines, 2 phenols, and many other volatile compounds in *furu*. High levels of volatiles are formed in the presence of ethanol during ripening.[30]

16.3.1.1.4 Health and Nutritional Benefits

Furu is mainly appreciated for its pleasant creamy taste and intense savory flavor, which are generally used to accent the otherwise bland flavors of rice or bread. Chinese people also consider it a easily digested and healthy food for children, the elderly and the infirm. This is probably due to its high content of calcium, peptides, free amino acids, peptides, and enzymes, resulting from the fermentation process. The water-soluble proteins in furu have been determined to be 6 to 7 times higher than that that in tofu.[31,32] Many physiologically active components, such as soybean peptides, B vitamins, nucleic glycosides, and aromatic compounds, which do not exist in unfermented soybeans are produced.[33] The vitamin B_{12} content, an essential nutrient for the nervous system, in *chou furu* (strong smell, stinky furu) has been shown to be much higher (9.8 to 18.8 mg/100 g) than that in red furu (0.42 to 0.78 mg/100 g),[32] suggesting a high activity of microorganisms during fermentation of the chou furu. The antioxidative and antihypertensive effects of furu have also been documented.[34,35]

A recent study[36] reported that a bacterial strain, *Bacillus subtilis* ZJU-7, with high poly-γ-glutamic acid (γ-PGA) production has been isolated from furu. γ-PGA is a water soluble polymer of D- and L-glutamic acid units linked by amide linkages between the γ-amino and γ-carboxylic acid groups. This compound exists naturally in other fermented soybean foods such as the Japanese fermented soybean food *natto*, where it is produced by *Bacillus natto* as a result of natto mucilage. The γ-PGA in natto was reported to increase calcium solubility both in vivo and in vitro by inhibiting the formation of an insoluble complex of Ca with phosphate during natto mucilage, thereby enhancing intestinal Ca^{2+} absorption.[37]

16.3.1.1.5 Safety Concerns

Because the manufacturing process involved in converting tofu to furu does not involve heat pasteurization, consumers often express concern about the safety of furu consumption. This issue has been addressed by Shi and Fung,[38] who evaluated the control of four food-borne pathogens *Escherichia coli O157:H7, Salmonella* Typhimurium, *Staphylococcus,* and *Listeria monocytogenese* during furu fermentation. Comparing the microorganisms in experimental furu, which had been inoculated with both pathogens and starter culture *Actinomucor elegans* and those present in commercial furu, they found that although the starter culture did not inhibit the growth of bacteria, the aging solution (10% alcohol + 12% NaCl) was effective in controlling these food-borne pathogenic bacteria after one month of aging without suppressing other natural microflora of furu. The total bacterial count for matured furu ranged from 4 to 6 log-colony-forming units (cfu)/g, and no pathogens were detected. They concluded that the end products of fermentation-controlled food pathogenic and spoilage microorganisms, and that consequently, furu is a safe fermented product even though its preparation does not include pasteurization.

16.3.1.2 Soy Sauce

16.3.1.2.1 Background and History

Soy sauce is a dark brown liquid obtained from a fermented mixture of soybeans and wheat that has gained popularity worldwide. With a salty taste and sharp savory flavor, it has served as an all-purpose seasoning for at least 2000 yr in China. Soy sauce was originally the liquid pressed from *jiang*. In Chinese food and culinary history, the earliest record of jiang appeared during the age known as the Spring–Autumn Period (772 B.C. to 481 B.C.). Confucius (551 to 479 B.C.) also mentioned the popular consumption of jiang in his time, in the *Analects of Confucius*, a collection of brief aphoristic fragments compiled many years after his death. The first time jiang was mentioned as being made of soy was during the West Han Dynasty (206 B.C to 24 A.D.) and the term "liquid jiang" (soy sauce) first appeared a few years later in the East Han Dynasty (25 A.D. to 220 A.D). Later the use of soy sauce as a popular household spice was clearly recorded by Lin Hong, writing during the Song Dynasty (960 to 1279 A.D.).[39] Although soy sauce originated in China, the technique of making it gradually spread to Japan, Korea, and other countries in Asian. The Japanese, however, have made the biggest contributions to improving manufacturing techniques and developing high quality soy sauce products by using selected starter cultures under controlled fermentation conditions. Today, the Chinese per

capita annual consumption has been estimated to be less than 5 kg compared with 12 kg per capita in Japan.

Before the mid-1950s, soy sauce in China was produced by natural fermentation through the exposure of raw materials to natural molds, bacteria, and yeasts in the open air with added high concentrations of salt solution to the mixture. The fermentation process proceeded at natural ambient temperatures for 6 to 12 months. Soy sauce produced following this method has an excellent flavor as a result of the hydrolytic actions of numerous microbial enzymes produced by multiple strains acting on the proteins, lipids, and carbohydrates in the substrate. The flavor and quality of the product depend on the natural microorganisms that coexist in the mixture under local environmental conditions such as temperature, humidity, fermentation time, water quality, raw materials used, etc. However, this process allows neither quality assurance and quality control standards, nor hygienic controls, and in order to meet the high demand for soy sauce domestically, in the second half of the 20th century, the manufacturing process of soy sauce began to use a controlled fermentation process that is primarily modeled on Japanese methods.[39] The quality of the soy sauce produced is now better controlled, and a variety of processing methods have been developed. According to Wu,[40] the soy sauce production in China is estimated to have reached 550 kilo tonnes in 2000 and was manufactured by a total of about 2,000 plants. Among these soy sauce manufacturing plants, about 300 produce more than 5,000 tonnes of soy sauce annually. The majority of these plants (80%) use the economic "low-salt solid-state" fermentation approach, which involves a short fermentation process at a higher temperature, compared with the natural fermentation method. A powdered form of soy sauce has also been developed to meet today's demand for convenient storage, transportation, and ease of use.[41]

16.3.1.2.2 Manufacturing Process

In general, there are two fermentation stages involved in the production of soy sauce. The first stage is aerobic *koji* fermentation, which uses fungi to break down the polysaccharides into simple sugars. The second stage is an anaerobic salt mash or *moromi* (in Japanese), where the mixture undergoes LAB and yeast (*Zygosaccharomyces rouxii*) fermentation.[1] Although the traditional method is still used at a domestic level, as noted before, many commercial soy sauce producers in China have adopted the low-salt, solid-state fermentation technique using starter cultures.[42] Briefly, a mixture of defatted soybeans (60%) and wheat (40%) are inoculated with *Aspergillus oryzae* or *A. soyae* to make koji and allowed to stand for 3 d at 25 to 35ºC and 27 to 37% moisture. The matured koji is then mixed with a smaller volume of brine (about half the usual amount) containing about three-quarters as much salt as used in the traditional method (salt content 17 to 19%) and the mixture is allowed to ferment at a relatively high temperature (40 to 45°C) compared to the lower temperature used in the traditional method. In the traditional method, the brine–koji mixture is fermented at room temperature for 12–14 months (home preparation) or at 35 to 40°C for 2 to 4 months (commercial preparation). Under such low-moisture, low-salt, and high-temperature conditions, it takes only 3 weeks to complete the fermentation process. The fermented mash is transferred to another tank, mixed with additional brine, and then heated to more than 80°C. This is followed by the separation of the

liquid in the tank through gravity. The soy sauce may be pasteurized or benzoic acid added before finally being clarified, bottled, and shipped. Compared with the traditional method, this method is more economical because of the significant reduction in processing time. However, the ratio of amino acid nitrogen to total nitrogen in the final product is not as high as in the traditionally fermented product, and the flavor characteristics of the soy sauce produced are also compromised to some extent.[6,39]

As the economic situation in China has continued to improve in recent years, consumer demand for high quality products has been ever increasing. The future trend in Chinese soy sauce production is therefore expected to gradually shift to the lower fermentation temperature–longer time process that more closely simulates the natural fermentation conditions to produce high quality soy sauce.[39]

The chemical composition of soy sauce varies with the raw materials used. In general, good quality soy sauce contains 1.0 to 1.65% total nitrogen (w/v), 2 to 5% reducing sugars, 1 to 2% organic acids, 2.0 to 2.5% ethanol, and 17 to 19% sodium chloride (w/v). About 45% of the total nitrogen consists of simple peptides, whereas 45% is in the form of amino acids.[43]

16.3.1.2.3 Classification of Soy Sauce

Based on the manufacturing methods and raw materials used, there are three general types of soy sauce produced in China: fermentation soy sauce, formulated soy sauce, and variety soy sauce. "Fermentation soy sauce" is the main category of soy sauce products, manufactured through natural or controlled fermentation of raw materials, while "formulated soy sauce" is a mixture of fermentation soy sauce (at least 50%) and the liquid acid–hydrolysate of plant proteins (no more than 50%). As the production time for the formulated soy sauce is much shorter, it has a lower cost than the fermented product. However, its flavor and nutritional value are inferior to the fermentation soy sauce. "Variety soy sauce" is a fermentation–based soy sauce with certain nontraditional raw materials added. This has led to the development of a number of specially formulated products, and has become popular among gourmet consumers who desire special flavors or compounds from a variety of untraditional raw ingredients in soy sauce, such as mung beans,[44] black beans,[45] hazelnuts,[46] seaweed, mushrooms, shrimps, and selected herbal ingredients. Although most of these products are promoted for the additional health value arising from the special ingredient materials, research on the actual health benefits from ingestion of these products is scarce in the literature.

Soy sauce is also classified as white soy sauce (*sen-chou*) or red soy sauce (*lau-chou*), based on its color. White soy sauce is light in color, a natural result of fermentation. This product has a superior flavor, and is often produced by a fermentation process using lower fermentation temperature and longer fermentation time, to fully develop the flavor components. White soy sauce is commonly used for both cooking and seasoning of prepared foods. Red soy sauce has a darker color and a higher viscosity compared with white soy sauce due to the addition of caramel to the product.[47] It is particularly desirable for cooking food where a dark color is preferred. *Aspergillu oyyzae* JP-DQ-1 has been reported to be the starter strain of choice for manufacturing red soy sauce, whereas *Aspergillu oyyzae* JP-W is more suitable for

manufacturing white soy sauce, based on the enzyme system produced by each during fermentation.[48]

16.3.1.2.4 Health Effects

In addition to its taste and aroma, soy sauce is now considered a functional seasoning because it has been found to contain several bioactive components showing a range of biological activities, including anticarcinogenic, antimicrobial, antioxidative, antiplatelet, hypoallergenic, and antiallergic activities, and the inhibition of an angiotensin I-converting enzyme. These collective effects of soy sauce are thought to make it a healthy and beneficial food in the human diet.[49]

16.3.1.2.4a Antioxidative Activity

Oxygen-related free radicals have been associated with aging and a number of chronic diseases such as cancer, atherosclerosis, neurodegenerative disease, and rheumatoid arthritis.[50] Soy sauce has been found to have a very high antioxidative capacity; the antioxidant activity reported by Benjamin et al.[51] was 66.8 neq/ml soy sauce. The antioxidant activity was found in both ethyl-soluble and ethyl-insoluble fractions, indicating the presence of multiple antioxidant components in soy sauce. Melanoidin, the main product formed as a result of the Maillard reaction during soy sauce production, is known to have a high peroxyl radical scavenging capability. As measured by the chemiluminescence method, melanoidin, along with other products in soy sauce, exhibited strong antioxidative properties.[52] This powerful antioxidant activity in vitro was especially marked in dark soy sauce.[53] Dark soy sauce was also reported to decrease lipid peroxidation in vivo in human volunteers.[54]

16.3.1.2.4b Anticarcinogenic Activity

Soy sauce has also been reported to exhibit anticarcinogenic activity in animals.[51,55] The multiple antioxidant components in both the ethyl-soluble and ethyl-insoluble fractions of soy sauce have also been found to possess anticarcinogenic properties. In benzo(α) pyrene-induced mouse forestomach neoplasia, for example, dietary soy sauce reduced neoplasm formation.[51,55] In a long-term (13 months) study, a diet containing 10% soy sauce significantly reduced the frequency and multiplicity of liver tumor in mice.[56]

Because soy sauce exhibits substantial antioxidant activity, and given that many antioxidants inhibit carcinogenesis, some of the antioxidants in soy sauce may be responsible for this anticarcinogenic effect. For example, a flavor component of fermented soy sauce, 4-hydoxy-2(or 5)-ethyl-5 (or 2)-methyl-3(2H)-furanone, is a potent antioxidant, and one of the active anticarcinogens.[57] The major group of antioxidants, melanoidins, exhibit protective effects on nitric oxide-induced DNA damage.[58] However, the mechanism by which soy sauce contributes to the inhibition of neoplasia remains to be elucidated. Although protease inhibitors from soybeans are recognized as being effective in cancer prevention in animals, they are substantially reduced in soy sauce during the fermentation and pasteurization process, and thus account for little, if any, of the anticarcinogenic effect observed.[51]

16.3.1.2.4c Antimicrobial Activity

Soy sauce with added preservative, p-hydroxybutyl benzoate, killed pathogens such as *Escherichia coli, Shigella flexneri, Salmonella* Typhimurium, *Salmonella*

paratyphi A, Salmonella enteritidis, and *Vibrio cholera* within 6 h, whereas without preservatives, it took 48 h to kill these microorganisms.[59] Masuda et al.[60] demonstrated the antimicrobial property of soy sauce. They inoculated five strains of *Escherichia coli* O157:H7 in soy sauce and incubated the samples at 4, 18, and 30°C. The soy sauce showed an increased inhibition activity of the growth of *E. coli* with increased incubation temperature. The number of cells decreased to an undetectable level within 9 d in all the soy sauce samples at 30°C, but did not decrease in the 0.1 M phosphate-buffered saline (pH 7.0) control solution under the same conditions. The antimicrobial action of soy sauce cannot be attributed to its high salt concentration or other individual ingredients alone, however, because a combination of soy sauce components such as 10% or 16% NaCl, 5% ethanol, lactic acid, or acetic acid at pH 4.5, sodium benzoate (0.6 g/kg), or p-hydroxybenzoic acid *n*-butyl ester (0,05 g/l) showed a synergistic anti-*E. coli* O157:H7 effect.[60] The antimicrobial activity of soy sauce is apparently attributed to the combined effects of salt, ethanol, pH, preservatives, and temperature.

16.3.1.2.4d Antiplatelet Activity

Besides the many identified bioactive components in soy sauce, two alkaloidal compounds, 1-methyl-1,2,3,4-tetrahydro-β-carboline and 1-methy-β-carboline, have been isolated from the basic extract of soy sauce that exhibit antiplatelet activity.[61] It has been observed that 1-methyl-1,2,3,4-tetrahydro-β-carboline inhibited the aggregation response induced by epinephrine, platelet-activating factor, collagen, adenosine 5′-diphosphate, and thrombin, and this inhibition effect was much greater than that of 1-methy-β-carboline. Both antiplatelet compounds are universally found in commercially available soy sauce, and therefore consumption of soy sauce may have a potent preventive effect on thrombus formation.

16.3.1.2.4e Hypoallergenic Effect

Soy sauce is considered as a hypoallergenic food for both wheat and soybean-associated allergic patients. The two major raw materials for soy sauce production are soybeans and wheat, and at least fifteen soybean proteins have been identified as allergens in sera of soybean-sensitive patients.[62] Among these, Gly m Bd 60 K, Gly m Bd 30 K, and Gly m Bd 28 K are three major soy allergens. However, it has been reported that the allergenicity of soybeans is greatly reduced in soy sauce.[62,63] Allergic reactions associated with the ingestion of wheat proteins are also a well-known public health issue. Wheat allergens have been identified from both the salt-soluble and salt-insoluble fractions of wheat flour.[64,65] The degradation of wheat allergens in both fractions from each brewing process between raw materials and the final soy sauce was demonstrated by Kobayashi et al.[66] The reduction of wheat allergens in soy sauce was explained by the solubilization of salt-insoluble proteins, and the degradation of salt-soluble proteins during fermentation.[67] Soy sauce possesses microbial proteolytic activity during fermentation, and proteins of raw material including soybeans and wheat are completely degraded into peptides and amino acids by microbial proteolytic enzymes after fermentation. Consequently, few or no allergens related to the raw material are present in soy sauce.

16.3.1.2.4f Antiallergic Activity

Soy sauce has also been reported to be a potentially promising seasoning for the treatment of allergic diseases. Polysaccharides in the cell wall of soybeans were first purified from soy sauce by Kikuchi and Yokotsuka,[68] and now are termed *shoyu* polysaccharides (SPS).[69] Polysaccharides contain a large amount of galacturonic acid, and are only slightly hydrolyzed by mold enzymes during soy sauce fermentation.[70] SPS are generally present in soy sauce at about 1% (w/v) level, and they exhibit potent antiallergic activities both in vitro and in vivo. SPS has been shown to inhibit hyaluronidase and histamine release from antigen-induced mast cells, and has a significant suppressive effect on the passive cutaneous anaphylaxis reaction in the ears of mice, an animal model for the study of type I allergy. In this study, 12 mg/kg body weight per day, corresponding to 1 ml/kg of raw soy sauce per day, was administered orally for 3 d.[69,71] Furthermore, double-blind placebo-controlled clinical studies have reported that oral supplementation of 600 mg SPS, corresponding to 60 ml soy sauce each day for 4 weeks, was an effective intervention for patients with perennial and seasonal allergic rhinitis.[63,71] Therefore, consumption of soy sauce may be beneficial for patients with allergic diseases due to both the hypoallergenicity and antiallergic activity of soy sauce. A comprehensive review of the immunological functions of soy sauce has been published by Kobayashi.[67]

16.3.1.2.5 Safety Concerns

It has been suggested that consumption of soy sauce may be a cause of the relatively high incidence of stomach cancer in Japan. Wakabayashi et al.[72] hypothesized that the presence of some precursors of bacterial mutagens, such as 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-caroxylic acid in soy sauce might interact with nitrite from the consumption of vegetables containing nitrite in the gastrointestinal tract to form one or more nitroso compounds, which are known to be carcinogenic. To test this theory, Benjamin et al.[55] investigated the effects of soy sauce and nitrite on benzo(α) pyrene-induced mouse forestomach neoplasia. Contrary to their expectations, they found that soy sauce in the diet plus nitrite provided in the drinking water significantly reduced forestomach neoplasm formation. This result contradicted the previous hypothesis that cancer is caused by the consumption of soy sauce and vegetables together.

The biogenic amines and chloropropanols in soy sauce have also been a concern, as biogenic amines such as histamine, tyramine, phenylethylamine, putrescine, and cadaverine are formed by enzymatic decarboxylation of amino acids, and have been shown to cause food poisoning, and have all been detected in fermented soy foods.[29,73,74] The amino acids liberated from the soy and wheat proteins during the fermentation process involved in making soy sauce could be the basis for the formation of biogenic amines by endogenous or microbial decarboxylases. The biogenic amine content in soy sauce is generally low and the amine composition is quite different, with tyramine as the dominating amine in soy sauce.[75,76] Even in cases of high consumption of soy sauce, the biogenic amine levels ingested from soy sauce are far below the harmful level. Therefore, the health risks associated with biogenic amines from the consumption of soy sauce can be excluded.[75]

The industrial production of soy sauce may have a concern due to the presence of chloropropanols. Chloropropanols can be produced by the acid hydrolysis of vegetable proteins, being derived from triacylglycerols in the residual oil in the protein matrix.[77] The formation of a number of chlorinated propanols, including monochloropropanols, dichloropropanols, and monochloropropanediol, has been observed.[78] Chloropropanols have been shown to be hepatotoxic in both animals and humans.[79,80] 1,3-dichloro-propan-2-ol (1,3-DCP) and 3-monochloro-propane-1,2-diol (3-MCPD) are the two most important chloropropanols in terms of both the levels present and their toxicity.[81] The European Commission has adopted a regulatory limit of 0.02 mg kg/1, based on a 40% dry matter content, for 3-MCPD in soy sauce and hydrolyzed vegetable protein (HVP).[82] The most likely sources of contamination of soy sauce by chloropropanols are the result of the addition of acid HVP in formulated soy sauce. A survey of chloropropanols in commercial soy sauces in the United Kingdom reported that although low levels of 1,3-DCP and 3-MCPD were found in a few retail products, none of the "naturally brewed" or "traditionally brewed" soy sauce tested in 2002 contained detectable chloropropanols.[83]

16.3.1.3 *Douchi*

16.3.1.3.1 Background and History

Douchi, or *touchi*, is a traditional Chinese soybean food fermented with *Aspergillus* spp., and is used mainly as a seasoning ingredient. The term *douchi* or *touchi* (bean *chi*) first appeared in the seventh century in China.[84] As the ancient literature records, douchi seems to have been well known and widely used during the early Han dynasty (206 B.C. to A.D. 220), and probably existed even earlier. Douchi products have been found among the artifacts from the Tomb of Western Han Dynasty of Mawangdui, located at Chang-shu in Hunan province.[85] Fermented whole soybean products similar to douchi spread throughout Asia, for example *tausi* or *taosi* in the Philippines, *taosi* in Malaysia, and natto in Japan.[84]

In China, douchi can be produced by either fungal strains, *Aspergillus* and *Mucor*, or by bacterial strains. Aspergillus-fermented douchi is the most popular, with production identifiable for more than 2000 yr.[86] Currently, bacterial douchi is made not by the use of pure microbial cultures, but by natural fermentation.[87] The imported Chinese douchi product sold in America is generally labeled "salted black beans" or "fermented black beans." Although neither term indicates that the food is made from soybeans, the "black" is actually a descriptor of the color of the soybeans resulting from the fermentation process. Besides salt and soybeans, the ingredients often include ginger root, five-spice powder, and orange peel.

Douchi can be either salted or unsalted. China's most famous *materia medica*, the *Ben-Cao-Kang-Mu*, compiled in 1578 by Li Shih-chen and republished in 1957, gives the most detailed descriptions to date of both salted and unsalted douche. It explains how black soybeans were to be used when making douchi for medical purposes, and that a liquid derived from salt-free douchi made from black soybeans was widely used in treating diseases. Li described the many ways that the different types of douchi and douchi liquid were used medicinally. By the time of the founding of the People's Republic of China in 1949, however, unsalted douchi was not widely

available. The three basic varieties of salted douchi that were available consisted of plain salted, salted with ginger, and salted with five-spice seasoning. In many cooking recipes, douchi is used as the basic salt seasoning, replacing or reducing the need for table salt as well as adding the rich savory flavor of douchi to the dish. Douchi has also commonly been used in folk remedies for the common cold, sore throats, fever, and diarrhea both in modern China and in Taiwan.[84,88]

16.3.1.3.2 Manufacturing Process

Douchi is produced from steamed soybeans. The soybeans are washed, soaked for 3 to 4 h at room temperature, then drained and steamed for 4 h to achieve 52% water content in the soybeans. After cooling to 28 to 30°C, the steamed soybeans are mixed with 5% fungus inoculum, *Aspergillus oryzae*, and incubated for 4 d under controlled temperature (28 to 30°C) to make koji. The koji is washed 2 to 3 times, then water, salt and spice are added to the mixture. After mixing with a dressing, douchi is fermented and aged by storing in sealed containers. At this stage the fermented douchi is called wet douchi, containing approximately 53% water, and can be consumed directly. Alternatively, the wet douchi can be sun-dried until the moisture content is reduced to 20% to make dry douchi.[89] Salt (NaCl) is typically added to douchi to select the microorganisms responsible for flavor and taste development, and to inhibit the growth of pathogenic and spoilage microorganisms for shelf life extension.[90]

16.3.1.3.3 Health Effects

16.3.1.3.3a Antihyperglycemic Effect

A water-soluble extract of douchi extract (DE) has been reported to strongly inhibit rat intestinal α-glucosidase, dose-dependently. DE elicited a significant antiglycemic effect at a minimum effective dose of 0.3 g.[91] The 50% inhibitory concentration (IC_{50}) value of DE in rat intestinal α-glucosidase inhibition using sucrose as a substrate is reported to be 0.34g/l.[92]

Fujita et al.[93] examined the antiglycemic effects and the safety of long-term ingestion of DE in subjects with borderline and mild type-2 diabetes in a double-blind randomized comparative study in Japan. All the subjects ingested *houji*-tea with or without 0.3 g of DE before each of three meals per day for 3 months. Houji tea was used to deliver DE because it is a common beverage habitually taken with each meal in Japan. The results of the experiment showed that the fasting blood glucose and HbA1c levels of subjects were significantly reduced at the end of the 3 months. Triglyceride levels were also significantly decreased at 3 and 6 months post-ingestion of DE. In addition, DE inhibited only α-glucosidase, without changing the activities of the gastrointestinal proteases amylase and lipase.

The anti-α-glucosidase activity of commercial douchi products collected from different parts of China, and douchi prepared in the laboratory using three strains of fungi, *Aspergillus oryzae*, *Actinomucor elegans,* and *Rhizopus arrhizus*, have also been studied. Various degrees of inhibitory activity against rat intestinal α-glucosidase were observed in all the commercial products. The anti-α-glucosidase activity of *douchi* fermented with *A. oryzae* were higher than those for either *A. elegans* or *R. arrhizus.*

16.3.1.3.3b Fibrinolytic Activity

Microorganisms are important sources of thrombolytic agents. Fibrinolytic enzymes have been found in traditional fermented soybean foods such as natto and *chung-kook-jiang* in Japan and Korea, respectively. Fibrinolytic enzyme (nattokinase) produced by *Bacillus* spp. has been purified from natto,[94–96] whereas subtilisin CK produced from *Bacillus* sp. Strain CK-11-4 was isolated from chung-kook-jiang,[97] and subtilisin DJ-4 produced by *Bacillus* sp. Strain DJ-4 was screened from Korean *doen-jang*.[97] Fibrinolytic activity has also been found in douche,[98] from which some fibrinolytic enzyme-producing bacterial strains were isolated and identified as *B. subtilis*. *Bacillius amyloliquefaciens* DC-4 was first screened from douchi[99] and a novel enzyme, subtilisin DFE (douchi fibrinolytic enzyme), produced by *Bacillus amyloliquefaciens* DC-4 was isolated from douchi and cloned by Peng et al. recently.[100,101] This enzyme, which belongs to the serine protease group and has a molecular mass of 28 kDa and pI of 8.0, displays thermophilic, hydrophilic, and strong fibrinolytic activity, with a high substrate specificity for fibrin. As thromboembolism is a frequently lethal complication of many medical diseases and surgical procedures, it has been suggested that fibrinolytic subtilisin DFE should be given orally as a thrombolytic therapy agent. However, whether or not the purified subtilisin DFE induces the lysis of the thrombi in vivo, as well as a consideration of the possible side effects, warrant further investigation.[100]

16.3.1.3.3c Antioxidant and Associated Activities

Fourteen antioxidants that act as DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavengers have been isolated from a commercial douchi product.[88] These included four phenol compounds (*p*-hydroxybenzoic acid, vanillic acid, syringic acid, ferulic acid), one isoflavonone (hihydrodaidzein), eight isoflavones (8-hydroxydaidzein, 8-hydroxyglycitein, 8-hydroxygenistein, 6-hydroxydaidzein, daidzein, glycitein, and genistein) and one novel 4-pyrone compound (3-[9E0-2-carboxyethenyl]-5-(4-hydroxyphenyl)-4-pyrone-2-carboxylic acid). The mushroom tyrosinase and soybean lipoxygenase inhibitory activities, extrogenic activity, and antimutagenic activity toward AF-2 in the Ames test were also examined for these DPPH radical-scavenging compounds.[88] Among these 14 compounds, 6-hydroxydaidzein had the highest mushroom tyrosinase inhibitory activity, with an IC_{50} value of 10 μm. At a concentration of 13 μM, isoflavones showed inhibitions ranging from 39 to 53% of AF-2 using *Salmonella* Typhimurium, whereas phenolic compounds did not show high antimutagenic activity. It is known that isoflavones show estrogenic activity, and have high estrogen receptor binding affinity.[102] The order of estrogenic activity of these compounds is reported to be as follows: genistein>daidzein>>3′+-hydroxydaidzein>8-hydroxygenistein, using a green fluorescent protein expression system.

16.3.1.3.3d Isoflavone Content

Isoflavones are known to be important functional compounds in soybean products. The main isoflavones found in soybeans are daidzein, genistein and glycitein, each of which can exist in four chemical forms: aglycones (daidzein, genistein, and glycitein), β-glucosides (daidzin, genistin, and glycitin), acetylglucosides (6″-O-acetyldaidzin, 6″-O-acetylgenistin, and 6″-O-acetylglycitin), and malonylglucosides (6″-O-malonyldaidzin, 6″-O-malonylgenistin, and 6″-O-malonylglycitin).[103] The processing

techniques used are thought to cause significant differences in the distribution of these isoforms in soybean foods.[104,105] The isoflavone glucosides have been reported to be hydrolyzed into their corresponding aglycones during the fermentation of soybean foods,[106,107] and isoflavones in the form of aglycones have been shown to display a higher bioavailability than isoflavones in the form of glucosides.[108,109] Douchi has a high content of isoflavone aglycones, with an aglycone content that increases from 1.82 to 95.02% during the transformation from cooked soybeans to douchi at 10% salt content. The enzyme β-glucosidase derived from the microorganisms involved in douchi fermentation is thought to be a key enzyme for the conversion of isoflavone forms during processing.[90] Because salt is a basic ingredient in producing douchi, the high NaCl content in douchi inhibits the β-glucosidase activity, thus preventing isoflavone glucosides from being converted into glycones. Consequently, low NaCl douchi is richer in isoflavone aglycones than douchi with a high NaCl content. From the health point of view, in terms of reducing sodium intake, and increasing the consumption of isoflavone aglycones, low-salt douchi therefore appears to be more desirable for future douchi product development.

16.3.1.3.4 Safety Concerns

The safety of 12-week douchi extract (DE) ingested by healthy subjects and its antiglycemic effects and lipid metabolism after long-term (6 months) ingestion in diabetic subjects were investigated.[110] Nine healthy subjects were given 1 g of DE before every meal (3g/d) for 12 weeks, and 18 type 2 diabetic patients ingested 0.3 g of DE before every meal (0.9 g/d) for 6 months. In this study, subjects complained of none of the side effects involving the gastrointestinal system, such as abdominal distension, abdominal pain, diarrhea, flatulence, and retching, that are a problem with most current therapeutic α-glucosidase inhibitory drugs.[111] These drugs have been proven effective in controlling hyperglycemia in patients with type 2 noninsulin-dependent diabetic mellitus. They inhibit the activity of the disaccharide hydrolases that convert disaccharides to monosaccharides, thus impeding digestion and the absorption of glucose, and consequently attenuating increases in postprandial plasma glucose levels.[112] However, DE treatment failed to induce any of these problems, and did not cause any hematological and biological abnormalities. In addition, there were no significant changes in body weight or BMI.[110] Therefore, DE may be proved useful as a way to safely control hyperglycemic conditions in type 2 diabetic patients.

16.3.2 Fermented Rice Products

Rice (*Oryzae sativa L. Gramineae*) is the most important staple food in China, which has long been recognized as one of world's largest producers and consumers of rice. As much as 80 to 90% of the daily caloric intake of people in China is derived from rice, and China contributes 38% of the world's rice production using 24% of the world's growing area.[113] In addition to the standard forms of rice cereal and rice flour products, many forms of fermented rice products are produced as alternative food sources, due to their nutritive values and special functional and sensory attractions. Fermented rice products such as *tian jiu-niang,* red mold rice, wine, and vinegar are described below.

16.3.2.1 Red Mold Rice

16.3.2.1.1 Background and History

Red mold rice (RMR), also known as *hongqu* (Chinese), red yeast rice, red fermented rice, red koji, red *kojic* rice, or *anka,* is fermented rice on which the food fungus species *Monascus* has been grown.[114] The use of RMR was first documented in the Tang Dynasty (618 to 907 A.D.).[115,116] RMR was described as "sweet in flavor and warm in property" by Li Shizhen, the great pharmacologist of the Ming Dynasty (1368 to 1644 A.D.), who also reported that RMR promotes digestion and blood circulation and can strengthen the spleen and dry the stomach.[117,118] It has traditionally been used in Chinese food as a preservative, food coloring and flavoring agent, and diet supplement, in addition to its use as a food medicine for improving digestion and enhancing blood circulation.[119] *Monascus,* especially *M. purpureus,* has also been used as a starter culture for manufacturing both red rice wine and red rice vinegars, as well as in red furu to enhance ripening during processing.[120] The Chinese Ministry of Health has included the RMR in their modern food additive standards as part of the Chinese diet.[121] RMR is sold in jars at Asian markets as a pasteurized moist aggregate, as whole dried grains, or as a ground powder. These products can be conveniently added to meat, fish, or soups during cooking to give an attractive color and enhanced taste. Typical consumption of RMR ranges from 14 to 55 g per person per day.[122] RMR has been used in the Asian-American community in the United States since World War II, and is now well known, having gained wide acceptance throughout the world for its serum cholesterol-lowering effect.[116] In the United States, RMR is marketed in the form of a food supplement called Cholestin ™ (Pharmanex, Inc.); in Singapore, it is available as Hypocol™ (NatureWise™, Wearness Biotech & Medicals PTE Ltd.); and in China, it is sold under the brand name Xuezhikang (Beijing WBL Peking University Biotechnology Ltd.).[123]

16.3.2.1.2 Starter Culture

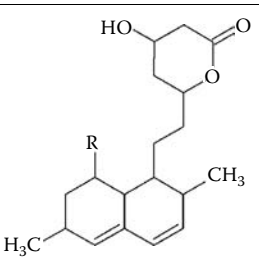
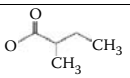
The fungus *Monascus* first became known in Western society when van Tieghem (1884)[124] noted the use of RMR powder by local populations in Java. The first species that was isolated from RMR was named *Monascus purpureus* by Went in 1985[125] in recognition of its purple color.[126] *Monascus* species belong to the group of ascomycetes and particularly to the family of *Monascaceae*. The genus *Monascus* is divided into four species: *M. pilosus*, *M. purpureus*, *M. rubber*, and *M. froridanus*, which comprise the majority of strains isolated from traditional Oriental food.[127–129] Today, more than 30 *Monascus* strains have been deposited with the American Type Culture Collection. The pigments produced by *Monascus* strains have also been extracted and purified as a natural food colorant. *Monascus* strains can be used to produce at least six major free pigments, divided into three groups: (1) orange pigments, namely monascorubrin and rubropunctanin, (2) yellow pigments, ankaflavin and monascin, and (3) red pigments, monascorubramine and rubropuctamine, as well as complexed pigments. These pigments link to proteins, peptides, amino acids, and nucleic acids in the product or cultural medium.[127,130,131] A number of toxicity studies of *Monascus* pigments have been reported, but their toxicities have not yet been clearly defined.[114,132]

In addition to pigments, a group of secondary metabolites produced by *Monascus* spp. and found in RMR are the major active functional compounds known as monacolins, which have the ability to reduce blood cholesterol levels in both animal models and in humans.[116,133,134] One of the main monacolins, Monacolin K (also known as mevinolin or lovastatin) was first isolated from *Monascus* rubber,[135] and also independently by Alberts et al.[136] from *Aspergillus terreus.* Monacolin K, is an inhibitor of the enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, which is a key step in cholesterol biosynthesis.[136,137] Monacolin K–related compounds, monacolin J and monacolin L, were isolated and reported in 1985,[138] then dihydromonacolin L and monacolin X were found,[139] and monacolin M has been reported to have been extracted from *M. rubber.*[140] All of these monacolins have been found to be effective as hypocholesterolemic agents. Monacolins exist in both lactone forms and hydroxyl acid forms.[128,141] There have been 7 monacolins isolated from *M. purpureus*-fermented rice by Ma et al.,[126] and 14 monacolin compounds including monacolin K, J, L, M, X and their hydroxyl acid form, as well as dihydromonacolin K, dihydromonacolin L. compactin, and 3α-hydroxy-3,5-dihydromonacolin L, among others, have been identified in ten commercially finished RMR products.[142] The structures of selected monacolins are summarized in Figure 16.4. It should be noted that the monacolin contents of these products differed considerably, not only in individual components but also in the total quantity present.[142,143] In addition to monacolins, *Monascus* spp. produces an antihypertensive substance, γ-aminobutyric acid (GABA).[128,136,144,145] GABA is the main suppressive nerve transmitter for the central nervous system.[146] Different strains of *Monascus* have been shown to produce different amounts of GABA,[145] and the production of these secondary metabolites can be affected by the composition of the medium,[127,147–149] as well as by environmental parameters such as agitation,[150] temperature,[131] and moisture content.[151]

16.3.2.1.3 Manufacturing Process

Traditional methods of manufacturing RMR cultivate *Monascus purpureus* on polished rice.[152] The rice is first soaked in water for about an hour, then steamed and cooled to about 55 to 58°C. Inoculation is done by mixing *M. purpureus* spores or powdered RMR with the cooked rice at levels of 0.4 to 0.6% by weight. The mixture is then incubated at room temperature for about 7 d. The fermentation causes an elevation of temperature, so during this stage the rice mix is turned several times to keep the temperature within the range 35° to 45°C. The temperature can be controlled by ventilation or by spraying with cold water at intervals. The rice grains gradually turn red in color, and they are fully cultured when each rice grain becomes bright red in its core, and reddish purple on the outside. The fully cultured rice is then either sold as a wet paste or dried to a water content of less than 10%, and sold as the dried grain. A fine powder of pulverized RMR grain is also available. China is the world's largest producer of RMR, with annual production reaching 7,000 tonnes in 2006 according to a report from the World Industrial and Commercial Organization Forum.[153]

RMR consists primarily of cooked nonglutinous rice, red fungi, and secondary metabolites of the fermentation process. Ma et al.[126] undertook a complete study of the chemical constituents in RMR and reported its ingredients to be: total carbohydrate (73.4%), fiber (0.8%), crude protein (14.7%), moisture (6.0%), pigments (0.3%),

Structure	Compound	R	Reference
	MK		135,136
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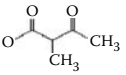
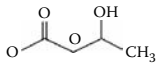
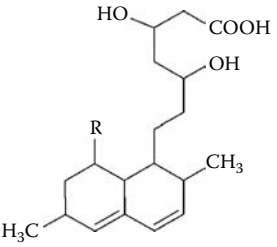
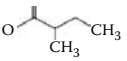
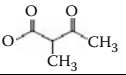
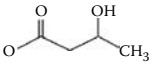
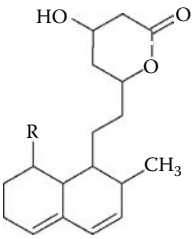
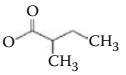
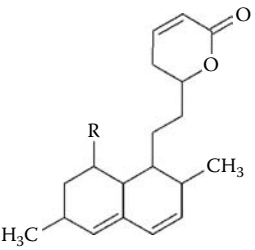
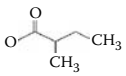
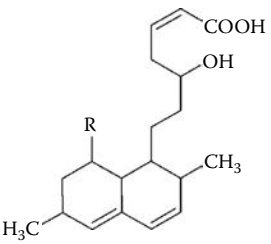
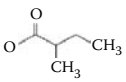
	ML	H	138
	MX		139
	MM		140
	MK acid form		141
	MJ acid form	OH	
	ML acid form	H	
	MX acid form		
	MM acid form		
	Compactin		195, 196
	DehydroMK		126
	DehydroMK acid form		197

FIGURE 16.4 Structure of monacolins in red mold rice.

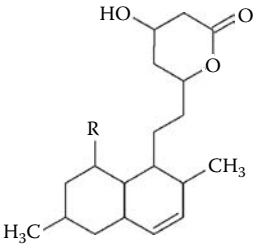
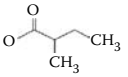
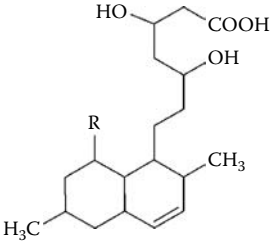
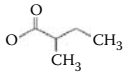
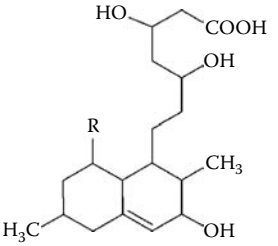
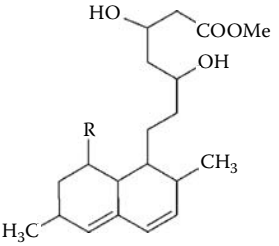
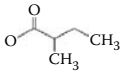
Structure	Compound	R	Reference
	DihydroML	H	139
	DihydroMK		
	DihydroMK acid form		198
	3α-hydroxy-3, 5-dihydroML	H	142
	Methyl ester of lactone ring opened MK		199
	Methyl ester of lactone ring opened ML	H	

FIGURE 16.4 Structure of monacolins in red mold rice. (continued)

ash (2.45%), phosphorus (0.4%), organic phosphorus (0.02 %), monacolins (0.4%), fatty acids (2.84%), vitamin C (0.03%), and vitamin A < 70 IU/100g. The trace elements found in RMR (μg/g) were: Ca (352), Mg (1092), Na (2370), Al (78), Fe (36), Mn (19), Cu (3), Zn (12), and Se (< 0.25). Of these trace elements, magnesium, and sodium were the most abundant metal elements. Gas chromatographic analysis of the fatty acids

revealed that the lipid content (%) of RMR was made up of palmitic acid (0.56), stearic acid (0.50), oleic acid (0.62), linoleic acid (0.74), arachidic acid (0.09), linolenic acid (0.36), total unsaturated fatty acids (1.43), and total fatty acid (2.84).[126]

16.3.2.1.4 Health Effects

16.3.2.1.4a Hypolipidemic Effect

Because RMR contains multiple functional compounds derived from the fermentation process, extensive studies have been done on both RMR products and the starter strains of *Monascus.* In several animal models of hyperlipidemia, including rabbits, quails, and hamsters, the RMR treatment group showed reduced serum total cholesterol and triglycerides, as well as the suppression of atherosclerosis by an atherogenic promoting diet.[132,154]

The lipid-lowering effect of RMR has also been assessed in several clinical trials. A multicenter, single-masked clinical study[134] involving 446 patients with hyperlipidemia demonstrated that oral treatment of 1.2 g/d of RMR (Cholestin 3™) preparation for 8 weeks reduced total cholesterol by 22.7%, low-density lipoprotein (LDL) cholesterol by 30.9% and serum triglycerides by 34.1%. The treatment also significantly increased high-density lipoprotein (HDL) cholesterol by 19.9%. In the experiment, 93.2% of these patients in the RMR group benefited from the RMR treatment, as assessed according to criteria established by the Ministry of Public Health of China. In addition, the total efficacy rate was nearly double that of the rate of 50.8% in the positive control group receiving treatment with a Chinese herbal medicine, *Jiaogulan* (*Gynostemma pentophylla*). Although mild side effects such as heartburn, flatulence, and dizziness were reported in a few patients, these symptoms resolved quickly.[134] Another double-blind, placebo-controlled, randomized 12-week clinical study[116] was conducted to examine the efficacy and safety of RMR supplement (2.4 g/d) in lowering cholesterol concentrations in an American population consuming a diet similar to the American Heart Association Step 1 diet. The results confirmed that there was a significant decrease in both total cholesterol and LDL-cholesterol with the RMR supplement. However, HDL cholesterol did not change significantly. No serious adverse effects in any of the 88 subjects were found in this study.[116] The findings from these clinical trials demonstrating cholesterol reduction conducted with a defined Chinese RMR preparation, however, may not be generalized to preparations that do not contain the same levels and distribution of monacolins.[143] Recently, RMR was used as a feed supplement (2 to 8% by weight) in hens' diet to produce low-cholesterol eggs.[155] The process of producing eggs with low cholesterol has been patented.[156]

The mechanism of the lipid-lowering effect of RMR is not yet well understood. Although it contains monacolins, which are HMG-CoA reductase inhibitors, the cholesterol-lowering effect is unlikely to be due solely to a single species of monacolin, but rather result from a combination of the actions of monacolins and other substances in RMR. The quantities of monacolins in RMR are inadequate to explain the magnitude of the lipid-lowering effects observed in these studies by comparison with evaluations of lovastatin.[116,157] RMR also contains significant levels of monounsaturated fatty acids, sterols (*b*-sitosterol, campesterol, stigmasterol, and their analogs), proteins, peptides and free amino acids, saccharides, isoflavone and its glycosides,

saponin, and sapogenin. It has been reported that considerable amounts of GABA have been found in RMR,[144] which is known to retard the elevation of systolic blood pressure[158] and also has diuretic effects.[146,149] Hence, the effective lipid-lowering effect of RMR may be attributable to a combination of these functional constituents.[154]

16.3.2.1.4b Amelioration of Impaired Memory and Learning Ability

A recent study reports the neuroprotection effect of ethanol extract of RMR (RE) against Aβ40 neurotoxicity in PC 12 cells. The in vitro results indicate that RE provides strong neuroprotection in rescuing cell viability as well as repressing inflammatory response and oxidative stress. The effects of dietary administration of RMR on memory and learning abilities were also investigated in this study in an animal model of AD rats infused with Aβ40 into the cerebral ventricle.[159] The RMR administration potently reverses the memory deficit in the memory tasks such as water maze and passive avoidance. Biochemical examination of the cerebral cortex and hippocampus of the animals show RMR is able to prevent Aβ fibrils from being formed and deposited in the hippocampus, and further decreases Aβ40 accumulation. This is the first report to use RMR to ameliorate the impairment of memory ability in an Aβ-infused rat model.[159]

16.3.2.1.5 Safety Concerns

Although RMR is authorized for food use in both China and Japan,[127] and has empirically been regarded as safe in Asia for centuries,[160] the discovery of citrinin (Figure 16.5) in RYR has led to concerns being expressed regarding the safety of using RMR in food. Citrinin, which was previously found mainly in *aspergillus* and *penicillium* genera,[161] is also produced by several strains of *Monascus*.[129,143,162] It has been detected in both solid and submerged cultured products of *Monascus* at levels from 0.2 to 122 mg/kg.[163] Citrinin is a potent nephrotoxin and hypatotoxin, and is known to cause functional and structural kidney damage and alterations in liver metabolism. However, no adverse effects have been reported as of yet, which might be attributable to the low concentrations applied in food technology.[129] Therefore, use of RMR is controversial and is still restricted in many countries.[132]

In light of the multiple health benefits that RMR and *Monascus* products offer, as well as the ill-defined toxicity of citrinin in these products, it would be acceptable to consume RMR as part of the daily diet as long as the citrinin content remains below the level that induces in vivo damage.[132] In Japan, the maximum allowed level of citrinin in RYR is 200 ng/g,[164] and only pigments of *M. purpureus* are authorized for food use.[127] In China and the European Economic Community, the allowable citrinin level in RMR is still not defined.[114] Most of the *Monascus* strains tested in

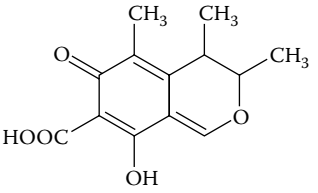


FIGURE 16.5 Structure of citinin.

China produced > 1 μg/g of citrinin and < 0.08 mg/g of monacolin in RMR.[114] Acute and chronic animal toxicity studies have shown that RMR is well tolerated, even at doses several times higher than the effective therapeutic dose.[132,134]

Efforts have been made to screen *Monascus* species to select or develop mutants of certain strains in order to maximize levels of monacolin K, while minimizing levels of citrinin. For example, *M. purpureus* NTU 568, mutated from *M. purpureus* HM 105 isolated from RMR, possesses a high concentration of monacolin K (9.5 mg/g) but only 0.94 μg/g of citrinin in RMR;[165] and another mutant strain *Monascus* spp. M12-69 can produce up to 2.52 mg/g of monacolin K and 0.13 ng/g citrinin in RMR under optimum conditions.[114]

In conclusion, RMR exhibits promising preventive and therapeutic properties and standardized manufacturing conditions should be established for RMR to be sold as a dietary supplement. It is important to ensure the consistent and equivalent content of active ingredients in RMR preparations, and to minimize the production of unwanted byproducts of fermentation such as citrinin. Studies on the long-term efficacy and safety of RMR supplements in a larger population will also be needed.

16.3.2.2 Rice Vinegars

16.3.2.2.1 Background and History

Rice vinegar is made from fermented rice or rice wine. Although similar in acetic acid content, its taste is usually milder than western white or cider vinegars. The use of rice wine and vinegar originated in China, where large scale rice cultivation dates back to 4,500 B.C. or before. The use of starters for making rice wine probably began around 4,000 B.C. and became common in the Spring–Autumn and Warrior periods (700 to 300 B.C.) in China.[166] Subsequently, the techniques for making rice vinegar spread to many adjacent regions, along with rice cultivation. Today in China, rice vinegars are grouped by color into three categories: black, red, and white. Among these, black vinegars, especially those that have been aged, are deemed most valuable.

Like winemaking, traditional vinegar makers produce vinegars with unique characteristics by utilizing and improving starters, formulations, and techniques that have been handed down from generation to generation. Today, some regions of China are still known for specialty vinegars created there in ancient times. Specialty rice vinegars, such as Zhenjiang aromatic vinegar and Zhejiang rosy vinegar, are particularly popular, and are recognized as being among the most famous of Chinese vinegars. Many popular Chinese dishes and medicines were developed using these specialty vinegars. In Chinese folk medicine, vinegar is used to treat a wide variety of disorders and complaints, including internal medicine, gynecological, dermatological, and traumatological conditions.[167] The Chinese saying that "food and medicine are of the same origin" represents a common attitude among Chinese people even today towards food, including rice vinegar and its functionalities.

16.3.2.2.2 Starter Culture

Rice vinegar production is characterized as a fermentation process that transforms glutinous rice and other ingredients through saccharification, alcoholic fermentation, and acidification phases into an aromatic, flavorful, aqueous liquid with acetic acid

as its primary ingredient. Preparing the starter culture is the first step for rice vinegar production. The most common rice vinegar cultures in China include small, large, and red starters. Traditional small vinegar makers use starter cakes made on site from previous batches, although some factories now use pure microbial cultures instead.

Traditional starters are typically rice or wheat cakes that have been cultured with molds, yeasts, and bacteria.[168,169] Before fermentation, the starter cakes are ground into a powder to facilitate subsequent mixing with steamed rice. The predominate fungi found in the small, large, and red starters belong to the genera of *Rhizopus* and *Mucor*, *Aspergillus*, and *Monascus*, respectively. These starters are used to create rice vinegars with unique characteristics. For example, small and large starters are used in combination to create the black-colored Zhenjiang aromatic vinegar,[169] whereas red-colored Zhejiang rosy vinegar is fermented using a red starter.[170] One unique aspect of starters in China is that the process changed from originally being made with cooked materials (Northern Wei, 386 to 534 A.D.) to most recipes using raw ingredients in the Song Dynasty (960 to 1279 A.D.). This development resulted in starters with higher and broader enzyme activities that not only promoted efficient fermentation, but also enriched the flavor of traditional rice vinegar.[171,172] The starter serves as an important source of enzymes that degrade rice to soluble products providing suitable substrates for later fermentation by yeast and bacteria.[173]

16.3.2.2.3 Manufacturing Process

Rice vinegar can be made using either a separate or concurrent fermentation process. In the separate method, rice saccharification and alcohol fermentation take place in distinct phases, whereas in the concurrent process, the two reactions occur in the same mash. Chinese rice vinegar is typically made by the concurrent process. This traditionally includes an initial step of gently polishing the rice to remove the hull and germ so that the fungal mycelia of the starters can easily penetrate and saccharify the rice.[173] After subsequent rinsing and soaking, the rice is steamed and then cooled to room temperature before a suitable starter is added to initiate rice saccharification and alcohol fermentation. At this stage, enzymes from the starter cake cause rice saccharification which, in turn, induces yeast growth, thus transforming the existing sugars to alcohol. In the case of zhenjiang aromatic vinegar, a small starter made from rice is used for the early saccharification and alcohol fermentation stage. This is followed by the addition of a large starter made from wheat to complete the later stage of saccharification and alcohol fermentation.[169] The final stage of the vinegar-making process is acetic acid fermentation, where acetobacter converts ethanol to acetic acid. Microorganisms (molds, yeasts, and bacteria) involved in the production eventually lyse, contributing to the product's rich content of nutrients. Chinese rice vinegar has high levels of amino acids, making it not only nutritious, but also overcoming the sharp taste from acetic acid, and thus making it an excellent complementary food.

Production of traditional rice vinegar is a labor intensive, time-consuming process. The operation is typically carried out in an open system, thus careful control of the temperature, humidity, and air transmission are required in order to discourage the growth of undesirable microorganisms. Furthermore, better quality rice vinegar requires at least 2 to 6 yr of aging to complete fermentation.[173] Consequently, modern

factories are continually incorporating new techniques to cut cost and increase production to meet the growing annual consumption in China, which has increased from 0.5 kg to1.5 kg per person during the period 1980 to 2000.[174]

16.3.2.3.4 Health Effects

The medicinal and health benefits of vinegar have been recognized in China for over two thousands years. In recent years, new scientific discoveries have confirmed that Chinese rice vinegar is an excellent dietary vehicle for conveying health benefits because its high amino acid content masks the sharp taste generally associated with acetic acid.

16.3.2.3.4a Vascular Disease Prevention

A recent study identified tetramethylpyrazine, a recognized phosphodieasterase inhibitor, in a popular Chinese rice vinegar, Zhenjiang aromatic vinegar.[175,176] The compound is also known to be a key component of *Ligustici Chuanxiong,* a herb that is widely used in traditional Chinese medicine to prevent cardiovascular and cerebrovascular diseases.[177,178] The study by He et al.[175] revealed the formation of tetramethylpyrazine in low concentrations (~5.8 μg/ml) during the acetic acid fermentation stage of vinegar production, and significantly increased concentrations during heating and aging. After 2 yr of aging, the zhenjiang vinegar was found to contain tetramethylpyrazine at a level > 500 μg/ml.

Other researchers have observed the hypertension prevention properties of dietary vinegar in animal models.[179,180] In the study by Nishkawa et al.,[179] for example, the antihypertensive effect of rice vinegar free from acetic acid was studied in spontaneously hypertensive male rats. A single oral administration of the vinegar extract reduced systolic blood pressure in test rats. After receiving the vinegar extract for three months, their blood pressure readings were significantly lower than those of the control group. Furthermore, Kondo et al. found that treatment with acetic acid (the main component of vinegar) alone significantly reduced blood pressure. They concluded that this reduction in blood pressure may have been caused by a significant reduction in rennin activity and a subsequent decrease in angiotensin II.[180]

16.3.2.3.4b Cancer Prevention

The presence of antioxidants in rice vinegar has received much attention in recent years due to their potential anticancer effects. Wu et al.[181] studied the source of antioxidant compounds in zhenijang aromatic vinegar and found that the amount of total phenolic compounds and flavones increased during acetic acid fermentation, and that both were related to 1,1-dipheny-2-picrylhydrazyl (DPPH) scavenging activity. The authors also speculate that the Maillard reaction, a chemical interaction between an amino acid and a reducing sugar in vinegar fermentation, is a major contributing factor to both product darkening and antioxidant formation.

A study of black rice vinegar known as *kurosu* also found significant levels of antioxidants in its ethyl acetate extract.[182] The extract (5 mg) inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced edema formation (14%), and myeloperoxidase activity (52%) in female ICR mouse skin. The extract also reduced the number of tumors per mouse by as much as 36% at 15 weeks after promotion.[182] Similarly, in a study on the effects of an ethyl acetate extract of Kurosu on the proliferation of a variety of human cancer cell lines, Nanda et al.[183] found the extract to

be a potential apoptosis inducer in Caco-2 cells. The primary source of antioxidants in various rice vinegars could be different, however. For example, the main active fraction of hengshun aromatic vinegar made in China accumulates primarily in the supernatant of an ethanol precipitate rather than in the ethyl acetate extract according to Wu et al.[40]

16.3.2.3.4c Postprandial Glycemia and Appetite Reduction

Exaggerated postprandial glycemia is believed to be associated with a heightened risk of chronic disease and disease complications.[184,185] Several recent studies have shown that adding vinegar or acetic acid to a diet may help reduce postprandial glycemia without altering the mealtime glycemic load. For instance, a study on the glucose response of healthy individuals who consumed test meals with known glycemic loads found that the addition of vinegar as a complementary food to a high-glycemic load meal, significantly reduced postprandial glycemia.[186] Similarly, others have reported the postprandial glycemic lowering effect of vinegar added to boiled potatoes, white wheat bread, or as a simple drink with breakfast.[133,187] The observed antiglycemic effect may be partially due to the suppression of disaccharidase activity by acetic acid, as demonstrated using Caco-2 cells.

In addition to its antiglycemic effect, vinegar also delays gastric emptying and increases satiety after meals.[187,188] It has been suggest that vinegar can significantly improve postprandial insulin sensitivity in insulin-resistant individuals similar to the physiological effect of acarbose or metformin.[186] It has been demonstrated that supplementing a meal based on white wheat bread with vinegar reduced postprandial blood insulin responses and increased the subjective rating of satiety in healthy volunteers.[189] This further shows the interesting potential of fermented and pickled products containing acetic acid.

16.3.2.3.4d Mineral Deficiency Prevention

Vinegar can enhance dietary mineral intake and absorption, which may in turn help prevent calcium and iron deficiencies and their associated illnesses, osteoporosis and anemia, respectively. A survey in China showed that the prevalence of anemia was unexpectedly low in some poor, rural areas of the northwest, despite dietary similarities between this region and others where anemia is endemic.[190] Scientists later found evidence that regionally specific use of iron pots, along with cooking and consumption of large quantities of rice vinegar and fermented cabbage, could be the reason for the lower prevalence of iron deficiency. The vinegar acid not only helps by releasing iron from cookware into the food, but also improves iron absorption, as shown in studies using animal and human intestinal cell models.[190,191]

Similarly, studies have shown that cooking beef or fish bones with vinegar can increase the calcium content of soup and positively influences calcium intake.[192,193] Furthermore, it has been suggested that dietary vinegar may help prevent osteoporosis. Their study showed that vinegar enhanced intestinal calcium absorption in ovariectomized rats by improving calcium solubility and reducing the bone turnover caused by ovariectomy.[194] Some dietary practices incorporating vinegar (such as the consumption of ginger vinegar soup recommended in traditional Chinese medicine for improving the health status of postpartum women) have now been found to convey beneficial concentrations of iron and calcium.[195]

16.3.2.2.5 Safety Concerns

Rice vinegar is considered a very safe condiment. The acidity of this product is generally mild and its sour taste naturally prevents excess consumption. Nonetheless, abnormal uses of vinegar products can be dangerous. For example, a report from Hong Kong shows that a woman attempted to use white vinegar to "soften" crab shell struck in her throat evidently suffered corrosive oesophageal injury.[196] This folklore application of vinegar to "dislodge" a foreign body in the throat should be discouraged. As a rule of thumb, patients should consult qualified medical doctors before using vinegar products to treat illness.

16.5 FINAL REMARKS

Food in China is not consumed simply to satisfying hunger, but also to enjoy its taste and flavor, and for its value in health promotion and treating diseases. Traditional fermented foods and beverages not only offer desirable sensory characteristics but many also convey health benefits, and thus they have played an important role in Chinese diets for millennia. The diversity of traditional Chinese fermented foods developed over thousands of years is immense. Although much of the art of the fermentation process has unfortunately been lost, a great deal has been preserved and flourishes today.

At present, industrial production of most traditional Chinese fermented food products remains small-scale, with uncontrolled processes and low efficiency. Although many fermentation processes are dependent on inoculation from a previous batch, starter cultures are commonly used for many commercial processes to ensure product quality. In order to meet the modern consumer's demand for large volumes of consistently high quality products, upgrading of traditional processes is unavoidable to ensure that traditional fermented foods continue to be available in order to maintain and strengthen China's cultural heritage, and allow domestic producers to compete successfully with imported products. Over the past decade, China has experienced explosive economic growth, while at the same time facing tremendous challenges in complying with international food quality and safety standards. Standardized systems, controlled operations, technically advanced processing techniques, and first-rate quality assurance and quality control are all involved in successfully up-scaling the food production process. Although fermentation enhances the nutritional quality of foods and contributes to food safety in general, under certain operational and hygienic conditions, some fermented foods may pose a safety concern. The principles of the Hazard Analysis Critical Control Point (HACCP) management system must be demonstrated and widely adopted by both large enterprises and small scale manufacturers of fermented foods in order to control the safety of raw ingredients and end products. A systematic and extensive exploration of the mysterious "medicinal diet" value of many traditional Chinese fermented foods will be of interest to food and nutrition researchers, manufacturers, consumers, and medical professionals. The health benefits associated with consumption of certain Chinese traditional fermented foods offers an interesting new area of investigation. The development of modern Chinese foods, based on these traditional sensory traits, is expected to result in economic, social, and health benefits. Traditional Chinese fermented foods

will continue to find a special niche in history, preserving the Chinese culture and remaining essential in the modern diet for generations to come.

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17 Tempeh

A Mold-Modified Indigenous Fermented Food

Daniel Y. C. Fung and Beth Ann Crozier-Dodson

CONTENTS

17.1 INTRODUCTION

Tempeh, or *tempe kedelee*, which originated in Indonesia, is made by the fermentation of dehulled, cooked soybeans with *Rhizopus oligosporus*. The mycelia bind the soybean cotyledons together into a firm cake. During the fermentation process, organoleptic properties developed in the product result in a clean, fresh, and yeasty odor. When sliced and deep-fat fried, tempeh has a nutty flavor, pleasant aroma, and a texture that is highly acceptable to most people around the world. Some people even consume tempeh in the raw state. Also, tempeh does not have the "beany flavor" associated with soybean products that are disagreeable to many Westerners. Due to increased populations of vegetarians in the world, soybean based foods are becoming more and more popular in the West. Indeed, tempeh has become known as the "vegetarian's burger," "veggie burger," or "tempeh burger." Unlike many indigenous fermented products such as *miso*, soy sauce, *natto, hamanatto, ang-kak*, and *sufu,* which are mainly used as flavoring agents, coloring agents, side dishes, breakfast items, and condiments, tempeh is used as a main dish in Indonesia. The purpose of this article is to present some of the manufacturing practices, properties, microbiological aspects, nutritional qualities, and chemical and biochemical changes in legume tempeh, and also the antinutritional factors associated with it. This review article is a synopsis of the paper by Hachmeister and Fung,[1] with additional materials on tempeh as a functional food.

17.2 MANUFACTURING PROACTICE OF LEGUME TEMPEH

Indigenous fermented foods constitute a large group of foods that are produced in homes, villages, small cottage industries, and even in larger commercial processing establishments. Other chapters of this book deal with many different fermented foods. This chapter concentrates on soybean tempeh production, microbiological aspects, and functional issues related to soybean tempeh.

17.2.1 Tempeh Fermentation

17.2.1.1 General Tempeh Preparation

The general process involves using soybeans that are soaked in water, the seed coat is removed, and the soybeans are drained and cooked, and then drained and cooled. They are then inoculated with spores of *R. oligosporus*, packed into trays,

and incubated 20 to 24 h at 30 to 38°C. Matured tempeh is ready for consumption either raw or cooked.[2] It should be noted that there are many ways to inoculate prepared soybeans for fermentation. Wrapping materials with previously manufactured tempeh can be used as an inoculum, pieces of successful tempeh (ragi tempeh) can also be used to inoculate the prepared soybeans similar to "back slopping" in sausage making. The most reliable method is to inoculate large numbers of spores of *R. oligosporus* into the prepared soybeans. U.S. Department of Agriculture (USDA) researchers in Peoria, Illinois, have done a great deal of work in developing starter cultures for making tempeh over the past 40 yr.

17.2.1.2 Homemade Soy Tempeh Production

Homemade tempeh[3] may be prepared by:

1. Placing whole, dry soybeans in water just brought to a boil
2. Soaking the soybeans in hot water for 8 to 16 h
3. Draining and dehulling (by hand)
4. Boiling for 30 minutes with added vinegar or lactic acid
5. Draining and cooling the soybeans (20 to 30 min)
6. Inoculating with tempeh starter (*R. oligosporus* spores)
7. Packing into containers
8. Incubating at 31°C for 22 to 26 h

The result is fresh tempeh cakes.

17.2.1.3 Small Factory Production of Tempeh

Small factory production[4] of tempeh is done by:

1. Size-grading raw dry soybeans
2. Heating for 10 min at 93°C
3. Dehulling in a Burr mill, 30 min at 100°C in dilute lactic acid
4. Boiling for 90 min in the soak water
5. Draining and cooling to 38°C
6. Mixing with the mold inoculum
7. Spreading on dryer trays
8. Covering the soybeans with a sheet of waxed paper and incubating at 38°C (75 to 85% relative humidity [RH]) for 18 h

The resulting tempeh is then dehydrated and packaged in plastic bags.

17.2.1.4 Tempeh Production on a Large-Scale Basis

Large-scale tempeh production[5] uses 10 kg whole dry soybeans. The soybeans are

1. Split with roller mill
2. Hulls separated with an aspirator

3. Washed and drained
4. Boiled 45 minutes (skiming off remaining hulls), drained and cooled with a centrifugal extractor
5. The hydrated beans (20 to 25 kg) are inoculated with 5 g of dry inoculum
5. 300 mL apple cider vinegar is added
6. Spread on perforated trays 2 to 3 cm deep
7. Covered with perforated plastic sheets and incubated at around 32°C for 16 to 24 h

17.2.1.5 Laboratory Tempeh Production

Two recipes have been published for the production of tempeh in the laboratory. Laboratory Production[6] Method 1:

1. Whole, clean soybeans are first dehulled with a Burr mill
2. The soybeans are then heated to boiling in water
3. They are soaked in the boil water for 22 h
4. The soybeans are then washed and remaining hulls removed
5. The soybeans are boiled for 40 min, drained, and cooled
6. The soybeans are inoculated with tempeh mold, wraped in cheese cloth, and incubated for 24 h
7. The inoculated soybeans are then transferred to perforated plastic bags (200 g/bag), and fermented 14 to 16 h at around 32°C

Laboratory Production[7] Method 2:

1. Whole, clean soybeans, are washed, and then soaked in excess water for 24 h at room temperature
2. The soybeans are boiled for 1 hour, dehulled, cooled and then dried
3. The soybeans are inoculated with *R. oligosporus* mold spores, packed into sterile petri dishes, and fermented for 18 to 24 h at 37°C

17.2.1.6 Laboratory Tempeh Production 3—Kansas State University Procedure[8]

1. Using a "Magic Bullet" blender or similar blender, small amounts of soybeans are blended in order to break the beans slightly and knock off the majority of the hulls. Prepare 3 lbs in this way. Do not over-blend the beans to fine particles as it will not allow *R. oligosporus* to have enough oxygen to grow; lactic acid bacteria will make the material anaerobic and no tempeh will be produced.
2. Wash and soak the soybeans overnight in twelve cups (2.84 L) of water and 35 mL of 85% lactic acid solution to prevent bacterial growth.
3. Change the water and boil the soybeans for about 1 h, stirring frequently with a strainer to remove floating hulls and to mash beans slightly.
4. Allow beans to cool to room temperature.

5. Add sterile water (ca. 5 mL each) to 2 to 3 test tubes that contain good growth (3 to 5 d old cultures) of *R. oligosporus* with heavy mycelium, and shake to dislodge spores into the water.
6. Pour the spores and water into the prepared soybeans in a big bowl and mix thoroughly with a spatula.
7. Use sterile-gloved hands to pack soybeans into sterile standard Petri dishes (100 × 15 mm). Do not pack too tightly as *R. oligosporus* needs air to initiate good growth.
8. Incubate for 24 to 36 h at 32°C in a laboratory incubator. Do not over incubate the tempeh because black spores will develop after the growth of the mold.
9. After fermentation, the soybean should appear as white patties with dense mycelium but no black spores. The mature tempeh easily comes out of the Petri dish.
10. Fry the patties in oil and serve while hot. Some people like to eat tempeh raw. Use of condiments (salt, pepper, catsup, salsa, etc.) are recommended (see Figure 1 and Figure 2).

17.3 TRADITIONAL TEMPEH FERMENTATION AND INDUSTRIAL PRODUCTION

17.3.1 Cleaning

Cleaning the soybeans is the first step in preparation for the production of tempeh. This step is carried out to remove dirt, weeds, seeds, damaged and possible decomposed beans, insects, worms, pebbles, sand, and other foreign matter.

17.3.2 Dehulling

R. oligosporus cannot grow on whole soybeans with hulls, and therefore dehulling is essential in the production of tempeh; dehulling enables the mold to reach the nutrients in the cotyledons. The soybeans can be dehulled by either wet or dry dehulling. Wet dehulling is generally done after precooking, which facilities hydration of the soybeans. This type of dehulling requires no mechanical devices other than hands or feet to rub the hulls from the cotyledons. Therefore, this method may not be feasible for large-scale tempeh production**.** In the dry dehulling process, the soybeans are heated for 10 min at 93°C to shrivel the cotyledons and loosen the seed coats. Dry dehulling is performed before any hydration procedures, and is a desirable, efficient method provided that suitable mechanical equipment is available. Burr, corn, or steel roller mills may be used to crack the hulls.[9] Following dry dehulling, the hulls can be separated from the cotyledons by the use of an aspirator, a gravity separator, or by a winnowing process.[9] Ko and Hesseltine[10] reported that abrasive dehulling of dry beans required less labor. By dropping the soybeans through an upward flow of air, the lighter hulls are carried upward and the heavier cotyledons fall to the bottom.[11]

17.3.3 Hydration and Acid Fermentation

Hydration is a process whereby soybeans are soaked in excess water for 12 to 15 h at room temperature in order to facilitate mycelial penetration. During this time, bacterial fermentation occurs, resulting in acidification. Many authors described the use of < 0.5% lactic acid or < 0.25% acetic acid. This will lower the initial pH, allowing the mold to grow and suppress bacterial growth. Mold is not inhibited until the pH falls to below 3.5.

17.3.4 Partial Cooking

Partial cooking has three functions: it destroys contaminating bacteria that could interfere with fermentation, it destroys antinutritional factors such as trypsin and chymotrypsin inhibitors, and it releases some of the nutrients required for mold growth. Cooking times vary from 10 min to 3 h. Boiling in excess water serves the purpose of partial cooking, which facilitates fungal penetration and digestion by humans.[12] Cooking by steaming for about 30 min at 100°C or 15 min at 95°C have been reported. During the boiling process, a heat-stable, water-soluble mold inhibitor is leached out into the water, which is discarded.[9]

17.3.5 Draining, Cooling, and Surface Drying

Traditionally, the boiling water is drained and the cotyledons are spread onto plated bamboo trays. Winarno and Reddy[12] suggested using a wire mesh or woven basket to drain the soybeans. Excess water favors bacterial growth and spoilage of tempeh. Winarno and Reddy[12] reported that the soybeans should be cooled to 38°C before inoculation. Cooled cotyledons should be dull in appearance, indicative of a relatively dry surface. Malaysians surface dry the soybeans by rolling them in a piece of cloth, whereas other manufacturers coat the beans with wheat flour.[7]

17.3.6 Inoculation

Traditionally, Indonesians used small pieces of soybean tempeh from a previous batch to inoculate soybeans (the ragi tempeh process). In the United States, pure culture (freeze-dried or suspended in water) inoculation is used to make tempeh. Excess inoculum promotes rapid and uniform fermentation. If too little inoculum is used, spoilage bacteria would be allowed to grow. For optimal fermentation, Wang et al.[13] recommended that 6 log spores per 100 g of cooked soybeans be used. Fermentation failure and excessive heat production were reported to be caused by insufficient packing density with pockets of air and heavy inoculation.[14] Detailed studies by Hesseltine along with his colleagues over 40 yr found over 40 different strains from 6 species of *Rhizopus* that were capable of producing acceptable legume tempeh.[15] Steinkraus et al.[16] originally believed *R. oryzae* to be the organism responsible for tempeh, however later Steinkraus[7] confirmed that *R. oligosporus* was the responsible mold. Shurtleff and Aoyagi[3] indicated that *R. oligosporus* is the principal species of mold used to make tempeh in Indonesia and North American because the mold has strong protease and lipase activities. However, they also indicated that other species

such as *R. oryzae*, *R chinensis*, and *R. arrhizus* can also be used to make soybean tempeh. According to Shurtleff and Aoyagi[3] *R. oligosporus* NRRL 2710 is the most widely used strain. NRRL 2710 along with NRRL 1526 and NRRL 2549 are available for research purposes from the USDA Northern Regional Research Laboratory (NRRL) in Peoria, IL.

Steinkraus et al.[16] suggested that a *Rhizopus* strain used for tempeh production should have the following characteristics:

1. Rapid growth at 37°C
2. High lipolytic activity
3. Production of strong antioxidants
4. Inability to ferment sucrose
5. Ability to produce the typical tempeh flavor, aroma, and texture
6. High proteolytic activity, resulting in the release of free ammonia after 48 to 72 h of fermentation

17.3.7 Fermentation Containers

Large wilted banana leaves, golden berry, or tropical almond leaves were reported to serve as excellent wrappings for traditional fermented tempeh.[7] Steinkraus et al.[16] developed 25 × 35 × 5 cm deep covered stainless steel pans for tempeh making, and later Steinkraus et al.[4] developed a small factory process in which 35 × 81 × 1.3 cm deep dryer trays were used. Leaves, plastic, glass, wood, or stainless steel can serve as appropriate container material as long as the following criteria are met:

1. Permits access of sufficient oxygen for mold grow
2. Not too much oxygen to promote sporulation and darkening of the mycelium
3. Temperature can be controlled
4. The soybeans maintain moistness during fermentation
5. No free water in contact with the soybeans
6. Fermenting tempeh remains clean and wholesome.

17.3.8 Incubation

The temperature, length of fermentation, and relative humidity are three crucial factors that dictate the outcome of tempeh fermentation. Fermentation temperatures ranging from 25 to 37°C; fermentation time is inversely proportional to temperature increase. Below are some time and temperature combinations suggested by various groups of researchers, assuming the inoculum level of 6 Log spores/100 gram of cooked substrate:

1. 25°C for 80 h
2. 35 to 38°C for 15 to 18 h
3. 25 to 37°C for 20 to 50 h
4. 32°C for 20 to 22 h

Another consideration is using pregerminated mold spores, which will reduce fermentation time by 17%. Relative humidity also influences the speed of fermentation. At 75 to 78% humidity, Steinkraus et al.[4] developed a fermentation pilot plant process requiring an 18 h incubation at 35 to 38°C. Conditions for producing high quality tempeh are quite variable. Temperature, relative humidity, fermentation time, oxygen availability, and other conditions must guarantee that the overall growth requirements of the mold are met. With proper control of these conditions, *R. oligosporus* grows at a rapid rate, and will yield tempeh in a minimum length of time.[9]

17.3.9 Harvesting, Storage, and Preservation

Tempeh should be harvested after the soybean cotyledons have been overgrown with mold and have knitted into a compact cake. Cotyledons should feel soft and pastry and not rubbery when pressed between the fingers. Fermentation is considered completed when the soybeans are covered with, and bound together by, the white mycelia of the mold. Successful raw tempeh should resemble a firm white cake (see Figure 17.1). Freshly made tempeh can be stored for several days at room temperature without adversely affecting the nutritional or organoleptic properties. Storage stability of tempeh can be extended by drying, frying, dehydration, freezing, blanching, steaming, and even canning. An interesting strategy called "deferred" fermentation was developed by Martinelli and Hesseltine.[17] In this method plastic tubing containing preinoculated soybeans was kept in a freezer to defer the fermentation process until the need for tempeh arose. They reported that the fermentation time for tempeh stored at –10°C and then thawed, was 36 to 38 h at 35°C. A preinoculated and packaged bag could be sold and kept frozen, and then when needed, allowed to thaw and then fermented at home at room temperature. This is similar to frozen dough technologies in the baking industry.

FIGURE 17.1 Mature tempeh after fermentation by *Rhizopus oligosporus* (Photo Credit: Joshua A. Reed).

17.4 USES AND PREPARATION OF TEMPEH

Tempeh is a very versatile product and can be used in combination with many different recipes and dishes. Tempeh can be served with grains and eggs for a breakfast item or in salads, sandwiches, burgers, sauces, or soups for a lunch or dinner. When using tempeh as an ingredient in recipes such as for salads, soups sauces, or casseroles, frying is a recommended step to ensure a crisp texture[3] Deep-fat frying and pan frying tempeh in vegetable oil or margarine yields a crisp, golden brown colored product (see Figure 17.2). Before frying, sliced tempeh can be dipped in soy or fish sauce, and a batter made from corn or rice flour and coconut milk.[7] According to Bates et al.[5] American vegetarians consume tempeh burgers of about 1.5 cm thickness. Furthermore, the fried tempeh can be served on a bun with tomato, onion slices, and lettuce.

17.5 ORGANOLEPTIC PROPERTIES OF LEGUME TEMPEH

Good quality tempeh should appear as a white, compact cake with a clean, fresh odor, yeasty odor and without a beany flavor.[12] Tempeh can be served in numerous ways such as fried in oil, baked, or used as a soup. Before eating tempeh, the bean cake is usually sliced and deep-fat fried until the surface is crisp and has a golden brown color; the flavor becomes nut-like and peppery due in part to the presence of free fatty acids.[12,18] Tempeh scored best at the end of the first phase of fermentation (30 h at 32°C), kept its food quality during the second phase (one additional day at 32°C), and deteriorates rapidly during the third phase.[19] The reasons given for tempeh's improved organoleptic qualities over unfermented soybeans have centered primarily on changes to the food due to the manufacturing steps (including fermentation).[15] Histological observations and the degree of penetration of hyphae of *R. oligosporus* into the soybean cotyledons are partially responsible for the rapid physical changes in soybeans during tempeh fermentation.[16,20]

FIGURE 17.2 Tempeh patties being fried and cooked in oil (Photo Credit: Joshua A. Reed).

17.6 MICROBIOLOGICAL ASPECTS OF TEMPEH

At first, microbiologists were interested in the type of filamentous fungi involved in the fermentation of tempeh.[10] Recently, much research has been directed to the total ecology of microbes involved in the fermentation of tempeh. It is not uncommon to have 8 Log colony forming units (CFU)/g to 9 Log CFU/g of bacteria and 7 Log CFU/g of yeast present during tempeh fermentation.[21] The exact roles of these organisms in tempeh fermentation are complex, and not completely understood; flavor developments, substrate modifications, synthesis of vitamins, and other growth factors influence tempeh quality and acceptability. The main mold in tempeh fermentation *Rhizopus* was not found to produce vitamin B_{12}. However, many bacteria grown along with *Rhizopus* have been found to produce vitamin B_{12}. One "unidentified Gram-negative rod shaped bacterium" was found to produce 148 ng of vitamin B_{12} per g of soybeans.[22] Normally, soybeans contain less than 1 ng of vitamin per gram. Effects of *Klebsiella pneumoniae*, *Enterobacter* spp., unidentified Gram-positive and Gram-negative rods on vitamin B_{12} production have been well studied.[12,23,24] More recently, Keuth and Bisping[25] reported the production of vitamin B_{12} by *Citrobacter freundii* or *K. pneumoniae* during tempeh fermentation, and proof of absence of enterotoxin producing genes by polymerase chain reaction (PCR) technology. *Enterococcus faecium* was reported by Moreno et al.[26] to produce two bacteriocins in Malaysian tempeh. Interesting, data were observed concerning the fate of folate during tempeh fermentation. Soaking and boiling of soybeans in preparation for tempeh fermentation causes major losses of folate. However, *Rhizopus* fermentation of boiled soybe caused an increase of 68 to 100% in under-conjugated and total folate contents respectively.[27]

17.7 IMPORTANT CONSTITUENTS OF SOYBEANS

17.7.1 Soybean Trypsin Inhibitors

Soybean trypsin inhibitors (SBTI) are undesirable to human health. According to Wang et al.,[28] fermented boiled soybeans showed higher trypsin inhibiting activity than extracts prepared from boiled soybeans. The extractable SBTI increased and reach a maximum after 48 hour of incubation; thereafter SBTI activity decreased. The increase in trypsin inhibitor was not synthesized by the mold, because no inhibitory activity was found in the culture filtrates of mold grown in laboratory media. An increase of trypsin inhibitor was also noted when heated soybeans were treated with a partially purified extracellular protease produced by *R. oligosporus*. Therefore, they concluded that an active trypsin inhibitor was liberated from a heat resistant, inactive, bound form by *R. oligosporus* protease. Once released, the inhibitor was readily inactivated by heat.

17.7.2 Gamma-Aminobutyric Acid

Gamma-aminobutyric acid (GABA) is an important compound that has a depressive neurotransmitter effect in the sympathetic nervous system. It has been reported that GABA retards the elevation of systolic blood pressure and improves discrimination

learning in rats. In Japan, highly purified GABA is used as medication for amelioration of the brain bloodstream.[29] The GABA content in aerobically fermented soybeans was about 30 mg per 100 g dry fermented soybeans, while the anaerobical cultivation resulted in about 370 ng per 100 g dry fermentation. After studying several strains of *Rhizopus* spp., all *R. oligosporus* and *R. oryzae* accumulated GABA in anaerobically fermented soybeans. Studies of GABA enriched tempeh-like fermented soybeans fed to animal models indicated that GABA systolic blood pressure in spontaneously hypertensive rats was significantly reduced by the GABA-tempeh as well as that with authentic GABA, when compared with the controls.[30]

Kim et al.[31] reported the purification and characterization of TPase, a fibrinolytic subtilisin-like protease from a *Bacillus subtilis* TP-6 culture isolated from Indonesian tempeh. The fibrinogen degradation pattern generated by TPase as a function of time was similar to that obtained with plasmin. On plasminogen rich fibrin plates, TPase did not seem to activate fibrin clot lysis. TPase converted the active plasminogen activator inhibitor-1 to the latent form.

17.7.3 Antioxidants

An excellent paper published by Gyorgy et al.[32] described the role of antioxidants produced in tempeh (fermented soybeans) compared with plain boiled soybeans. They concluded that the antioxidants liberated in tempeh likely act by protecting and preserving the biologically available vitamin E in soybeans, and not by direct biological action. More recently, Berghofer et al.[33] studied the antioxidative properties of faba bean-, soybean-, and oat-tempeh. They studied the antioxidative potential expressed by the protective factor against the oxidation of lard or sunflower oil. All three raw materials showed pronounced antioxidative potential, but native flour from oats had an exceptionally strong antioxidative effect. Tempeh fermentation increased the antioxidative effects in all three raw materials, where the highest increase was obtained with flour derived from faba bean. The influence of conditions of fermentation on antioxidative activity was also tested during the production of tempeh from oats. Fermentation time, as well as the water/oat flour ratio, were found to have a highly significant influence ($P < 0.01$).

Dried tempeh is known to be significantly more stable to lipid oxidation compared with unfermented soybeans. Esaki et al.[34] isolated a new antioxidant from the methanol extract of tempeh and identified it as 3-hydoxyanthranilic acid (HAA), which is effective in preventing autoxidation of soybean oil and soybean powder. HAA also exhibited strong antioxidative activity in both water/ethanol and rabbit erythrocyte membrane host systems. HAA was not found in unfermented soybeans, but was produced during the incubation with *R. oligosporus* IFO 32002 and 32003. Hoppe et al.[35] isolated and purified a substance at Rf 0.58 that was identified as 5-(delta-tocopheroxy)-delta tocopherol, which was thought to be the main tempeh antioxidant. But they could not show that this compound had antioxidative activities. They concluded that the antioxidative effect of tempeh oil seems to be the result of a synergist effect of tocopherol (present in the soybeans), and amino acids liberated during fermentation process by *R. oligosporus*.

17.7.4 Isoflavonoids

Many papers have been published concerning the production of isoflavonoids in fermented and unfermented soy products in relation to various aspects of human health. According to Hutchins et al.,[36] "Interest in isoflavonoids phytoestrogens and lignans has been generated because of their antiproliferative effects *in vitro*, and the discovery of their presence in human urine and plasma. Mammalian lignans and isoflavonoids are diphenolic compounds whose structure and molecular weight are similar to steroid estrogen, and possess some estrogenic activity. Lignans and isoflavonoids influence steroid hormone metabolism, inhibit tyrosine-specific protein kinase, protein histidine kinase, and topo-isomerase I and II activity, reduce angiogenesis *in vitro*, inhibit malignant cell proliferation, and have mild antioxidative and antihemolytic properties." In their experiments they found that the availability of the isoflavones increased with consumption of tempeh compared with consumption of soybean pieces, despite lower amounts of isoflavones in the fermented soybean products.

To produce a tempeh-like functional food containing a high level of isoflavone with a high absorptivity, Nakajima et al.[37] analyzed changes in the composition of isoflavone during tempeh fermentation, and the difference in isoflavone content depending on the soybean variety and particular tissue. As a result, by adding soybean germ (hypocotyl) that contained a large amount of isoflavone, they were able to prepare a new isoflavone-enriched tempeh in the form of a granular fermented soybean-based food, which can serve as a nutritious supplement for the elderly.

Soybeans and soy products have been established as an important source of isoflavones. Many research works have been performed on this subject, and much more work will be performed in this area in the future.[37–41]

17.8 NUTRITIONAL QUALITY OF TEMPEH

The nutritional quality of tempeh is a vast subject, and will be dealt with only briefly here. Various tests with animals are frequently used to evaluate the bioavailability of different types of nutrient in foods. The protein quality of tempeh can be measured by methods such as the protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV), amino acid score, and chemical score. Protein quality is dependent on the supply of essential amino acids as well as the digestibility. Tempeh is easily digested and is even tolerated by patients suffering dysentery and nutritional edema.[10] Tempeh- and milk-based formulas were evaluated in the rehabilitation of children with chronic diarrhea. Recovery from diarrheal disease was reported to be faster with tempeh-based formula and resulted in better weight gain.[42] According to Shurtleff and Aoyagi[3] soybean tempeh contains 19.5% protein, 56% of which is actually usable protein. On a dry matter basis, tempeh contains approximately 55% crude protein, 14% fat, 28% carbohydrates, 3% fiber, and 3% ash.[12] Subsequent processing, such as pan frying or deep-fat frying in oil, will affect the nutrient composition through increased fat content.[3] Soybean researchers feel that the improvement of the tempeh PER during fermentation can be attributed to the better availability of amino acids from the tempeh, the greater digestibility of the tempeh protein, differences in the substrates or substrate mixtures, and conditions of production. Hackler

et al.[43] found that the nutritional value of tempeh decreased as the time of fermentation increased. However, these researchers discovered that the PER value remained constant throughout the course of fermentation times (0, 12 , 24, 36, 48, 60, and 70 h). Zamora and Veum[44] reported that fermentation of cooked soybeans inoculated with *R. oligosporus* improved the digestibility and NPU in rats fed diets containing tempeh. According to Hackler et al.,[43] deep-fat frying tempeh for longer than 5 min at 196 °C caused a decrease in the protein quality. Stillings and Hackler[45] concluded that consumption of deep-fat frying tempeh decreased the weight gain in rats. Wang et al.[46] reported that deep-fat frying the tempeh reduced the PER by 2.0%. Although some researchers have reported slight improvements in PER and digestibility, most investigators have found that rats do not utilize protein from tempeh any better than from the cooked substrates. No differences were observed in the growth rates of rats (PER or NPU) when autoclaved, unfermented soybeans and soybean tempeh samples were fed. Tempeh prepared from a mixture of soybeans and groundnut had a better protein quality than soy tempeh. Different conclusions have been reached by various research groups in determining the nutritive value of tempeh in rat feeding tests.

The bioavailability of minerals such as iron and zinc has been determined by rat feeding tests. Zinc availability was 1.22 times better in soybean tempeh than in boiled soybeans. The availability of zinc improved by fermenting the soybeans with *R. oligosporus*. Processing steps such as boiling, soaking, and fermenting decrease the level of phytic acid in legumes, thus preventing the chelation of minerals by phytic acid. More recently, Kasaoka et al.[47] studied the effects of tempeh on iron bioavailability and lipid peroxidation in rats. They concluded that fermented soybean tempeh increased liver iron, compared with unfermented soybeans, without promoting lipid peroxidation in iron-deficient anemic rats. The synthesis of vitamin B_{12} by certain bacteria has received much attention, especially in societies in which vegetarianism is practiced. Okada et al.[48] found that the vitamin B_{12} of tempeh increased 11.4-fold during storage. However, pasteurized tempeh produced in the United States that contained 0.12 ug/100 g and tempeh-burger containing 0.06 to 0.11 ug/100g had decreased vitamin B_{12} after storage.[49] Since a daily requirement of 0.1 to 1.0 ug of vitamin B_{12} for adults has been suggested,[50] a moderate daily consumption of tempeh (100g) would fulfill the daily requirement for vitamin $B_{12.}$

17.9 CHEMICAL AND BIOCHEMICAL CHANGES IN TEMPEH

The chemical and biochemical changes in tempeh have been reviewed in detail by Hachmeister and Fung.[1] Key observations are summarized in this section.

During a 72 h fermentation of tempeh, total soluble solids and soluble nitrogen increased from 13 to 28% and 0.5 to 2.5%, respectively, while total nitrogen remained fairly constant. Van Buren et al.[51] found that the solubility of solids reached 52% in a 72 h fermentation period and 28% in ethanol. The ethanol-soluble components were mainly lipids, where the water-soluble components were crude protein, lipids, and nitrogen-free extract (NFE). The NFE fraction is primarily composed of readily available carbohydrates, such as sugars, dextrins, and starches. The initial unfermented, soaked soybeans had a pH 5.0, but increased to pH 6.5, 6.8, and 7.0 during fermentation with free ammonia being noted in the later phases

of fermentation. *Rhizopus* spp. produce carbohydrases, lipases, proteases and other enzymes. The production of pectinase and amylase by the mold are not conclusive. Production of carbohydrases such as polygalacturonase, endocellulase, xylanase, arabinase, and alpha-D-galactosidase have been reported. Although carbohydrase activity of *R. oligosporus* is minimal during fermentation, substantial amounts of lipase and protease are produced. Lipase activity in tempeh fermentation was maximum at 24 h of fermentation, and was fully inactivated by heating at 60°C for 10 min. The production of extracellular lipase was found to be maximum at 25°C after 3 days at pH 6.5. Also, some strains of *Rhizopus* were found to be able to degrade aflatoxin B1 to a nonfluorescent compound aided by mono-oxygenase systems.

17.9.1 Changes in Protein and Amino Acids

Although no large differences exist in protein content between soybean tempeh and unfermented soybeans, there is an increase from 1 to 85 times the amount of free amino acids during fermentation. Lysine and methionine in horsebeans and tryptophan in soybeans increased considerably during tempeh fermentation. Stillings and Hackler[46] observed an increase in free amino acid content and in ammonia as fermentation time increased. The effect of tempeh fermentation on total nitrogen content is negligible but increases of free amino acids takes place. The essential amino acid index is hardly affected during a 24 h fermentation period, but longer fermentation times result in losses of threonine of 8.9%, lysine of 25%, and arginine of 13.5%.

Deep frying under 5 min had little effect on the amino acid composition of tempeh, except reduction of heat-sensitive lysine and cystine was observed. Although the effect of tempeh fermentation on total nitrogen content is negligible, during processing steps including dehulling, soaking, and cooking, a loss of nitrogen occurred. Dehulling and soaking account for 46.7% of solid loss and 48.2% of nitrogen loss. Cooking results in a 50.8% nitrogen loss. Fermentation of whole soybeans only accounts for 12.6 and 86% of solid loss and nitrogen loss, respectively. Differences between constituent concentrations of whole beans and unfermented controls are due to processing techniques such as dehulling and boiling in acidified water, whereas differences between unfermented controls and tempeh are attributable to the effects of fermentation. As a result of protein metabolism, a gradual pH increase can be observed from the initial pH of 4.5 to 6.0 after 26 h at 28°C or 18 h at 3 °C. Tempeh fermented for 48 h at 28°C or 30 h at 38 °C resulted in the pH leveling off at around 7.5 to 8.0.

17.9.2 Changes in Carbohydrates

Sucrose, stachyose, and raffinose, the main di- and oligosaccharides in soybeans decrease in concentration by 84, 65, and 50%, respectively, during soaking. Glucose, fructose, and galactose are present soak water, with glucose serving as the main substrate for microbial growth. Processing of soybeans into tempeh brought about favorable changes, including reduction in levels of starch and flatulence-causing oligosaccharides such as raffinose and stachyose. Processing of soybeans resulted in a much greater loss of soluble carbohydrate materials than did fermentation. Slight

increases in the concentrations of the unspecified monosaccharides and raffinose during fermentation are due to the attack by alpha-galactosidase on stachyose and by other chemical interactions between the other sugars. Therefore, monosaccharides and raffinose, which are end products of enzymatic degradation, increase with fermentation; sucrose and starch concentrations are reduced by fermentation.[52] Calloway et al.[53] found that tempeh was virtually nonflatulent when fed to humans. One of the reasons for this important phenomenon is the fact that stachyose, raffinose, and other flatulence-causing carbohydrates are reduced by fermentation. Sucrose concentrations also were lower in fermented tempeh than in raw soybeans.

17.9.3 Changes in Lipids

Rhizopus oligosporus has strong lipase activities hydrolyzing over a third of the neutral fat of the soybean during a 3-day fermentation period. Of the free fatty acids (FFA) that are liberated, only linolenic acid was used by *R. oligosporus*. Glycerides in raw soybeans are broken into free acids during the first 30 h of fermentation. Free fatty acids liberated from lipids during fermentation include palmitic, stearic, oleic, linoleic, and linolenic acid, with linoleic acid predominating. Murata et al.[54] found an increase in oleic acid content, but a decrease in linoleic acid content after fermentation of the soybeans. Total FFAs composition rose from 39 mg/100 g of unfermented soybeans to 10,678 mg/100g of tempeh during a 90 h fermentation. Stearic, oleic, linolenic, linoleic, and palmitic acids increased 559, 557, 250, 232, and 138, respectively in the fermented tempeh. Although frying the tempeh resulted in an increase of crude fat from 8.7 to 26.5%, this technique decreased the levels of the free fatty acids, including palmitic, stearic, oleic, linoleic, and linolenic acids.

17.9.4 Changes in Minerals

Concentrations of calcium, phosphorus, iron, copper, zinc, magnesium, and manganese tended to increase with fermentation time using *R. oligosporus,* but also resulted in a large decrease in potassium concentration. van der Riet et al.[52] concluded that the observed decrease in potassium concentrations was due to leakage of this element during fermentation; hence, significant levels of potassium were found in the "sweat" water that formed inside the plastic bags.

According to Haytowitz et al.,[55] a 100 g serving of tempeh provides 56, 19, and 16% of the Recommended Dietary Allowance (RDA) for iron, phosphorus, and calcium, respectively. The percent RDA is based on the RDA for males 10 to 22 yr of age. Research from Shurteff and Aoyagi[3] shows that a 100 g portion of soybean tempeh supplies 28, 24, and 14% of the RDA for iron, phosphorus and calcium, respectively. This improvement could be the result of decreased levels of phytic acid which decreases the bioavailability of minerals such as calcium, zinc, iron, and magnesium, because of its strong chelating properties. For this reason, the bioavailability of other minerals such as iron, calcium, and magnesium also may improve with tempeh fermentation.

17.9.5 Changes in Vitamins

According to Haytowitz et al.[55] 100 g of tempeh contains about 62% of riboflavin, 25% of niacin and 20% of pyridoxine of the RDA requirement for adult males 19 to 22 yr of age. van der Riet et al.[52] found that fermenting soybeans reduced thiamine concentrations to undetectable levels; however, riboflavin and nicotinic acid concentrations increased significantly as a result of synthesis by *R oligosporus*. Despite the thiamine concentration slightly decreasing wth fermentation, rioflavin, nicotinic acid, pyridoxine, and pantothenic acid concentrations increased during the conversion of soybeans into tempeh. Besides thiamine with some reduction, all the other vitamins increased from 1.2 times to 4.6 times, except cobalamin (B_{12}), which increased from 0.15 ng to 3.90 ug, an astonishing 26,000 time increase. Since foods derived from plants are thought to be devoid of vitamin B_{12}, soybeans would not be expected to contain vitamin B_{12}. Steinkraus[7] indicated that vitamin B_{12} is produced by *Klebsiella pneumoniae*, which is a desirable, and possibly essential microorganism in the natural fermentation process of tempeh. Truesdell et al.[49] reported that tempeh and tempeh burgers contained from about 0.06 ug to 0.12 ug/100 g of vitamin B_{12}. Liem et al.[21] reported vitamin B_{12} value of 0.4 to 6.2 ug/100 g of tempeh from Canada. The discrepancy may be due to extraction techniques, pasteurization, assay microorganisms used, or sources of tempeh.

17.10 ANTINUTRITIONAL FACTORS ASSOCIATED WITH LEGUMES

Although the mold responsible for producing tempeh does not produce aflatoxins, contamination with *Aspergillus flavus* and *A. parasiticus* is possible. Fortunately, *R. oligosporus* can hydrolyze aflatoxins thus preventing the accumulation of these mycotoxins in tempeh.

Reduction of ANF can be achieved by the following methods for specific ANF: flatulence-producing factors reduced by soaking an boiling; protease inhibitors reduced by temperatures of 110°C for 40 min; tannins reduced by soaking and heating; phytic acid reduced by dehulling, soaking, and fermentation; hemagglutinins reduced by cooking and heating, and favism-inducing factors reduced by heat. Other ANF in legume seed include favism-inducing factor (such as vicine and convicine), goitrogens, lipoxygenase, saponins, isoflavones, and others. Raw soybeans are goitrogenic because they interfere with iodine metabolism and thyroid function which leads to goiter.

17.11 CEREAL GRAIN TEMPEH

This article has been concentrated on soybean tempeh. Other substrates can also be used to make tempeh like products. Shurtleff and Aoyagi[3] reported that wheat-soybean, rice-soybean, millet and millet-soybeans tempehs could be produced in a fashion similar to soybean tempeh. Major steps in soy-wheat production include preparation of the soybeans and wheat, hydration of the soybeans, boiling, draining, cooling, and inoculation with a suitable strain of *Rhizopus* and incubation. Articles by Hesseltine et al.,[56] Wang and Hesseltine,[57] Wang,[58] Wang et al.,[46] provided

invaluable information on many steps involved in making tempeh from different substrates. Boiling time for various substrates such as soybeans, hard wheat, white wheat, barley, oats, rye, corn, sorghum, peanuts, and rice were provided. Sensory evaluation of cereal grain tempeh products were presented and organoleptic evaluation of fermented cereal grain tempeh products after frying was also provided. Readers are referred to those articles for details. At Kansas State University Ms. Kathleen A. Hachmeister developed a series of tempeh-like products she coined as "Hack-Snak" as part of her M.S. degree in 1993. She made tempeh-like products by mixtures of wheat/sorghum grain, wheat and yellow corn, triticale and sorghum grain and red milo and yellow milo. Details of that study were published in the paper by Hachmeister and Fung.[1]

In summary, tempeh is a versatile, inexpensive, nutritious, and delicious mold modified as a traditional fermented soybean food, which is gaining acceptance in Western culture, especially within the vegetarian communities. Advantages of tempeh include excellent digestibility, increased vitamin B_{12} content, content of bacteriocins, increased folate content, reduced levels of trypsin inhibitors, production of fibrinolytic subtilisin from *Bacillus subtilis* in tempeh, antioxidative properties, content of GABA, HAA, and isoflavonoids etc. compared to raw or boiled soybeans. Besides potential function food benefits, there are true nutritional benefits following consumption of tempeh. The intent of this review was to highlight some of the important points related to tempeh production, and the role of tempeh play in human health. Tempeh may not be a miracle food, but it certainly is a fermented food of great interest to contemporary society.

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18 Thai Fermented Foods

Microorganisms and Their Health Benefits

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CONTENTS

18.1 INTRODUCTION

Fermentation is the oldest biotechnology employed in manufacturing foods in Thailand. Traditional fermented products have played an essential role in the Thai diet and are consumed either as main dishes or as condiments. A variety of fermented foods have gained popularity not only because of their own unique characteristics in sensory quality, but also because of their nutritional value. Some of these foods

have become more economically important as commodities for both domestic consumption and export. Today, the interest in fermented items continues to contribute to the food industry by inspiring new products and lowering costs, and as a result an improvement has been seen in the microbial processes on which food producers have long relied. In Thailand, there are over 60 traditional fermented products that are consumed all over the country.[1] Thai fermented products can be categorized according to their source, main processing techniques, ingredients added, and microorganisms associated with the food. Although some products are produced by similar processing, some ingredients used can be varied, leading to differences in appearance, flavor, taste, as well as sensorial properties. In addition, several products are very similar to products found in other Asian countries.

18.2 PRODUCTION OF FERMENTED FOODS

Most of the traditional fermented products have been prepared using microorganisms that produce physical, nutritional, and organoleptic modifications of the starting materials. However, the majority of fermented foods are still produced traditionally at both the cottage and small-scale levels,[2] where the fermentation processes vary from simple to complex.[1] Over the years, the specific environmental conditions during natural fermentation have caused a gradual selection of microorganisms responsible for the desired final product. A number of fermented foods are still homemade, but some are produced at commercial factories.

18.2.1 Fermented Fish Products

Nam-pla (fish sauce), *ka-pi* (shrimp paste), and *bu-du* are produced from fish and contain a large proportion of salt. Most are made from marine fish in the coastal provinces, especially the east coast. There are many kinds of fermented fish that contain salt and carbohydrates, such as *pla-ra, pla-som, pla-chao, som-fak*, and *pla-chom*. The fish used are mainly freshwater fish, but freshwater shrimp are also used (see Table 18.1). Carbohydrates from cooked rice, roasted rice, etc., are added as a carbon source for the fermenting microorganisms, which consist mostly of lactic acid bacteria (LAB). These fermented food products have a sour taste, due to lactic acid and other acids formed during fermentation. The roasted rice also gives a brown color to these foods. There are two types of fermented rice used: *khao-mak*, which is mold and yeast fermented, and *ang-kak* rice, which is fermented using the mold *Monascus purpureus,* which gives a red color to the *pla-paeng-daeng.*

18.2.2 Fermented Meat Products

Fermented pork (*nham*) is made from minced red pork meat mixed with pork rind, garlic, pepper, salt, chilli, and trace amounts of potassium nitrate. The mixture is wrapped with banana leaves or plastic sheets and fermented for a few days. *Sai-krog-prieo* (fermented sausage) and *mam* (fermented beef or pork sausage or beef/pork liver) are produced from shredded pork or beef meat with fat, cooked rice, salt, sugar, pepper, and spices. All ingredients were packed into pig intestine, tied with string at intervals, and fermented spontaneously.

TABLE 18.1
Fermented Fish, Components, NaCl (%), Fermentation Period, and Distribution of Bacteria in Some Thai Fermented Foods

Fermented fish	Components	NaCl (%)	Fermentation peroid	Genera
Nam-pla	Fish and salt	22.8–26.2	12–18 months	*Tetragenococcus, Lentibacillus, Halobacillus, Halobacterium, Halococcus, Natrinema*
Bu-du	Fish, salt, and brown sugar	14.9–27.5	3–6 months	*Tetragenococcus, Staphylococcus*
Ka-pi	Planktonic crustaceans or small shrimp and fish	11.5–31.9	4–6 months	*Tetragenococcus, Virgibacillus, Lentibacillus, Salinicoccus*
Tai-pla	Fish bowels and salt	13.5–25.3	10–20 d	*Tetragenococcus*
Pla-ra	Fish, salt, roasted rice powder	7.8–17.9	6–10 months	*Lactobacillus, Weissella, Piscibacillus, Bacillus, Salinivibrio, Lentibacillus, Tetragenococcus, Virgibacillus, Enterococcus*
Pla-paeng-daeng	Fish, salt, red mold rice, cooked rice	4.5–9.2	4–5 d	*Tetragenococcus*
Pla-som	Fish, salt, cooked, rice, garlic	2.3–5.9	5–7 d	*Lactobacillus, Pediococcus, Enterococcus*
Som-fak	Fish, salt, cooked, rice, garlic	2.5–4.8	3–5 d	*Lactobacillus, Pediococcus*
Pla-chom	Fish, salt, garlic, and roasted rice powder	3.8–4.8	3–5 d	*Lactobacillus, Tetragenococcus, Enterococcus, Staphylococcus*
Pla-chao	Fish, salt, and khao-mak	4.4–9.5	10–20 d	*Lactobacillus*
Kung-chom	Shrimps, salt, and roasted rice powder	4.6–11.0	3–5 d	*Tetragenococcus, Enterococcus, Staphylococcus*
Hoi-dong	Mollusces and salt	6.6–15.1	4–5 d	*Lactobacillus, Tetragenococcus*

18.2.3 Fermented Plant Products

Naw-mai-dong (fermented bamboo shoot) is fermented by adding brine to sliced pieces of bamboo shoot, which are packed in jars. *Phak-gard-dong* (pickled green mustard) is fermented with the mixture of green mustard and brine and packed tightly in jars. *Miang* (fermented tea leaves) is produced in the northern part of Thailand. The steamed tea leaves are wrapped tightly in individual bundles and packed into containers (small baskets for miang made from young tea leaves, or large underground cement wells for miang made from mature tea leaves). The tea leaves are pressed tightly, weighted down, covered with banana leaves or plastic sheets, and spontaneously fermented for a week or many months.

18.2.4 Fermented Grains and Cereal Products

Fermented glutinous rice (*khao-mak*) is produced by loog-pang (a starter cake containing mold and yeast) as a starter; LAB can also be found in this food. A starter of *Aspergillus oryzae* is used for production of *koji* in soy sauce fermentation. LAB also contribute to the fermentation of rice noodle (*khanom-jeen*) and soy sauce. Rice wine, such as *sa-to, nam-khao*, *kra-chae*, and *ou*, is produced with loog-pang[3] by the microorganisms *Saccharomyces cerevisiae, Saccharomycopsis fibuligera, Aspergillus oryzae, Amylomyces rouxii, Rhizopus* spp., and *Mucor* spp.[3,4] In soy sauce fermentation, *Aspergillus oryzae* is also important for producing koji.[3] Although yeasts are also found in these fermented products,[5] LAB are responsible for both souring and ripening.[6] Red yeast rice, red koji, or *angkak* obtained using a *Monascus* culture on rice, has long been used as a natural food colorant.[7] Alkaline-fermented soybean foods are very common in northern Thailand. *Thua-nao* fermentation mainly relies on opportunistic microorganisms; Gram-positive, endosporeforming bacteria, have usually been found as the predominant microorganism.[8,9] Microorganisms involved in thua-nao fermentation have been isolated and shown to be *Bacillus subtilis*.[8,10] Hara et al.[11] reported that four strains of aerobic, Gram-positive, and spore-forming rods isolated from thua-nao produced in Thailand were taxonomically similar to *Bacillus subtilis* (*natto*). Protease and amylase produced by the bacteria decompose the protein and insoluble sugar in the raw soybeans, thus increasing the nutritional value as well as the nutrient availability of the soybean.[12, 13]

Because the natural fermentation method is difficult to control, and there are risks of accompanying microflora causing spoilage and unsafe products, starter cultures have now become more important in the production of many Thai fermented foods. For example, the first starter formula for *nham* fermentation was developed by Wiriyacharee et al.[14] A mixture consisting of lactobacilli, pediococci, and micrococci, along with fixed amounts of various ingredients, has been successfully and exclusively utilized in the industrial production of nham. Further developments of starter formula for nham have been carried out by Valyasevi et al.[15] to improve the control of the microbial processes, and to maintain product uniformity. Selected isolates from the dominant genetic groups were evaluated to determine both ability of the starter culture bacteria to ferment the starting materials, and the sensory quality of the final product. Among the bacteria selected and commonly found in fermented meat products, *Lactobaciilus plantarum* has been shown to be one of the most effective strains

as a nham starter culture.[15,16] At present, some of these starter cultures are being tested by various nham manufacturers. Besides nham, the cultures can be applied to various other kinds of fermented meat products, i.e., fermented pork sausage, fermented chicken sausages, fermented pork spare ribs, etc. Three formulations of the starter cultures are being marketed in Thailand.

18.3 MICROORGANISMS INVOLVED IN FERMENTATION

LAB and allied bacteria have been isolated from various food products (fermented fish, meat, vegetables, or plant products) in Thailand. They occur naturally with other microorganisms, and are responsible for souring and ripening. Although yeasts and other bacteria are also isolated, their numbers are much smaller than those of LAB. LAB are perhaps the most widespread and desirable microorganisms in food fermentations.[17] They convert most available carbohydrates to lactic acid, with small amounts of acetic acid, resulting in a lowering of the pH.[18]

Homofermentative strains of *Lactobacillus pentosus, Lb. plantarum, Lb. sakei, Lb. farciminis, Lb. acidipiscis, Lb. thailandensis*, *Lb. camelliae,* and other *Lactobacillus* sp., *Pediococcus siamensis, P. pentosaceus, P. acidilactici, Tetragenococcus halophilus,* and *T. muriaticus* strains occur in a variety of fermented Thai foods. Heterofermentative strains of *Lb. vaccinostercus, Lb. fermentum, Lb. brevis,* and other *Lactobacillus* sp., *W. confusa*, *W. thailandensis,* and *Leuconostoc* sp. can also be found. The DL-lactic acid producing *Lb. pentosus* and *Lb. plantarum* strains that contain *meso*-diaminopimelic acid in the cell wall are the predominant rod-shaped LAB found in fermented Thai foods. *P. pentosaceus* strains are the major coccal bacteria although *T. halophilus* strains occur in products containing high concentrations of salt. Recently, L-lactic acid–producing *Lb. farciminis* and *Lb. acidipiscis* have been found in salt fermented fish.[19,20,21] *P. acidilactici* and *Leuconostoc* sp. are the minor LAB in many fermented Thai foods. The LAB are widely distributed and they are not generally specific for one kind of fermented product. The concentration of salt often influences the flora found in the food. The lactobacilli containing *meso*-diaminopimelic acid in their cell wall, however, are usually found in fermented tea leaves (*miang*). The tannin in miang may have an effect on the growth of these cultures.

Other bacteria present include *Halobacterium salinarum, Halococcus* sp., *Natrinema, Salinivibrio, Salinicoccus, Bacillus, Lentibacillus salicampi, Ln. Juripiscarious, Ln. halophilus, Ln. kapialis, Piscibacillus salipiscarius, Staphylococcus carnosus*, *S. piscifermentans, Enterococcus hirae, E. faecalis, E. casseliflavus*, *E. camelliae* and other *Enterococcus* sp., as shown in Table 18.2.[6,22–26] *H. salinarium, Hbc. thailandensis,* and *Halococcus thailandensis* strains are important for fish sauce fermentation.[6,27–29] Recently, a unique proteinase was isolated from a moderately halophilic *Halobacillus* sp. SR5-3 isolated from fish sauce. This same proteinase was also described, as a 49 kDa-proteinase from *Filobacillus* sp. RF2-5, that may be involved in the degradation of fish protein during fermentation at high salt concentrations.[30,31] Staphylococci from fermented fish, which are coagulase-negative and haemolysin-negative, produce a small amount of lactic acid, but do not seem to play a role in the fermentation and ripening of foods.[32,33] Enterococci,

TABLE 18.2
Fermented Meat and Plant Products, Components, NaCl (%), Fermentation Period, and Distribution of Bacteria in Some Thai Fermented Foods

Fermented product	Components	NaCl (%)	Fermentation peroid	Genera
Nham	Minced red pork meat, pork rind, garlic, salt, cooked rice, pepper, chilli, KNO_3	2.5–2.8	3–4 d	*Lactobacillus, Pediococcus*
Sai-krog-prieo	Ground pork meat, fat and rind, cooked rice, salt, sugar, pepper, spices	1.1–1.9	2–3 d	*Lactobacillus, Pediococcus, Enterococcus*
Mam	Ground beef/pork, beef/pork liver, salt, garlic, roasted rice, cooked rice	2.3–7.1	3–4 d	*Lactobacillus, Pediococcus, Enterococcus*
Phak-gard-dong	Green mustard, salt, palm sugar, rice wash water	1.6–7.3	3–4 d	*Lactobacillus, Pediococcus, Enterococcus*
Phak-sian-dong	Young *Gynandropsis pentaphylla*, salt, sugar	0.6–2.5	2–3 d	*Lactobacillus, Pediococcus*
Naw-mai-dong	Bamboo shoots, salt, water, or rice wash water	0.5–6.4	1 month	*Lactobacillus*
Hom-dong	Spring onion, salt, sugar	1.5–2.3	4 d	*Lactobacillus*
Miang	Steam tea leaves	0.1–1.5	3–4 months	*Lactobacillus, Pediococcus, Enterococcus*
Khao-mak	Glutinous rice, *loog-pang* (starter)	0.1	3 d	*Lactobacillus, Pediococcus*
Khanom-jeen	Rice, water	0	1–2 d	*Lactobacillus, Pediococcus, Leuconostoc, Enterococcus*
Soy sauce, Tao-cheow	Soy bean, flour, starter mold	19.2–21.7	3–6 months	*Aspergillus oryzae, Lactobacillus, Tetragenococcus*
Thua-nao	Soy bean paste (Thai *natto*)	5.1	3–4 d	*Bacillus*
Tao-hu-yi	Soy bean curd	12.6–19.6	40–60 d	*Monascus*

especially *E. faecalis* and *E. faecium* strains, are common organisms in the intestinal tract of man and other animals, and it is difficult to keep them out of foods. Their presence in fermented foods such as those found in Thailand may indicate inadequate sanitary practices in food production and handling.[34]

18.4 POTENTIAL HEALTH BENEFITS OF THAI FERMENTED FOODS

For ages, the consumption of fermented food products has been associated with good health and longevity. Nevertheless, only a few foods have significant scientific research to back up these claims. Nearly all food fermentations are the result of more than one microorganism, either working together or in a sequence. For the maximal growth, these microorganisms should be able to secrete hydrolytic enzymes, assimilate, and/or convert some hydrolyzed substrates into structural components and secondary metabolites. It would be reasonable to suggest that fermented foods may contain various functional components either originating from the ingredients or formed during fermentation. Therefore, the health promoting benefits of food fermentation could be attributed to five main features:

1. Enhanced digestibility
2. Increased bioavailability
3. Increased micronutrient content, e.g., vitamins and cofactors
4. Probiotic and prebiotic properties
5. Microbial products such as enzymes, metabolites, and bioactive peptides released after enzymatic digestion of food proteins[35]

18.4.1 Enhanced Digestibility

The digestibility of nutrients in foodstuffs is often limited by the unwanted presence of antinutritional factors (ANFs), such as phytic acid, trypsin inhibitors, nonstarch polysaccharides (NSPs), oligosaccharides, lectins, and saponins.[36] These factors have been documented to cause gastrointesinal disturbances, intestinal damage, increased disease susceptibility, and reduced growth performance. Due to differences in their structure and their biological effects, the maximum destruction of ANFs may require different processing treatments. The action of microorganisms during the preparation of fermented foods has been shown to improve the digestibility of some dietary nutrients through destruction of ANFs. Moreover, microorganisms contain certain enzymes that are incapable of being synthesized by humans. Thus, these enzymes can hydrolyze complex food components into simple units, which are then readily digested or absorbed by humans.

Because fermentation increases the quantity of soluble proteins in foods, it may improve the amino acid profile, and because it reduces the levels of certain antinutritional effect, fermented foods are often more efficiently utilized than unfermented foods.[37,38] For example, the protein nutritive value of the soybean product thua-nao, as indicated by the protein efficiency ratio (PER), biological value (BV), and true digestibility (TD), was significantly higher than nonfermented soybean.[39]

18.4.2 Increased Bioavailability

The term *bioavailability* involves both absorption and utilization of the ingested nutrients within the body. Absorption is often limited by the chemical nature of the nutrients and interaction with other food components. The biological properties of soybean isoflavones include preventing chronic diseases, acting as phytoestrogen, antioxidant, and antitumoral activity.[40–44] However, the bioavailability of soybean isoflavones in humans depends on their metabolism capacity, and on the occurrence of hydrolysis by the action of β-glucosidase enzymes produced by the intestinal microflora. The β-glucosides have less estrogenic activity when compared with their respective aglycone forms. The aglycones have lower hydrophilic capacity and lower molecular weight, resulting in better absorption.[44,45] The β-glucosidases (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) are a heterogeneous enzyme group, which hydrolyse β-glucosidic links of oligosaccharides and other glucosides conjugates releasing glucose. These enzymes are widely distributed in plant sources and have a high specificity for isoflavones.[46] Several works report that β-glucosidases from microbial sources hydrolyze the β-glucosides of soybean into aglycones.[47–50] The enhanced effect on antioxidative activity varied with the starter microorganism. Berghofer et al.[51] reported that the antioxidative activity of fermented soybean products produced with filamentous fungi such as *Aspergillus* and *Rhizopus*, was significantly higher than in nonfermented soybean. It was suggested that the liberation of aglycones of isoflavone glucosides such as daidzein and genistein by the catalytic action of β-glucosidase during fermentation resulted in the increased antioxidative activity.[52]

18.4.3 Micronutrient Synthesis

The fermentation processes can also result in increased levels of micronutrients in the final product. The production of vitamins by LAB provides a very attractive approach to improving the nutritional composition of fermented foods.[53] Folic acid is an essential cofactor in bacterial metabolism, and many bacteria used in food fermentations possess the biosynthetic capability to produce folate.[54–56] Similar results have been shown for vitamin B_{12} (cobalamin) production in thua-nao[39] and a tempeh-like soy product cofermented with *Propionibacterium shermanii* 1250.[57] Vitamin B_{12}, an essential cofactor in fatty acid, amino acid, carbohydrate, and nucleic acid metabolism, is exclusively produced by microbial synthesis, and is also present in foods such as red meat and milk as a result of rumen microbial action.[58] Thai fish sauce also contains relatively high concentration of vitamin B_{12} (1.91 μg per 100 mL), compared to fermented soybean.[59] As a common condiment for Thais, its high content of cobalamin is believed to protect the Thai population from megaloblastic anemia, an anemia caused by vitamin B_{12} deficiency.[59]

18.4.4 Probiotics and Prebiotics

Probiotics are defined as the live microbial supplements that can benefit the host.[60] These potential health promoting effects are mainly caused by the ability to prevent gastrointestinal tract infections, stimulate the immune system, and maintain

the balance of intestinal microflora. A number of studies have shown that Thai fermented foods (*pla-ra, pla-paeng-daeng, pla-som,* nham, *sai-krog-prieo, phak-gard-dong, phak-sian-dong*) contain potential microorganisms with some probiotic properties such as acid resistance, bile tolerance, and antagonism to some pathogen growth.[61,62] The most common microorganisms found are LAB, especially *Lactobacilli,* which are also resident microflora in the gastrointestinal tract of most animals.[63,64] A number of scientific studies indicate that ingestion of certain microbial cultures exerts health benefits.[65–67] However, these isolates have not yet been tested for their effectiveness and safety for direct human consumption. Nevertheless, some of these selected strains have also been shown to be effective in keeping a favorable balance of microflora in the gastrointestinal tract, and improving animal performance (chicken and piglets).[61] The exact mechanism of the probiotic effect might be associated with the ability to resist the unfavorable conditions arising during transit through the gastrointestinal tract of animals and then exerting an antimicrobial activity.

In addition to the probiotic effect, fermented products especially from plant origin could have excellent prebiotic functions. A prebiotic is generally referred to as a nondigestible food ingredient that beneficially affects the host by stimulating growth and/or activity of certain bacterial components of the intestinal microflora. Prebiotics are neither hydrolyzed or absorbed in the small intestine, but are a selective substrate for microorganisms in the colon. In addition to known prebiotic oligosaccharides such as fructo-oligosaccharides, soybean oligosaccharides raffinose and stachyose,[68] hydrolysis products of plant cell wall polysaccharides[69] and extracellular polysaccharides (EPS)[70] can be a novel source of prebiotics.

18.4.5 Microbial Products

18.4.5.1 Fibrinolytic Enzymes Produced by *Bacillus* Strains

Fibrinolytic enzymes are agents that dissolve fibrin clots. Fibrinolytic enzymes can be found in a variety of fermented foods, such as Japanese natto,[71] Korean *chung-kook-jang* soy sauce,[72] and fermented shrimp paste.[73] Although the source of these enzymes has yet to be determined, they are likely produced by *Bacillus* strains found in the food product. Recently, several strains of *Bacillus* isolated from *tao-hu-yi*, *tao-cheow, pla-chao, poo-dong* (fermented crab), and *ka-pi* have also been found to strongly produce a fibrinolytic enzyme. The identification and isolation of bacteria and enzymes are under in progress in our laboratory. Because large quantities of enzymes can be conveniently and efficiently produced, these enzymes should be useful for thrombolytic therapy by providing an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease. In addition, these enzymes have significant potential for food fortification and nutraceutical applications; their use could effectively prevent cardiovascular diseases.

18.4.5.2 Antimicrobial Substances

Fermentation of foods is often caused by the action of LAB. It is possible that LAB exert an antibacterial effect due to inhibition of pathogens in situ, which is relevant

for food product safety. The fermentation end-products, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and bacteriocins contribute to antagonistic activities.[74,75] Bacteriocins are proteinaceous in nature. Because of their antagonistic activity against specific sensitive bacteria, the bacteriocins produced by LAB have gained much attention as potentially useful food additives to be used against food-borne pathogens. In the extensive screening studies covering various fermented foods, numerous bacterial isolates have been reported to possess bacteriocinogenic activity.[76,77] Bacteriocins generally vary with regards to their mode of action, molecular weight, genetic origin, biochemical properties, and spectrum of activity. Bacteriocins produced by LAB display a high degree of heterogeneity, which is divided into four distinct classes. Examples of these bacteriocins that are important in the context of Thai foods include plantaricin W produced from *Lactobacillus plantarum* PMU33 isolated from *som*-fak,[78] antilisteria bacteriocin from *Lactobacillus plantarum* N014 isolated from nham,[79] nisin Z produced from *Lactococcus lactis* WNC 20 isolated from nham,[80] enterocins NKR-5-3A and B produced from *Enterococcus faecium* NKR-5-3 isolated from *pla-ra,*[81] and weissellicin 110 from *Weissella cibaria* 110 isolated from *plaa-som*.[82]

18.4.5.3 Fermented Red Rice

Fermented red rice is a common food ingredient used to enhance the color and flavor of various Thai foods. It also has been known as a food preservative and a folk medicine for digestive and vascular functions in China and Japan.[83] In addition to the edible pigments with a polyketide structure, various species of *Monascus* have also been shown to produce other bioactive metabolites with different properties (see Figure 18.1). Monacolins produced by *Monascus ruber* and *Monascus purpureus* Went are reported to exhibit a cholesterol lowering action by specifically inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.[83,84] Monoascidin A produced by *Monascus purpureus* exhibited antibiotic action not only against bacteria but also against yeasts and some filamentous fungi.[85] Monankarins A-D produced by *Monascus anka* showed monoamine oxidase (MAO) inhibitory activity.[86]

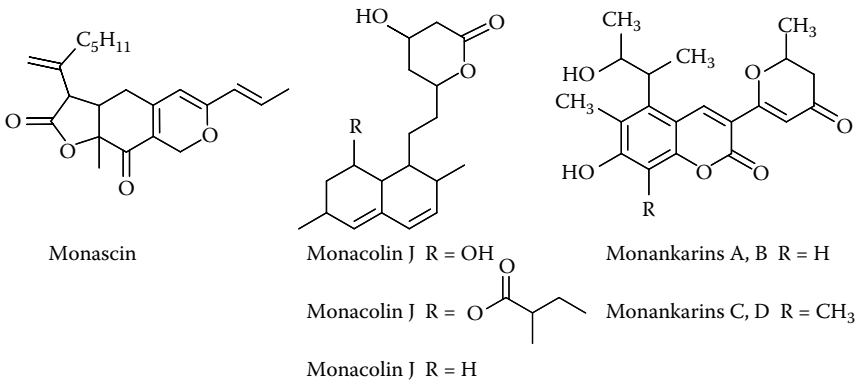

FIGURE 18.1 Bioactive metabolites produced by various species of *Monascus*.

18.4.5.4 γ-Aminobutyric Acid or GABA

GABA is an inhibitory neurotransmitter found in the nervous systems of widely divergent species and also in the retina.[87] People with too little GABA tend to suffer from anxiety disorders. GABA and their agonists have been used in some medicines and are also commercially added in some functional foods for the improvement of brain metabolic function and hypertension.[88,89] GABA production is known to occur in cheese production, and LAB, especially lactobacilli, were found to have the ability to produce GABA from glutamic acid by the catalytic activity of glutamate decarboxylase (EC 4.1.1.15) (see Figure 18.2). Recently, Sukontasing[90] reported that 5 *Lactobacillus* isolates from the Japanese-style pickle, pla-som, pak-sian-dorng, and pla-ra also possess glutamic acid decarboxylase activity and produce GABA. Consuming fermented foods containing these microorganisms may exert their therapeutic effects exclusively via the action of GABA. The genes encoding for glutamate decarboxylase of these LAB strains have been successfully cloned, and expressed in *E. coli* for GABA production on a commercial scale. [90]

18.4.5.5 Bioactive Peptides Putatively Derived from Fermented Foods

Several fermented functional foods derive their activity from the release of bioactive peptides that are released after enzymatic proteolysis of food proteins. For example, fish sauce, which contains a complex mixture of peptides, may contain a number of peptides with interesting biological activity. Although little is known about bioactive peptides in fish sauce, an in vitro experiment with human monocytes revealed some proliferation stimulation by addition of medium size peptides (500–3000 Da) isolated by ultrafiltration of commercial fish sauce made from anchovy.[91]

Moreover, many fish sauces traditionally made from anchovy, salmon, sardine, or bonito contain many peptides inhibiting angiotensin-I-converting enzyme (ACE).[92,93] ACE performs an important physiological role in controlling blood pressure; the enzyme catalyzes both the production of the vasoconstrictor angiotensin II and inactivation of the vasodilator bradykinin.[94] Therefore, inhibition of the ACE results in an overall antihypertensive effect. From the study of Ichimura et al.,[92] ACE inhibitory peptides isolated from various fish sauces were mainly proline-containing dipeptides, with proline in the c-terminal position. Due to the unique structure of proline as an imino acid, peptide bonds containing proline residues are often resistant to hydrolysis by common peptidases. This may be the reason for the survival of proline-containing dipeptides after long-term fermentation. Orally administered

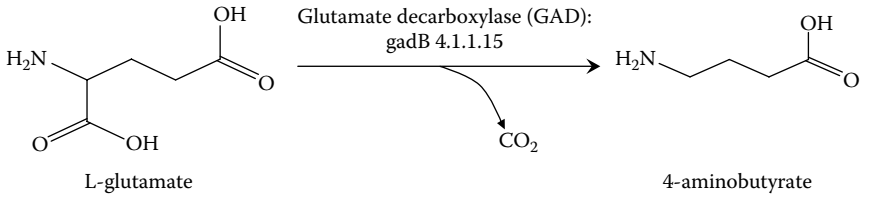

FIGURE 18.2 Chemical structure of GABA and the enzymatic reaction catalyzed by glutamate decarboxylase.

Lys-Pro showed a tendency to lower the blood pressure of spontaneously hypertensive rats.[92] Although fermented fish sauce itself may not be directly used for a physiologically functional food because of its high concentration of sodium chloride, the peptides from fish sauce may be useful as a natural alternative for the ACE inhibitory drugs. [95–97]

18.5 CONCLUSION

Compared to other fermented foods, only a few studies on the composition and health promoting properties of Thai fermented foods have been done so far. Nevertheless, examples like those provided in this chapter serve to highlight the potential of exploiting fermented foods as a basis for functional food development. Studies on health promotion activity in vivo would greatly facilitate the development of a completely new generation of fermented functional foods tailored to contain clinically proven health promoting metabolites and/or microorganisms. Owing to the lack of scientific and technological know-how, fermented foods are generally evaluated on the basis of qualitative attributes such as odor and flavor. Such studies show the inconsistency of quality obtained for different batches. Therefore, the development of uniform processes as well as the use of biotechnology has been encouraged to ensure the quality, consistency, and safety of the food products. This review detailed many examples of fermented functional foods that are currently manufactured in Thailand; their health promoting properties could be due to live cultures or to metabolites produced through fermentation. Future possibilities to exploit the potential health promoting properties of such foods will continue to expand, as we gain a greater understanding of the microorganisms used and how they, or their metabolites, can directly interact in a positive way with the human host.

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19 Production of Probiotic Cultures and Their Addition in Fermented Foods

Claude P. Champagne and Henrik Møllgaard

CONTENTS

19.1 INTRODUCTION

The original aim of fermentation by lactic acid bacteria (LAB) was to preserve foods by acidifying them. Today, there is increasing evidence that the consumption of LAB may have beneficial effects on human health. A fraction of the LAB used

in fermentation can be considered as being "defined, live microorganisms which, administered in adequate amounts, confer a beneficial physiological effect on the host" and can be termed *probiotics*.[1]

In North America, up to 93% of consumers believe certain foods have health benefits that may reduce the risk of disease.[2] This has created a strong demand for functional foods (FF). Although the FF market represents only 1.6% of the food and drinks market, it was expected to rise 9.5% between 2005 and 2006 alone.[3] Worldwide, the functional dairy sector (which is overwhelmingly linked to probiotics) is the largest FF market, accounting for nearly 33% of the broad market, whereas cereal products have over 22%.[3] These data show the importance of probiotics. As a result, a strong demand exists for the supply of these cultures, and specialized producers have responded. One of the goals of this chapter is to describe some of the steps in the industrial production process of probiotic bacteria.

Food processors generally do not propagate the probiotic cultures at the plant, and prefer the simpler approach of carrying out a direct inoculation with commercial products. Although this appears simple to carry out, there are critical manipulations to ensure adequate storage and inoculation of the probiotic bacteria. This chapter will also address the parameters food manufacturers must consider in the process of adding probiotics to their food matrix.

The treatment below is not an attempt to extensively review the literature on the production of probiotic cultures, but rather to present the current opinion of the authors on the field.

At present, the use of genetically modified organisms as probiotics is unacceptable to the consumer in most countries. Accordingly, this topic will not be treated, in spite of the large potential of the technology.

19.2 COMMERCIAL PROBIOTIC CULTURES

19.2.1 Species

The spectrum of species that are considered probiotic is constantly expanding as clinical studies are published. Originally, lactobacilli (*Lb. acidophilus, Lb. rhamnosus, Lb. paracasei (previously named casei), Lb. plantarum, Lb. reuteri*) and bifidobacteria (*B. lactis, B. bifidum, B. longum, B. adolescentis, B. animalis*) were the most commonly encountered. However, there are now strains from the genera *Pediococcus, Propionibacterium, Enterococcus, Bacillus, Streptococcus,* and even *Saccharomyces,* which have documented health effects. It is not our aim to list them all in this section as other chapters will present the various species used in specific applications. Nevertheless, it must be stated that not all strains from a given species share the same properties, and it is prudent to indicate the specific strains used in a product. This is accomplished by providing a code, which is typically a combination of letters or numbers (for example, GG, La-5 or RO11). Numerous reviews are available in the literature that verify the effect of probiotics.[4–5] The health claim of many strains is a controversial topic that needs further attention in the coming years.

19.2.2 Selection of Strains

Before their testing in human clinical assays, probiotic strains undergo numerous in vitro tests aimed at characterizing some of their physiological properties. The most basic property of a probiotic culture delivered via a fermented FF is its ability to survive the initial challenges of the gastrointestinal (GI) tract. Survival of stomach acid and bile salts are critical in this testing (Table 19.1). Sophisticated units have

TABLE 19.1
Some Selection Criteria of Probiotic Bacteria Based on Potential Health Effects, Physiological Properties, and Technological Properties

Potential health benefits	Microbial physiology	Technological demands
Prevention/reduction of symptoms of:	*Tolerance to:*	*Producers of cultures:*
Diarrhea	Acid	Inexpensive cultivation
Infections (*h. Pylori* ulcers, urinary)	Bile	Ease of concentration to high cell densities
Small bowel bacterial overgrowth	Lysozyme	Stability in dried or frozen form
Inflammatory bowel disease	Antibiotics	*Users of cultures:*
Necrotizing enterocolitis	Oxygen	Growth in the food matrix
Irritable bowel syndrome		Can be produced at a large scale (ex. Starter tanks)
	Production of:	
Constipation	Lactase	Compatibility with other lactic cultures
Coronary heart disease/ hypercholesterolemia	Bile salt hydrolase	Bacteriophage resistance
Cancer (colorectal, cervical)	Antibacterial factors	Survival to processing steps (freezing, heating, high shear, oxygen)
Improvement of:	*Other properties:*	Tolerance to preservatives
Lactose assimilation	Noninflammatory-promoting	Flavor and sensory properties
Food digestibility	Antimutagenic and anticarcinogenic	Stability during storage
Immune response	Nonpathogenic	
Blood pressure	Appropriate cytokine stimulation	
Oral health	Adhesion	
Lifespan		

Source: Data taken from Champagne C.P., Roy D. and Gardner N., *Crit. Rev.Food Sci. Nutr*., 45, 61–84, 2005; Collins, J.K., Thornton, G. and Sullivan, G.O., *Int. Dairy J.*, 8, 487–490, 1998; du Toit, M., Franz, C.M.A.P., Dicks, L.M.T., Schillinger, U., Haberer, P., Warlies, B., Ahrens, F., and Holzapfel, W.H., *Internat. J. Food Micro*., 40, 93–104, 1998; Huis In't Veld, J.H.J. and Havenaar, R., *Microecol. Ther.,* 26, 43–57, 1997; Kailasapathy, K. and Chin J.C., *Immunol. Cell Biol*., 78, 80–88, 2000; Ouwehand, A.C., Salvadori, B.B., Fondén, R., Mogensen, G., Salminen, S., and Sellars, R., International Dairy Federation (IDF), Brussels, Document #380, pp. 4–19, 2003; Shah, N.P., *Biosci. Microflora*, 19, 99–106, 2000; Suskovic, J., Brkic, B., Matosic, S. and Maric, V., *Milchwissenschaft*, 52, 430–435, 1997.

been designed to simulate the GI tract[6–9] and are used to characterize the strains on these criteria. Furthermore, genetic tools are now available to characterize the acid and bile sensitivity of strains.[10]

Subsequently, in vitro cell culture methods serve to ascertain potential properties of probiotics on the immune system.[11] These analyses are mainly based on evaluating the physiological properties of the strains (Table 19.1), and provide useful data in screening of the many LAB strains available. It must be stressed that physiological characterization not only tests for potential benefits of the strains, but also examines potential dangers the cultures could present (Table 19.1).

Following in vitro assays, promising probiotic cultures undergo testing in animal models, which include mice,[20] rats,[21] pigs, and poultry.[22] The clinical assays on humans are subsequently carried out.

In the past, some promising probiotic cultures were discarded as they could not be efficiently produced under industrial conditions, because they behaved poorly in food processing conditions, or because they were unstable during storage (Table 19.1). As will be seen in the section on production technologies that follows (Section 19.4), many steps in the fermentation, recovery, and stabilization procedures may generate sublethal or even lethal damages to the cells. Viability losses especially occur during food processing, where freezing, heating, or other conditions affect cell structures. However, both the starter manufacturers and the food processors are rising up to the challenge, and are devising novel methodologies to produce and maintain viability of probiotics during production in foods.[23] This will be addressed further in this chapter.

19.3 COMMERCIAL LACTIC STARTER CULTURES

19.3.1 Species

Many fermented FF based on a lactic fermentation are a blend of traditional starter cultures and selected probiotic strains. Thus, when discussing fermented FF, the interactions between the starter culture and the probiotic bacteria must obviously be considered.

Regional or specialty fermented foods of low production volume are still carried out as spontaneous fermentations. There is an amazing variety of such fermented foods around the world.[24] In this situation, the fermentation is basically carried out via the development of the contaminating lactic flora of the raw materials or of the equipment used in processing. In these instances, no commercial starters are added.

At the other end of the spectrum of fermented foods are the highly industrialized processes of commercial starter culture production. The most extensive user of such cultures is the dairy industry (Table 19.2). However, other sectors are increasingly taking advantage of the benefits of starter inoculation (Table 19.2) because of the ability to control the process, include phage resistance, and ensure repeatability of processes.

19.3.2 Selection of Strains

The selection criteria for starter cultures differ considerably from those of the probiotic bacteria (Tables 19.1 and 19.3). One of the main goals of a lactic fermentation

TABLE 19.2
Some Commercial Starter Cultures Used in Lactic Fermentations of Foods

Field	Products	Cultures
Dairy	Cheese, sour cream, cultured butter, yogurt, kefir	*Lactococcus lactis (many subspecies), Streptococcus thermophilus, Leuconostoc cremoris, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lactobacillus paracasei*
Meat	Dry sausages	*Lactobacillus plantarum, Lactobacillus sake, Lactobacillus brevis, Pediococcus acidilactici, Pediococcus pentosaceus*
Grains	Sourdoughs, breads	*Lactobacillus sanfrancisco, Lactobacillus plantarum, Lactobacillus brevis*
Vegetables	Sauerkraut, pickles, olives	*Lactobacillus plantarum, Leuconostoc mesenteroides*
Fruits	Beverages, including wine	*Oenococcus oeni*

TABLE 19.3
Main Selection Criteria of Lactic Starter Cultures

Fermentation properties	Resistance/sensitivity
Utilization of sugars (homo/heterofermentative)	Bacteriophage
Citrate fermentation	NaCl (behavior during pressing; Cheddar)
Gas productions	Temperature (behavior during cooking[a])
Acidification rate	Phosphates (culture media)
Proteolytic and peptidolytic activity	pH inhibition (excessive acid production during storage of yogurt)
Flavor production	Osmotic pressure (UF milks)
Production of polysaccharides	Lysozyme
Production of bacteriocins or other inhibitors	Oxygen
Association with other strains	

[a] "Cooking" does not refer to food preparation but rather to a technological step used in some cheese manufacturing processes where over-optimal growth temperatures are used (for example 37 to 40°C in Cheddar or 50 to 54°C in Emmenthal) to control acid production and wheying-off rates.

Source: Data adapted from: Dave, R.I. and Shah, N.P., *J. Dairy Sci.,* 79, 1529–1536, 1996; Porubcan, R. S. and Sellars, R. L., *Microbial Technology. Vol. 1, Microbial Processes*, 2nd Ed., Academic Press, New York, 1979, pp. 59–92.

is to help preserve the food matrix against spoilage microorganisms as well as to prevent the development of pathogenic bacteria. The LAB accomplish this by (1) generating a drop in pH to levels where the undesirable bacteria not only do not grow, but could actually die, (2) producing antimicrobial compounds such as H_2O_2 or bacteriocins, and (3) removing the carbon source and oxygen needed by the bulk of food-spoiling microorganisms. The LAB will also contribute flavors and modify texture. One must therefore keep in mind that a lactic-fermented food does not solely

contain bacteria that have demonstrated probiotic properties. Unfortunately, little data are available on the specific health benefits of the starter cultures typically used by industry, other than improved safety of foods, and it cannot be assumed that all fermented FF contain probiotic bacteria. Thus, the distinction between the typical strain used as a starter culture, and a probiotic strain is rather vague, and it may change when more knowledge is obtained.

19.3.3 Starter–Probiotic Relationship

A common feature of lactic starter cultures is their ability to rapidly grow and acidify the food matrix, with the exception of the malo-lactic cultures whose role is the opposite, i.e., to de-acidify wine. As a result, probiotic cultures, which are primarily selected for their health effect rather than acid-producing properties, face strong competition for substrates. This explains why fermentations using probiotics alone may be up to three times longer that those using a starter.[27] As a consequence, probiotic bacteria populations in the fermented FF are much lower in the presence of starters than when the probiotics are grown alone.[27] This is an important aspect to consider when assessing the "health" value of a fermented FF. It is believed that a minimum number of probiotic bacteria is required to have the clinical expression of the probiotic effect. One must therefore examine critically the numbers of probiotic bacteria found in the fermented product resulting from a mixed probiotic/starter blend, and ascertain if the required numbers of viable probiotics were indeed attained. Enumerating probiotic bacteria within a product containing many LAB presents challenges, but a number of differential plating techniques are available,[28–31] and real-time polymerase chain reaction (PCR) is expected to be critical for the determination of the population of a given strain in complex mixtures. Flow cytometry is another alternative method, which in the future may speed up the enumeration using strain-specific antibodies.

19.4 INDUSTRIAL PRODUCTION OF CULTURES

The manufacturing processes for starters and probiotics are fundamentally the same. Therefore, this section generally applies to both groups of bacteria; when some practices are specific to a given culture, this will be stated.

The flow scheme of a typical process appears in Figure 19.1. The four basic steps are (1) preparation of the growth medium, (2) biomass production, (3) biomass concentration, and (4) biomass stabilization.

It is of crucial importance to take a holistic view of the process. Thus, all steps will contribute to the final quality of the product. It is especially important to base the process development on a fermentation of good quality, as a biomass of inferior quality never will render a product with suitable properties.

Most starter products for FF are sold on a specification of the number of colony-forming units (cfu). However, the analysis of cfu suffers from several drawbacks. First of all, the reproducibility is relatively low. Secondly, it does not necessarily reflect the active biomass, which will confer functional activity to the product. The problem is that the many LAB appear as chains or clumps. Usually the problem is

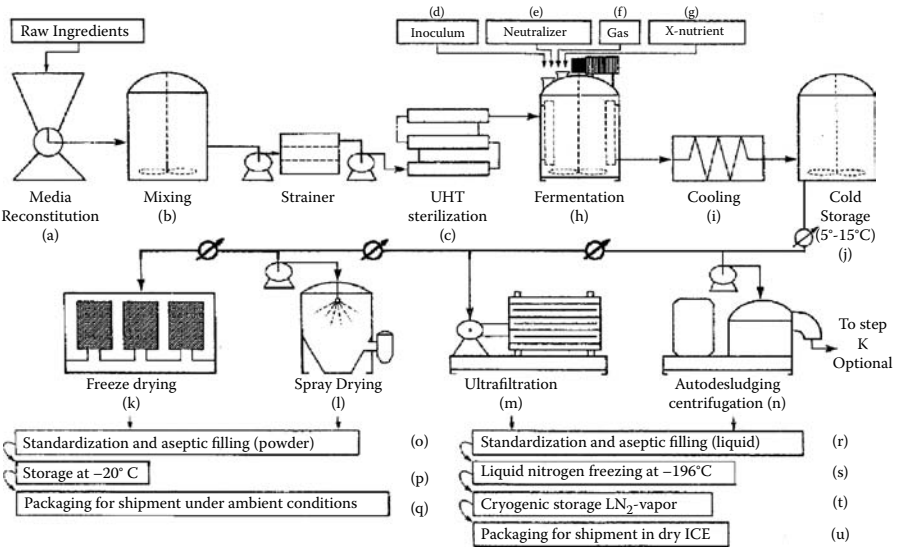

FIGURE 19.1 Flow scheme for the production of concentrated lactic acid bacteria and probiotics. The strainer prior to the UHT unit serves to remove particles which would be stuck in the UHT unit. The storage of the concentrate could also be done at –50°C. (From Porubcan, R. S. and Sellars, R. L., in *Microbial Technology. Vol. 1: Microbial Processes,* 2nd ed., New York: Academic Press, 1979, pp. 59–92.

not so large with freeze-dried products, as many chains break apart during processing, but in frozen products, cfus become almost meaningless. One reason for retaining the cfu is that it is accepted by the food authorities, but a more meaningful analysis parameter needs to be found.

19.4.1 Media

The LAB require numerous growth factors,[32] and growth media may therefore be quite complex. Unfortunately, there is no single low-cost medium that can be universally used to grow all LAB and probiotics. Therefore, media most often need to be adjusted to specific strains. A list of potential ingredients is found in Table 19.4.

The type of carbohydrate in the medium is important inasmuch as it is linked to subsequent activity in the food substrate. Thus, it is recommended to grow a LAB on lactose if it is to be subsequently used in milk fermentation. Indeed, cells grown on glucose require a lag period before showing growth or activity if inoculated in lactose-based milk. Because lactase is an inducible enzyme, this lag period is essentially due to the time required by the cell to synthesize lactase (β-galactosidase). The type of carbohydrate in the growth medium can also affect subsequent survival to freeze drying (FD).[33]

The LAB have a certain number of requirements for amino acids. The LAB generally prefer short peptides over free amino acids as a source of amino acids,[34–36] and the preferences of lactococci differ from those of lactobacilli.[37] Automated

TABLE 19.4
Main Ingredients Used in the Large-Scale Production of Lactic Cultures

Simple ingredients	Complex ingredients	Buffers	Antifoam agents
Organic:	*Soluble/clear solutions:*	*Organic:*	Silicone based
Monosaccharides (e.g., glucose)	Yeast extracts	Citrate	Glycol based
Disaccharides (e.g., lactose)	Casein hydrolysates	Acetate	(Oil based)
Tween	Whey protein hydrolysates	*Inorganic:*	
Vitamins (ascorbic acid)	Peptones of animal origin	Mainly phosphate salts	
Inorganic:	Peptones of plant origin		
A balance of Na and K salts and especially ammonia if it is not used for pH control	*Insoluble/opaque solutions:*		
Salts of Ca, Mn, Mg, Mo, Fe, Co, other trace metals	Skim milk		
	Whey		
	Whey protein concentrates		
	Grain hydrolysates		
	Dextrins		

Source: Adapted from Porubcan, R. S. and Sellars, R. L., in *Microbial Technology, Vol. 1, Microbial Processes*, 2nd ed., Academic Press, New York, 1979, pp. 59–92.

spectrophotometry can ascertain what is the most appropriate amino supplement,[38,39] as well as enable the screening of up to 50 media in a single assay.

Control of the pH of the fermentation is critical for biomass production of LAB. External pH control is most often applied, but buffering may be used also. In the latter case, theoretically, the higher the buffering capacity of the medium, the higher the final biomass attained. However, some lactobacilli do not tolerate high levels of phosphate as well as do lactococci, presumably because it binds Ca++, Mg++ or Mn++ ions,[40] and thus phosphate levels in the media need to be adjusted as a function of the species involved. Trace elements are also required for growth, and the reader is pointed to the review of Boyaval[41] for more details.

It is noteworthy that the composition of the growth medium affects biomass levels and the acidifying activity of the cultures. Conditions that enhance the intracellular accumulation of protective compounds (e.g., against reactive oxygen species and osmotic stress) not only improve survival to freezing and drying, but also influence stability during storage.[33] This stresses the need for a holistic view on the process.

19.4.2 Fermentation Technologies

The addition of buffers is very helpful in improving biomass levels in media fermented by LAB, but the constant addition of a neutralizer/alkali is even better.

External pH control can increase biomass levels by a factor of up to 10 as compared to unbuffered systems.[42–44] The type of alkali also influences biomass. Ammonium hydroxide is typically used.

Batch fermentation is often used in industrial scale for production of LAB. The reason for this is that lactate accumulation is the factor that typically stops the anaerobic fermentation. In other industrial fermentations of, for example, enzymes, the catabolic products of the carbon source is carbon dioxide, which does not accumulate in the medium. Accordingly, a much higher level of sugar can be metabolized. In contrast with lactate accumulation, the amount of sugar that can be metabolized is so small that a simple batch process can be applied.

The fed batch concept can be used to lower exopolysaccharide production during the fermentation. This reduces the viscosity of the fermented medium, and facilitates subsequent recovery.[45] Fed batch can also be used to adapt cells to a specific carbon source or for adding stressful compounds (acid, salts) at the end of the fermentation, in order to induce special technological or physiological properties in the cells (e.g., increased resistance to freezing, drying, heat, or acid conditions). Thus, the cells are adapted ensuing cell preservation and improved product use.

Some fermentation processes carried out under pH control have the disadvantage of generating starters with lower specific (on an equal cell number basis) acidifying activities.[42,43,46] When this occurs, it is usually a minor inconvenience, considering the much greater benefit of high biomass levels.

Lately, some *Lactococcus* strains are being produced by aerobic fermentation after addition of heme.[47] However, this concept is only applicable to a limited number of LAB harboring a rudimentary respiratory chain and sufficient resistance to reactive oxygen species.

19.4.3 Concentration Technologies

A high cell concentration is an advantage with respect to loss of active biomass during cell preservation.[48] Accordingly, the product of the fermentation is concentrated by either centrifugation or filtration.

It can be a problem to dry LAB in their fermentation broth because it contains organic acids and lacks protective carbohydrates; both these conditions may lead to low viability levels following freezing or drying.[49] Therefore, the fermentation broths can be either filtered or centrifuged in order to separate the cells from this spent broth and, at the same time, concentrate the biomass prior to the stabilization step (Figure 19.1).

Key processing parameters in centrifugation are temperature, gravitational force (g), and structure of the centrifuge unit. The centrifugation is strongly affected by the fermentation, especially the exopolysaccharide content, which may almost render concentration impossible for some strains. Generally, the concentration is a compromise: on the one hand, a high degree of concentration is desirable in order to make a highly active product and minimize storage. A highly concentrated product also minimizes the cost of freezing and FD. On the other hand, packing of the cells may be a problem in the centrifuge, and for subsequent handling.

In filtration processes, microfiltration or ultrafiltration units can serve the purpose. Key parameters in filtration processes are type of membrane, membrane pore size, temperature, filtration pressures, and number of recycling passes. Effective cleaning is also a factor of huge importance.

The choice between the two types of concentration must be based on the specific species and local set-up in the factory. Centrifugation is a heavy initial investment, but relatively cheap to run. In contrast, filtration equipment is relatively cheap. Both technologies are heavily affected by bacterial exopolysaccharide, but the effect may be strongest with filtration.

Unfortunately, these operations may generate lethal or sublethal damages to the cells, which result in lower acidifying activity[50] or lower survival during FD.[51] For this reason, some valuable LAB cultures are difficult to produce using the traditional processes. Production by microencapsulation in alginate beads offers an alternative process to address this problem.[52]

19.4.4 Preservation Technologies

LAB are generally commercialized in dried or frozen form (Figure 19.2). In most cases, dried products are prepared by FD because the LAB are heat sensitive and do not show high survival levels to spray–drying (SD).[53,54] The most common form of frozen cultures is in 1 to 2 L cartons or larger bags, which contain beads of approximately 2 mm of diameter. For some dairy applications, cans (between 50 and 400 ml) are also marketed.

Survival to freezing is a critical point in processing of LAB and probiotics because some products are sold in the frozen form as such and because freezing is the first step in FD. When marketed in cans, the concentrated cultures are inserted

FIGURE 19.2 Some of the forms by which the lactic acid and probiotic cultures are distributed to the food manufacturers. Courtesy of Chr. Hansen.

into the container, sealed, and plunged in a liquid nitrogen bath. The frozen beads marketed in the cartons are prepared by dripping concentrate into liquid nitrogen. These small frozen particles of pure cultures can be mixed in the frozen state to obtain multiple-strain cultures of defined composition.

In order to enable high survival rates to freezing, the following parameters must be controlled: pH of the medium, content of protective compounds, and freezing rate. As mentioned earlier, an acid medium does not enable high viability levels. For example, for lactococci, a pH of 6.6 gives approximately 10% higher residual activity of starters as compared to those frozen at pH 5.2.[55] However, this will vary between strains. Numerous studies have examined the composition of the cryoadditives and reviews are available.[33,49] Carbohydrates are considered one of the most important ingredients of the medium in this respect.[33] The cryoadditives also function during the subsequent storage step (see following text). The freezing rate is critical in providing high viability levels. Generally speaking, rapid freezing rates, such as those enabled by the use of liquid nitrogen (–196°C) are preferable.

Sensitivity to the drying processes is variable, and distinct strains of the same species can present different survival levels.[33] As was the case with survival to freezing, carbohydrates in the drying medium enhance survival to FD.[33] Unfortunately, in most studies it is difficult to ascertain if the protective effect was related to protection during freezing or during drying. Alcohol derivatives of sugars (sorbitol, xylitol, adonitol, mannitol) seem to be particularly effective in protecting cells, not only to the FD process, but also during subsequent storage. Surprisingly, little is published on the effects of the drying process on cell viability. The prehistory of the biomass (e.g., medium, stress, and harvest time) as well as the concentration, are factors of importance.

It must be stated that FD is not the only method of drying of LAB. Thus, SD has been carried out, but its success is very variable. Some strains such as *Streptococcus thermophilus* withstand the high temperatures, but lactococci do not.[53] In SD, the following process parameters will affect survival levels: type of atomization, air pressure, and outlet temperature. An important factor also seems to be adaptation of the cells. A short heat stress prior to SD can significantly improve viability levels.[56] Although SD has strong potential, its use is currently rather limited for the production of stable LAB and probiotics.

19.4.5 Encapsulation

Encapsulation has proven very successful in improving the survival of probiotics in FF.[23,57–59] Although encapsulation in alginate gels protects cells during freezing, heating, and storage in acid foods,[52,59] there are few commercial products on the market based on this technology. Producers of probiotics have preferred the spray-coating technology to market their products. Spray-coating is carried out by vaporizing a protective compound on the surface of a probiotic-containing dried particle (Figure 19.3). The spray-coated products are very effective in enhancing survival of the cells in the gastrointestinal (GI) tract, ideally releasing the biomass at a predetermined site. There is increasing evidence that coating also helps probiotic bacteria to better survive heat processes as well as storage at room temperature.[60]

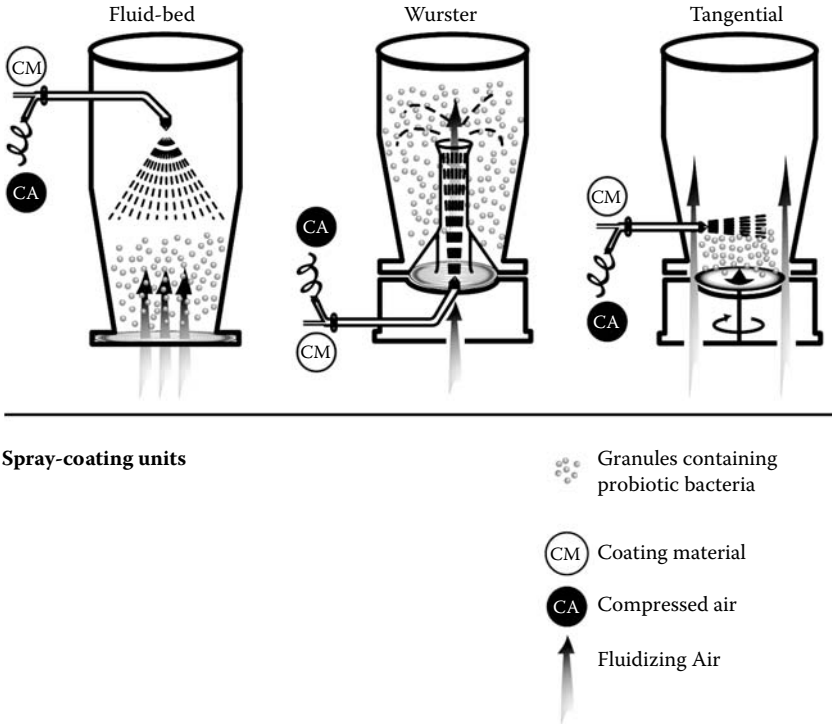

FIGURE 19.3 Spray-coating methods for encapsulation of probiotics. The technologies principally differ from the type of air fluidization and the site in the vessel where spraying of the coating material is carried out. In food applications the coating is mostly lipid-based (waxes, fatty acids, specialty oils), but proteins (gluten, casein) or carbohydrates (cellulose derivatives, carrageenan, alginate) can also be used.

19.5 SOME RECOMMENDATIONS FOR USE IN THE FUNCTIONAL FOOD INDUSTRY

The goal of this section is to address the use of starters or probiotics at the site of production of fermented FF. Obviously, the manufacturers' production, packaging, and shipping practices are critical in ensuring viability of probiotics or activity of starters. But some food manufacturers are unaware that how they handle the cultures at the production site will also affect their viability or their activity. For example:

- Is the storage temperature of a frozen culture important, as long as it does not thaw?
- Can cultures in powder form remain stable at room temperature?
- Can products be opened and contents be used over a period of a few days or even a few weeks?
- Should the cultures be added directly to the food matrix or should they be rehydrated beforehand?
- Does the method of addition to the food matrix have a strong impact on viability or activity?

When faced with these questions, the "golden rule" FF processors should try to follow is to apply the manufacturers' recommendations. These instructions range from storage conditions to inoculation levels. Below, a few of the reasons for these recommendations will be presented. But sometimes, equipment failure or errors by personnel will occur, and some data on what to expect in these circumstances will be presented.

19.5.1 Storage

During storage, the key concept is water availability. Water is required for life-based activities such as fermentation. When there are high-water activity (a_w) levels, growth rates are high, but so are death rates. Thus, to prevent viability losses during storage, one must have low water activity levels. This can be achieved by removing water (drying) or by making it unavailable through freezing or through the addition of "binding" compounds such as salt or sugars.

Frozen products must be kept at −40°C or below.[61] Although it would seem that as long as the product is frozen, stability would be maintained, this is not the case. The reason is that water starts to crystallize at 0°C, but not all of the contents are frozen at 0°C, and some water mobility remains. As the temperature drops below 0°C, the water become less and less mobile and the system will eventually reach a "glass transition" temperature (T_g) that enhances stability. The glass transition temperature depends on the biomass concentration, and obviously on the composition of the matrix. The storage temperature needs to be below the glass transition temperature. There are few published data on the effect of the medium composition and storage temperature on starter activity. Data on one specific starter culture showed that the loss of activity following storage at −21°C for 3 months was 30%, whereas it was only 19% at −31°C;[55] the T_g of this blend is unknown.

Freeze-dried products are generally considered more stable than frozen cultures, but this depends on the storage conditions. Some freeze-dried cultures can be kept at −20°C for a year without showing noticeable viability or activity losses. Even at 4°C they are quite stable (Figure 19.4). However, it can also be seen that significant viability losses occur if stored at room temperature. Data from Figure 19.4 show that cultures under the specific conditions of this study were about 10 times less stable at room temperature than at 4°C, and approximately 100 times less stable than at −20°C. Although these data provide an indication of the effect of temperature, it must be kept in mind that the effect of storage temperature on viability losses will be strongly affected by strain, a_w level, and matrix composition.

19.5.2 Opened Products

It must be stressed that the viability levels stated by the manufacturers for a given time period can only apply if the frozen product is not thawed, or if the sachet of a freeze-dried culture is not opened. Some small manufacturers of FF may wish to use one sachet of culture for many productions over a few days. In this strategy, the sachet is opened, a sample taken, it is refolded, and restored at 4°C until used again. It is important to mention that the stability of an opened sachet is much lower than that of the original product. This is because during the time a sample is taken, the powder

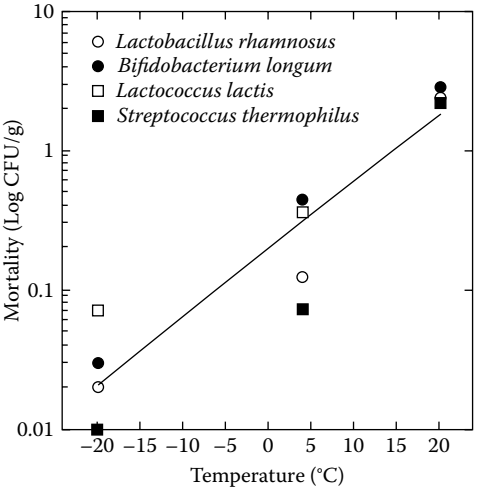

FIGURE 19.4 Effect of temperature on losses in viability of various LAB and probiotic cultures after 1 yr of storage. (From Champagne, C..P., Mondou, F., Raymond, Y., and Roy, D., *Food Res. Internat.,* 29, 555–562, 1996).[62]

absorbs humidity from the air and the a_w in the product increases. For example, in a lactose-based product, it only takes an increase in moisture from 2.5 to 3% (e.g., a moisture increase of only 0.5%) to generate a change in a_w of 0.10 to 0.45.[63] Data with *B. longum* show very high drops in stability during storage when a_w values are higher than 0.30.[60] It must therefore be remembered that very small increases in powder moisture due to absorption of the air humidity can strongly reduce the stability of the culture from an opened sachet. Using a culture from an opened sachet presents risks, and is generally not recommended; freeze-dried products which have been opened should be used within a few days, even if restored at 4°C. This also explains why sachets that have been inadvertently perforated will not keep their activity and should be discarded. Another contributing factor of importance is oxygen in the air, which is a strongly detrimental for some of the probiotic strains.

With respect to frozen products, a thawed culture simply must be used immediately. They cannot be refrozen with success at the plant, and even storage at 4°C generates losses of activity within a few hours.

19.5.3 Rehydration

Increasingly, producers of fermented FF abandon the preparation of liquid starters at the plant and carry out direct inoculation of the manufacturing vat. In this case, dried cells are added directly to the food matrix and must rapidly be active. The conditions in which the culture is rehydrated are critical to its viability.

The temperature of the rehydration medium is arguably the most important factor affecting viability. If the manufacturer wishes to carry out a direct-to-the-vat inocu-

lation, he must make sure that the food matrix is at an appropriate temperature. A general rule is to rehydrate the cells at the temperature that is close to their optimum growth temperature. Thus, with mesophilic lactococci, rehydrating at 22°C gives much higher viability levels than at 4°C or 37°C.[83] This temperature effect has also been seen with thermophilic bacteria, albeit at a higher level. Viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* were five times lower when the culture was rehydrated at 4°C as compared to 37°C.[64] Such an effect on the viability of bifidobacteria is also reported,[63] and this is presumably the case for many other probiotic bacteria. It therefore appears desirable to rehydrate mesophilic cultures at temperatures between 20 and 30°C, whereas for probiotics and thermophilic bacteria, temperatures between 35 and 40°C seem best.

If direct-to-the-vat inoculation does not create any technological problems, it is recommended to proceed in this fashion. But there are instances where (1) it will be difficult to evenly distribute the culture in the matrix or (2) the food matrix has a very low pH (e.g., fruit juices or wine) or contains toxic compounds (e.g., spices) that generate high viability losses. In these circumstances, it might be preferable to rehydrate the culture in a preliminary step, and subsequently add the cell suspension to the food matrix.

In addition to showing great care with respect to hygiene, manufacturers must standardize the procedure. Three parameters must be controlled to ensure high viability of the rehydrated cell suspension: temperature, composition of the medium, and rehydration time. The considerations on medium temperature that were discussed earlier also apply here. The second parameter in a rehydration procedure is medium composition. Water is not recommended,[65] especially if it is chlorinated. A salt solution can be acceptable,[66] but a medium containing a relatively high concentration of solids, particularly carbohydrates, is preferable[67,83] The third parameter is rehydration time. When a concentrated culture is rehydrated in a small volume, cells die after a period of time. Viability losses will vary between strains, but the data of De Valdez et al.[67] suggest that the rehydration period of a highly concentrated cell suspension should not extend much above 15 min. As an example, extending the rehydration period of a concentrated *Lb. acidophilus* ATCC 4356 culture from 15 to 30 min reduced viability levels from 68 to 4%.[67] Once this short rehydration period is over, the cell suspension should be blended with the food matrix.

19.5.4 Process Adaptations Due to Probiotics

If the probiotic bacteria do not grow well in the food matrix, or if they do not compete well against the starter bacteria of the fermented FF, a certain number of actions can be attempted to promote their growth. Examples of what has been proposed for fermented dairy products are presented in Table 19.5. Many of these strategies could extend to other fermented FF, and some will be presented in other chapters of this book.

TABLE 19.5
Some Process Adaptations to the Manufacture of Fermented Functional Dairy Foods in Order to Promote the Growth of Probiotic Bacteria

Adaptation	Example of application	Reference
Changes in the food matrix		
add antioxidants (ascorbic acid, antioxidants)	Yogurt	68,69
add enzymes (proteases, lactase)	Fermented milks	70,71
add prebiotics or other growth supplements	Yogurt, fermented milks	72–74
Modify starter culture use		
reduce starter inoculation level	Yogurt	75
select starter which can scavenge oxygen	Yogurt	76
apply stress conditions to starters	Fermented milks	77
delay inoculation with the starter	Yogurt	78
Technological adaptations		
deaeration of milk	Yogurt	79
fermentation temperature to better suit probiotic	Yogurt, cheese	80,81
grow probiotics separately (cream dressing)	Cottage cheese	82

19.6 CONCLUSION

One aim of this chapter was to show the extent of the selection process of probiotics as well as the complex technology required to supply probiotic cultures to industries that produce fermented FF. The great challenge of growing probiotic cultures at a manufacturing plant can only be tackled by using a holistic approach and having qualified personnel and sophisticated quality control laboratories.

In large scale productions units of fermented FF, probiotics are already added through direct-to-the-vat inoculation of commercial preparations. Although the traditional starters are still added, it is to be expected that some may gradually be replaced by probiotic cultures for the main fermentation task. As probiotic bacteria may generate different flavor profiles from those of the traditional starters, this will provide an opportunity for the development of new lines of fermented FF products.

Many small producers of fermented foods throughout the world still rely on spontaneous fermentations based on the contaminating flora. Although many species in such fermented foods have the potential to be probiotic,[24] health attributes can only be expected if strains having documented clinical effects are used. To achieve this, direct inoculation of proven strains using proper rehydration techniques will be required. Even for such an apparently easy task as adding a probiotic containing powder to a food matrix, a number of parameters must be controlled if the cultures are to be viable in the food product and express their beneficial effects when consumed.

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20 The Future for Fermented Foods

Edward R. Farnworth

CONTENTS

20.1 INTRODUCTION

Fermented foods are consumed in every country of the world, and as the chapters in this book show, there is growing scientific evidence that many fermented foods are good for health or contain ingredients that are good for health. Foods that improve or change the intestinal microflora are of particular interest because of our increased knowledge of the role the intestinal microflora population plays in health and disease resistance. In the future, more fermented foods with health promoting properties will become available on the market, many directed towards consumers with very specific health and metabolism needs.

The desire to buy and consume foods that impact favorably on the gastrointestinal (GI) tract will be driven both by consumer demands and the advancement of knowledge in both microbiology in general, and the human intestinal microflora in particular. It is becoming more evident that each individual has a unique microbial microflora and as more sophisticated and rapid methods are developed that can characterize this population, the nutrition and health implications of maintaining or altering it will become more evident.

In the future, fermented foods will become even more important in our diet and the maintenance of health, as we identify different microorganisms that can be used in the production of probiotic foods. Probiotic foods will be made that target specific age groups who have specific metabolic requirements (newborns, adolescents, seniors), people in specific disease states (irritable bowel syndrome, Crohn's disease, intestinal cancers) or those who have had their microflora compromised (irradiation patients, intestinal surgery patients, postantibiotic treatment). These advances will occur as we understand more about the role the intestinal bacteria play in human health, and we are able to identify the mechanisms involved in the interaction between food bacteria passing through the GI tract and the host intestinal bacteria.

New processes in food technology will allow for the incorporation of microorganisms in a wider range of foods and beverages. At this time, it is still not clear whether all microorganisms must be alive when they are consumed to exert their beneficial effect, however, it is apparent that many conditions during food processing (extremes of temperature, humidity, pH) challenge the viability of most microorganisms.[1] New ways will be found to add and protect microorganisms during manufacturing, packaging, and storage. Or alternatively, new strains of microorganisms will be identified with characteristics that make them better ingredients in food products. In addition, new technologies and processes such as controlled release (by pH, by time, by enzymatic action) encapsulation will be developed that enable live bacteria to be delivered to very specific locations along the GI tract. In the future, novel foods that contain both probiotic bacteria and prebiotics (substrates required by targeted bacteria) that have been termed synbiotics will be available to the consumer.[2]

Foods that are produced by, or contain microorganisms when they are consumed can impact on many disease states that plague humans today. As we start exploring beyond Earth, other problems will arise. Health and metabolism problems have already been identified during our first ventures into space. In the future, astronauts may consume probiotic foods to protect them during space travel and aid in their adaptation when they return to earth.

20.2 ADVANCES IN MICROBIOLOGY

Our knowledge of what endogenous bacteria are contributing to the host will advance only when they are fully identified and enumerated. It has been estimated that even with our current knowledge of the growth requirements of bacteria, only about 60% of the bacteria observed in the human GI tract can be isolated and grown and identified outside the body.[3] Welling et al.[4] quote data from an experiment where total *Bifidobacterium* spp. were counted using plate culturing (3.87×10^{10}/g) compared to nonculturing techniques (2.71×10^{11}/g). Suau et al.[5] used *Fusobacterium prausnitzii* as an example of a bacteria that is believed to be one of the prominent species in the human gut, but which is often reported as not detected, possibly due to problems with traditional enumeration techniques. Often more than one bacterial species is capable of growing on a specific media, again adding to an inaccurate count of the microbial population. These technical shortcomings lead to an incomplete picture of gut microbiota.

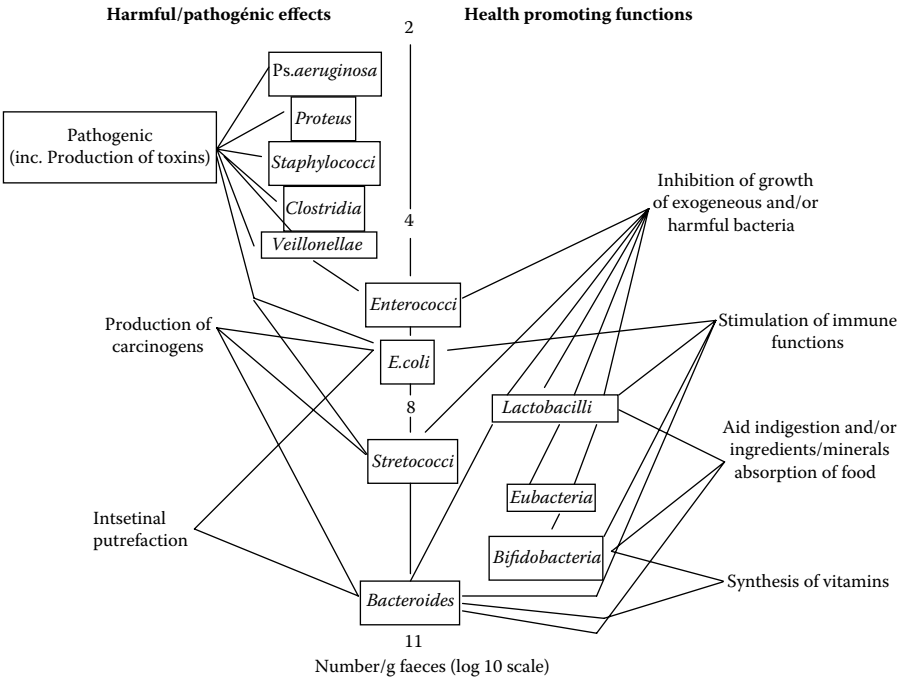

FIGURE 20.1 Bacterial population in the human gastrointestinal tract. (From Gibson, G.R., and Roberfroid, M.B., Dietary modulation of the human colonic microbiota: Introducing the concept of probiotics. *J. Nutr.* 1401–1412, 1995. With permission.)

Various reports have been made as to the population of the human microflora. Figure 20.1 lists what are believed to be the most common species. Figure 20.1 also shows one estimate of the size of individual bacterial species families. However, the population estimates appear to vary depending on the methodology used, and can vary between individuals. Advances in molecular biology has meant that a large number of bacterial groups in the gut ecosystem can now be identified and counted unequivocally—even many bacteria that are not culturable using classical techniques.

20.2.1 New Methods of Identification and Enumeration of Bacteria

Ribosomal 16S rRNA has been used in molecular phylogeny, where the different degrees of variability in the nucleotide makeup allows for the construction of phylogenetic trees and the establishment of evolutionary links between species.[6] Using the many bacterial nucleotide sequences that have been elucidated, complimentary DNA-probes can be made that will hybridize uniquely with a specific 16S rRNA sequence.[7] Probes generally need only be around 20 nucleotides in length. Depending on the uniqueness of the 16S rRNA being studied, probes can be customized to identify bacteria at different phylogenic levels (domain, family, genus, species). The fluorescence in situ hybridization (FISH) method uses synthesized oligonucleotide probes that have been labeled with fluorescent dyes that target the 16S rRNA of

various bacteria (see Figure 20.2). Table 20.1 lists some of the nucleotide sequences that have been used to study human intestinal bacterial populations.

The FISH method is not without its own problems. Some counting of nontarget organisms can occur, and sensitivity may limit applications.[8,9] It has been also pointed out that in some cases, such as with Gram-positive bacteria (e.g., lactobacilli) penetration of the cell wall may be difficult and require pretreatment of the sample

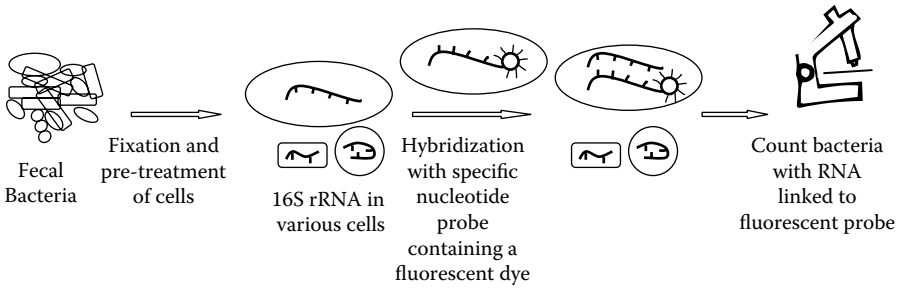

FIGURE 20.2 The FISH method.

TABLE 20.1
Nucleotide Sequences Used in F.I.S.H. Analyses of Fecal Bacteria

Nucelotide Sequence	Bacterial Species
HIS 150	Clostridia
5′-TTA TGC GGT ATT AAT CT(CT) CCT TT-3′	(Clostridium perfringens/histolyticum subgroup)
Bif 164	Bifidobacteria
5′- CAT CCG GCA TTA CCA CCC-3′	
Bac 303	Bacteriodes
5′- CCA ATG TGG GGG ACC TT-3′	
Lab 158	Lactobacill-enterococci
5′- GGT ATT AGC A(CT)C TGT TTC CA-3′	
COR653	Coriobacterium and (Harmsen) collinsella
5′ – CCC TCC C(A/C)T ACC GGA CCC – 3′	
ATO291	Atopobium cluster (Harmsen)
5′ – GGT CGG TCT CTC AAC CC – 3′	(includes coriobacterium group)
E.had579	*Eubacterium ventiosum (Schwiertz)*
5′ – GCA TCC ACC ATA CCG TTC AG – 3′	
S-*-Fprau-0645-a-A-23	*Fusobacterium prausnitzii (Suau)*
5′ – CCT CTG CAC TAC TCA AGA AAA CA – 3′	

Sources: Schwiertz, A., le Blay, G., and Blaut, M., *Appl. Environ. Micrbiol.*, 66, 375–382, 2000; Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., and Welling, G.W., *App. Environ. Microbiol.*, 64, 3336–3345, 1998; Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H.F., and Welling, G.W., *Appl. Environ. Microbiol.*, 61, 3069–3075, 1995; Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.-H., *Microbiol.*, 142, 1097–1106, 1996; Harmsen, H.J.M., Wildeboer-Veloo, A.C.M., Grijpstra, J., Knol, J., Degener, J.E., and Welling, G.W., *Appl. Environ. Microbiol.*, 6, 4523–4527, 2000. With permsission.

with enzymes.[4,8] In spite of these problems, bacterial species such as bifidobacteria can be enumerated as accurately as with traditional counting techniques,[10] and as has been shown recently, with replicate sampling and counting, the variability of data using F.I.S.H. can be much less than that obtained by plate counting methods.[11]

A DNA-RNA hybridization technique has been used by Sghir et al.[12] to estimate the relative proportions of the various intestinal bacterial populations in 27 human fecal samples. Individual bacterial groups are enumerated using a group specific probe, and this count was compared to that obtained with a universal probe (Bact 338) that hybridizes to a conserved rRNA sequence that is found in the majority of bacterial cells. 70% of the 16S rRNA hybridized by the universal probe was hybridized by the sum of six oligonucleotide probes (Bacto1080, Clept1240, Erecc482, Bif412, Lacto722, Enter1432). Total *Bacteroides* made up 37% of the total fecal bacteria probed, whereas *Lactobacillus* + *Streptococcus* + *Enterococcus* were found to contribute to 1% of the total. The Bifidobacterium group accounted for less than 1% of the total. Sghir et al.[12] emphasized the simplicity and specificity of this method and suggested it would be useful in nutrition studies where changes in the major microbial species could be monitored.

The FISH method is particularly suited for monitoring the effects of dietary changes on larger (> 10^6 cells/gram) populations of intestinal bacterial species.[11,13,14] Publications are now appearing in the literature using this method.[15–17] As more probes become available in the future, the application of this technique will grow.

Denaturing gradient gel electrophoresis (DGGE), which is based on the principles of temperature gradient gel electrophoresis (TGGE), is the most recent technique to be used to study the intestinal microbial population.[18,19] Figure 20.3 is a schematic of this procedure. The bacterial mixture is first digested to obtain 16S rRNA fragments. The 16S rRNA's are amplified using polymerase chain reactions (PCR), and then separated by DGGE. The DGGE uses a polyacrylamide gel that contains a gradient of urea and formamide that partially denatures (melts) the migrating 16S rRNA fragments and alters their structure and rate of migration. Bacteria with different 16S rRNA produce different 16S rRNA fragments, which in turn produce

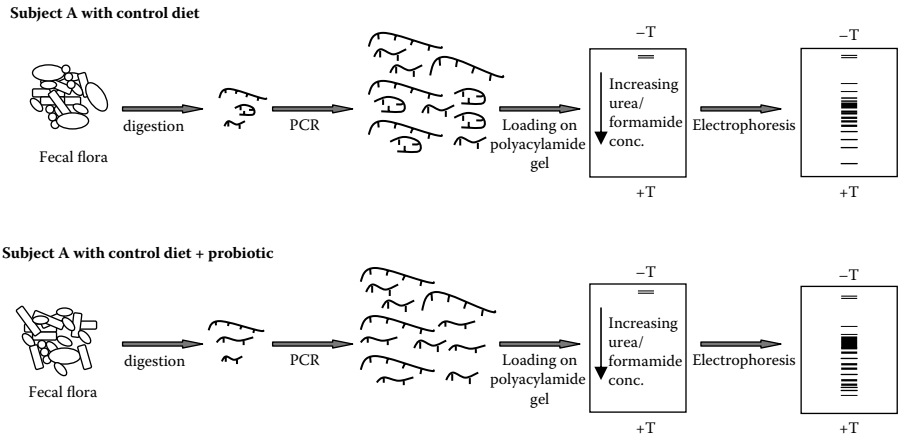

FIGURE 20.3 The DGGE method.

different migration patterns during electrophoresis. A characteristic pattern of 16S rRNA sequences from the original samples is obtained.[19] If there is a need to identify bacterial species, bands in the gel produced by DGGE can be cut out and the DNA fragments used to identify the bacterial species.

Tannock's group have used DGGE together with species-specific PCR primers to study fecal samples from mice, pigs, and humans.[20,21] They have shown that the DGGE method is able to identify bacteria down to the species level, and that in several cases, bacteria identified using DGGE were absent from traditionally plated samples. Changes in the DGGE pattern were used to identify the contribution of dietary bacteria to the total fecal population and to follow the time course of an administered probiotic bacteria (*Lactobacillus rhamnosus* DR20).

With the advent of techniques capable of identifying and enumerating bacteria at the species level, it is becoming apparent that there is a great variation between the microbial ecosystems of individuals, which means in the future, probiotic products may have to be tailored to specific individual needs.

20.2.2 Bacteria-to-Bacteria Communication

The population of bacteria that inhabits the gastrointestinal tract is large and diverse, and over time a symbiosis is established that results in a relatively stable population.[22,23] Endogenous bacteria occupy specific niches, and bacteria that is ingested in probiotic foods often are unable to displace these resident bacteria. Bacteria have a limited number of options to use in order to repel other microorganisms that may be competing for niches along the GI tract. It has recently been shown that bacteria that are part of a large population of like-bacteria are able to act in unison against a potential danger posed by foreign bacteria. Bacteria communicate with each other, and they do not need to be in physical contact to do so.[24] Some bacteria use this communication system as a means of estimating the number of like-bacteria close by and then signaling the production of protective bacteriocins. Wirth et al.[24] have used the term *bacterial pheromones* to describe these chemical compounds that are produced within the bacteria, diffuse freely across the cell membrane, and accumulate in the surrounding medium. If the pheromone accumulates above a critical concentration extracellularly, an intracellular response regulator is activated. This system of communication signals bacteria to produce antibiotics and virulence factors.

Acylated homoserine lactone (AHL) was one of the first signal molecules to be identified and is perhaps the best understood.[25] The enzyme that makes AHL (Lux I-type protein) and the protein that detects it and responds by activating specific genes have been identified.[26–28] In one study, it has been shown that a bacterium that resides on wheat has an AHL system that it uses to determine when to make antibiotic that helps it suppress its microbial competitors, as well as protect its host.[28] This bacteria-to-bacteria communication mechanism has been termed *quorum sensing, autoinduction,* or *population density–responsive gene regulation*. It is believed that this attribute enables related bacteria to be competitive in a mixed bacterial population. It is also possible that the bacteria are exchanging more than just population density information.

It is evident that as these forms of communication between bacteria become better understood, it will be possible to choose probiotic bacteria that will be better able to compete with endogenous intestinal bacteria. At the present time, a major weakness of probiotic products is that the bacteria consumed are not able to displace endogenous bacteria and that after the feeding regime is stopped, the food bacteria cannot be detected in the GI tract of the host. Bacteria that can sense the presence of pathogenic bacteria and are capable of releasing bacteriocins could also be used in place of broad-spectrum antibiotics to kill pathogenic bacteria that cause disease and infection.

20.2.3 Bacteria-to-Intestinal Cell Communication

The diverse bacterial population that inhabits the GI tract have the opportunity to interact with food bacteria setting up cell-to-cell communication. However, even more important is the fact that the cells that line the GI tract come in contact with the contents of the digesta including both beneficial (probiotic) bacteria and enteric pathogens. Recent reports indicate that a complex host cell–bacteria communication occurs between the intestinal cells and GI tract bacteria. This bacteria-epithelial cross-talk is best understood in the case of pathological bacteria. It appears that a variety of mechanisms are used by the foreign bacteria to talk to cells.[29] This first step is followed by processes that allow the bacteria to adhere and invade mucosal cells.

Researchers have shown that by using a group of mice reared in a germ-free environment and then exposing these mice to either intact mice microbiota or a selected group of organisms, the metabolism of the cells lining the intestine can be altered. In normal mice, the upper portion of the crypts and their associated villi in the mouse ileum produce fucosylated intestinal glycoconjugates. Bry et al.[30] have shown that germ-free mice lose the ability to produce these fucosylated glycoconjugates, but that it can be restored by inoculating the germ-free mice with gut flora from conventionally reared mice or with *Bacteroides thetaiotaomicron*, a bacteria that is normally found in mice and human intestines. The B. *thetaiotaomicron* were only effective in restoring the fucosylation when their concentrations reached $> 10^7$ CFU/ml ileal contents. It is believed that the bacteria communicate with the mammalian cells without direct binding and that soluble mediators are involved in the cross-talk mechanism.

Lu and Walker[29] speculate that some of the beneficial effects that have been attributed to probiotic bacteria such as disease prevention, immune system stimulation, and strengthening of the epithelial barrier are the result of biochemical communication between the probiotic bacteria and enterocytes. If this is true, and the mechanism of communication can become understood, probiotic products could be designed that are capable of altering targeted metabolic pathways in intestinal cells.

20.3 THE ROLE OF INTESTINAL BACTERIA IN HUMAN HEALTH IN THE FUTURE

Many of the beneficial functions of probiotic bacteria have not been defined yet. However, researchers have come to identify some bacteria as “good” and some detrimental to the host (Figure 20.1). It is the goal of all probiotic and prebiotic research

to produce the most favorable balance of GI microflora and thereby improve the metabolism, digestion, health, and disease resistance of the host.

20.3.1 New Probiotic Products

At the present time three bacterial species dominate in terms of interest, research into their possible beneficial effects, and number of products already on the market. These bacteria include *Bifidobacterium* (including *bifidum*, *breve*, *infantitis*, *longum,* and *adolescentis*), *Enterococcus* (including *faecium* and *faecalis*), and *Lactobacillus* (including *acidophilus*, *paracasei*, *rhamnosus,* and *reuteri*).[31] Tables 20.2 and 20.3 are lists of probiotic products currently on the market that contain *lactobacillus* and bifidobacteria. Although lactic acid producing bacteria are dominant today, in the future, other characteristics may become important. For example, some propionic acid– producing bacteria produce vitamin B_{12}—a characteristic that would be important in the design of foods for vegetarians, who have few sources of vitamin B_{12} in their diet. Also very little attention has been paid to the potential benefits of incorporating yeasts and molds in to probiotic products.

Many of the probiotic bacteria of interest are "of human origin," and therefore there is a tendency to believe that these bacteria are better suited to be included in human food, if the ultimate target is the human intestine. However, at this time, there is little scientific evidence to support this assumption. Very often potential probiotic species do not grow well in milk, and given the fact that a large majority of commercially available probiotic products today are milk based, this presents problems in terms of maintaining viability until the product is consumed. In the future, other food matrices besides milk will be used as the basal ingredient in probiotic foods.

TABLE 20.2
Some Fermented Milk Products Containing *Lactobacillus acidophilus*

Product	Microorganism
Acidophilus milk	*Lb. acidophilus*
Acidophilus paste	*Lb. acidophilus*
Acidophilus buttermilk	*Lb. acidophilus, mesophilic starter*
Acidophilus natural buttermilk	*Lb. acidophilus, mesophilic starter*
Acidophilus yoghurt	*Lb. acidophilus, Streptococcus thermophilus, Lb. delbrueckii* subsp. *Bulgaricus*
Biogurt	*Lb. acidophilus, S. thermophilus,*
Acidophilus-yeast milk	*Lb. acidophilus,* lactose–fermenting yeasts
Acidophilin	*Lb. acidophilus, Lactococcus lactis,* kefir culture
LC1	*Lb. acidophilus La 1*
ABS Ferment	*Lb. acidophilus, Bifidobacterium, L. casei*
LA-7 plus	*Lb. acidophilus, B. bifidum*
Vita	*Lb acidophilus LA-H3, B. Bifidum LB-H1, L. casei LC-H2*

Source: Kalantzopoulos, G., Detection and enumeration of probiotic cultures, in *Encyclodedia of Food Microbiology*, Batt, C.A. and Patel, P.D., Eds., Academic Press, N.Y., 1999, 1373–1379. With permission.

TABLE 20.3
Some Fermented Milk Products Containing Bifidobacteria

Product	Micro-organism (s)
Bifidus milk	*Bifidobacterium bifidum* or *B. longum*
Bifigurt	*B. bifidum, Streptococcus thermophilus*
Biogarde	*B. bifidum, Lb. acidophilus, S. thermophilus*
Biokys	*B. bifidum, Lb. acidophilus, Pediococcus acidilactici*
Special yogurt	*B. bifidum(B. longum), Lb. acidophilus , S. thermophilus, Lb. delbrueckii* subsp. *Bulgaricus*
Cultura	*B. bifidum, Lb. acidophilus*
Mi-Mi	*B. bifidum, B. breve, Lb. acidophilus*
Progurt	*Lactococcus lactis biovar diacetilactis, Lactococcus lactis* subsp. *cremoris, Lb. acidophilus* and/or *B. bifidum*

Source: Kalantzopoulos, G., Detection and enumeration of probiotic cultures, in *Encyclodedia of Food Microbiology*, Batt, C.A. and Patel, P.D., Eds., Academic Press, N.Y., 1999, 1373–1379. With permission.

20.3.2 Products for Specific Consumers

20.3.2.1 Infants

Studies of the bacterial profile of individuals has shown that, as the diet changes from a milk-based one during infancy to a more varied one as we age, the population of gut bacteria changes. The newborn is inoculated by maternal bacteria, and this leads to the colonization of large numbers of facultative anaerobes such as *Esherichia coli* and enterococci.[32] This, in turn, produces a highly reduced environment that favors the growth of strict anaerobes. At this stage of life, diet plays an important role in determining the microbiological population of the GI tract. Both breast-fed and bottle-fed infants have a microbiota dominated by bifidobacteria. However the bottle-fed infants have a characteristic colon microflora that contains higher counts of other bacteria including bacteriodes, eubacteria, enterobacteria, peptococci, and streptococci.[33,34] The long-term health implications of these differences is not known at this time. However, much of the interest in bifidobacteria as possible probiotic bacteria stems from these observations.

The regulations governing the composition of milk replacers and infant food are very strict in most jurisdictions.[35] At the present time, regulations related to the microbiological composition of infant foods are limited to measures to ensure that infant formulas are not contaminated by pathogenic bacteria.[36] Human milk and the breast-fed infant are used as standards, and it is apparent that current milk replacers do not contain any live bacteria or any prebiotic material specifically added to encourage the establishment of a pattern of bacteria in the formula-fed infant that is closer to that of the breast-fed infant. It is conceivable that in the future, milk replacers and infant formulae will contain live bacteria that when ingested by the infant, may produce a microbial population more like the breast-fed baby.

The intestinal microbial population, once it is established, is resistant to change. Therefore, targeting infants with specific beneficial bacteria may prove to be the best time to change the endogenous bacterial population. As time passes, the endogenous microbiota becomes more established and bacteria ingested in probiotic products have been shown to have a very low capacity to displace endogenous bacteria, thrive, and grow.[37] The factors that allow the members of the endogenous microbiota to establish and maintain their regional habitats is largely unknown at this time.

20.3.2.2 Aging Population

It has been shown that as humans age their intestinal bacteria population changes. For example, it is generally agreed that *Bifidobacterium infantitis* and *B. breve* predominate in the infant, but *B. longum* and *B. adolescentis* are most common in the adult and *B. adolescentis* most common in the elderly. These changes in the intestinal population are in due to diet, but other factors including environment, disease state, use of drugs and antibiotics can also be important.[38] Although there is no accepted "ideal" pattern of GI bacteria, it is believed that it would be beneficial, in the long term, to increase or maintain high levels of bifidobacteria and lactobacilli. It is believed that with proper intervention, digestive system related-problems such as diarrhea, constipation, and gastroenteritis could be alleviated. In addition, as the metabolism and function of these bacteria becomes better understood, it is anticipated that other benefits will be realized, including an improved resistance to GIT infections, improved immune response, prevention of cancer, production of vitamins, and increased calcium uptake.

At the present time a multinational, multidisciplinary study is being carried out in Europe to characterize the "typical" gut microflora of seniors and young adults.[39] This basic information is intended to be used as a baseline to which the effects of future interventions can be compared. Only by gathering such extensive data will it be possible to establish any relationships between the makeup of the gut microflora population and health and disease status. It is believed that with the use of probiotic products (those that contain live bacteria) prebiotic products (those that contain specific nutrients, in particular complex carbohydrates), and synbiotic products (those that contain both probiotics and probiotics), new food products can be developed that maintain a healthy gastrointestinal microflora, even as life expectancy increases.

In the future there will be products specifically designed for particular age groups, those on special diets such as vegetarians, those with specific metabolic requirements, and even different probiotic products for men and women.

20.3.2.3 Astronauts

The preoccupation of nutritionists today is, of course, focused on the various sectors of the population here on Earth. Our experience with extraterrestrial travel has only a 41-yr history,[40] but since the initial flights, astronauts have been under special scrutiny, and already several nutrition/metabolism-related problems have been noted as a result of weightlessness, nutrient intake changes, and stress. [41–46]

The nutrition/metabolism problems that affect the performance of astronauts include: gastrointestinal disorders, bone calcium loss, radiation damage, and immune response changes.[41,42,47–50] As space flights get longer in duration, health/metabolism problems will become even more important and may, in fact, put limits on how long we can live away from Earth.[42,45,51] Some of the properties of fermented foods noted in this book may present partial solutions to these space travel related problems.

20.3.2.3.1 Intestinal Problems—Diarrhea/Constipation

Intestinal disorders of any sort could be very debilitating during space travel. Even a short bout of diarrhea could present many practical problems in the confines of a space suit or space ship. Preflight precautions are taken in the preparation and packaging of food to avoid food-related infections, but it is inevitable that, in spite of the high health status of the astronauts, diarrhea may strike space travelers. Several studies have been reported in which probiotic products containing particular strains of bifidobacteria, streptococcus, or lactobacillus have been successfully used to prevent diarrhea and to reduce the severity and duration of a variety of diarrheas.[52–55] Hove et al.[54] list 12 studies in which the consumption of fermented milk products or particular bacterial strains prevented or reduced the severity of diarrhea in infants and adults. The inclusion of probiotic products with a specific bacterial composition may be one way to reduce problems of diarrhea in space.

It is a general feeling that constipation is a problem that plagues astronauts, although it is not well documented.[56] The cause of this disorder is not clear, but it may be a combination of an inadequate water intake, confined living space, limited facilities for personal hygiene, and lack of gravity. In the case of the latter, there has been much speculation about the effects of zero gravity. Usually, it is assumed that peristalsis is responsible for facilitating the passage of food down the digestive tract. However, it is not clear whether this activity is assisted by, or is in some way dependent on, gravity. Changes in the perceived taste, texture, and bulk of meals may also be contributing to digestive problems. In any case, it is common practice for astronauts to use mild laxatives during their missions to ensure proper bowel function.[56]

Probiotic products have a long history of effects at the intestine level in terms of regulating bowel function.[57] Both stool frequency and stool softness were observed to increase when chronically constipated patients were given a strain of *E. coli* daily.[58] Geriatric patients were found to have increased defecation rate and decreased use of laxatives after consuming as little as 200 mL of acidophilus milk daily.[59] Relief of constipation and stimulation of intestinal motility are listed as beneficial effects of probiotic products[60,61] Whether the positive effects of probiotics would be great enough to overcome the other constraints imposed during space travels will have to be investigated.

The role of probiotics is often stated as to establish a balanced intestinal microbial population, and so it is clear that including probiotic products in the diet of astronauts may be one way to ensure proper intestinal function and reduce the possibility of diarrhea and constipation during space flights.

20.3.2.3.2 Calcium Metabolism

It has been well documented that during space flight, calcium metabolism is altered; calcium is lost from skeletal bones, serum levels of calcium rise, calcium excretion

is increased, and some astronauts go into negative calcium balance.[47,48] This is a particular problem for the weight-bearing bones. Up to 0.4% of the total body calcium is lost per month, and there are concerns that this rate may not decline as the time in space increases.[48] It appears that after return to earth, calcium uptake by bones occurs and blood levels and excretion rates return to normal values, but it is not clear at what point bone loss during space travel will be too great to replenish.

Dairy products in general are considered as good sources of dietary calcium, and fermented milk drinks have been shown to be a good way to increase calcium intake.[62] The question of whether calcium absorption is improved after eating fermented foods is not clear. Early results using rats show (implied) improved calcium absorption.[63] Feeding yogurt has been shown to increase serum calcium levels,[64] but others have found no effect.[65] However, as Recker et al., pointed out, the availability of calcium in fermented milk products may depend on the bacterial culture used to prepare the product.[65]

It is generally assumed that fermented foods can bring about a lowering of the pH in the GIT, which should improve calcium absorption, and therefore including fermented milk products in the diet of astronauts may help improve calcium balance during long space voyages.

20.3.2.3.3 Radiation Damage

The effects of exposure to larger quantities of high energy radiation during space travel were anticipated as space craft ventured beyond the Earth's atmosphere and its geomagnetic field that protect against high energy-charged particles. Based on dosimeter readings taken inside and outside space craft, it has been possible to calculate the amount of radiation astronauts are exposed to, and to calculate their probability of contracting radiation-induced cancer and radiation-induced cataracts.[49,66] This aspect of space travel could have a major impact on astronaut health even when they return to Earth, and therefore ways of reducing the effects of this exposure need to be found.

The antimutagenic properties of yogurt fractions, fractions of milk fermented by *Lactobacillus helveticus* L89, and a mixture of *Bifidobacterium longum*, *Lb. gasseri,* and *E. coli* have been demonstrated using in vitro tests.[67–69] In these experiments, the ability to prevent damage by known chemical- and food-derived carcinogens and mutagens was tested. Both the bacteria themselves and fermentation products that presumably contain active ingredients formed during fermentation have been shown to be protective.

In animal models, both yogurt and kefir have been shown to suppress the growth of implanted tumours,[70–73] indicating that either the bacteria in these products or some product of the fermentation of milk is capable of slowing the progression of tumors.

Exposure to radiation produces several forms of acute injury. A 10,000 rad exposure produces rapid death as a result of damage to the central nervous system. At lower doses (approx. 1000 rad), changes occur to the gastrointestinal bacterial population that also result in death, but over a period of days to weeks. Dong et al.[74] were able to show that feeding mice *Lactobacillus GG* before exposure to 1400 rads of radiation changed the intestinal flora and prolonged the life of the mice.

It would appear that the consumption of fermented milk products may be a way to reduce the progression of cancer/tumors that result from exposure to ionization during space travel, and may also be a way to restore the balance of intestinal microflora in astronauts who have been exposed to large dose of radiation. Whether fermented foods can also prevent the initial inter- and extracellular damage produced by radiation is not clear at this time.

20.3.2.3.4 Immune Function

Access to medical treatment during any voyage in space is extremely limited, and so it is of utmost importance that astronauts be in good health. To date this has been achieved by selecting young, healthy individuals as candidates for space travel and monitoring their immunological response before, during, and after space travel. Both in vivo and in vitro experiments have shown that cellular immune response is depressed during space flight.[75] As space voyages get longer in duration, it is evident that every means possible must be used to keep astronauts healthy and disease free; maintaining or enhancing immune function will be important.

The impact of diet on the immune system is becoming clearer, and there is a growing consensus that the consumption of probiotics can improve immune status.[76] The host immune system appears to be enhanced by activating macrophage function and increasing the activity of natural killer cells and T-cells. Again, it is not clear whether it is the bacteria themselves or some metabolite(s) produced by the bacteria that is stimulating the immune function. Even if the exact mechanism is unknown, it may be prudent to include probiotic products in the diets of astronauts as a way to enhance their immune function as protection against disease and infection.

20.4 REGULATION AND HEALTH CLAIMS FOR FERMENTED FOODS

The number of fermented foods appearing in the marketplace will increase in the future, and it is evident that consumers will want more information about what they are eating—in particular, what is in their food, and how will it affect both short-term and long-term health. Most counties around the world have started to address the issue of health claims for functional foods,[81,82,83] and in the future, many fermented functional foods will carry government-approved claims (structure/function, risk reduction, and treatment). As with any functional food, fermented functional foods will only be given a health claim that is supported by sound scientific evidence.[84] Because most jurisdictions make a clear distinction between foods and drugs, it is unlikely that any fermented food product will ever carry a disease prevention claim.

It is acknowledged that designing experiments to show the efficacy of fermented foods present unique problems.[85] Of equal importance is the selection of appropriate biomarkers that can be used to measure the in vivo effects of consuming fermented foods.[86] Both of these factors may slow down the number of submissions by food manufacturers to food health regulatory officials. However, as has been shown in many of the chapters in this book, a wide variety of diseases and health conditions can be affected by the consumption of fermented foods; these effects go well beyond improving gut health. In the future, consumers will find more food choices that are good for their health.

20.5 CONCLUSIONS

As our knowledge of the role microorganisms play in human nutrition, immune function and disease resistance increases, so will the number of fermented products on the market. In some products it will be necessary to ensure that the microorganisms consumed are alive, but in other products, metabolites and/or fermentation products may be the active ingredient. Fermented foods will be available for specific niche consumers.

Fermented foods have been part of the human diet for centuries and it may well be that they will become important in the diets of future space travelers.

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Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

Y

Z

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