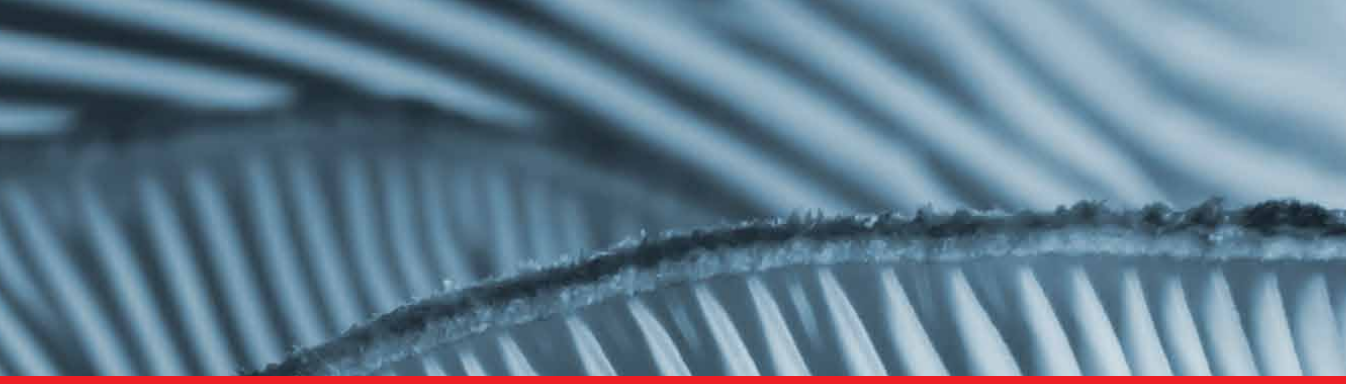

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Current Issues and Advances in the Dairy Industry

Edited by Salam A. Ibrahim

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Food Science and Nutrition

Volume 1

Aims and Scope of the Series

The significance of food is undeniable, especially in light of the impending challenge facing humanity: ensuring there will be enough food to meet the basic needs of a population expected to reach approximately 10 billion by 2050. These food-related challenges align with some of the United Nations' sustainable development goals, with a target to achieve them by 2030. One thing is certain: food should be not only nourishing and safe but also tailored to the diverse needs of individuals throughout their lifetimes, all while meeting consumers' sensory expectations. Understanding the diverse chemical composition of food, often referred to as biodiversity, and how these components can contribute to human health by considering factors like bioaccessibility, bioavailability, and bioactivity at the organ level, is crucial for grasping and promoting a healthy diet. Thanks to the continuous evolution of analytical methods and interdisciplinary research, significant strides have been made in the field of food science and nutrition.

Meet the Series Editor

Maria Rosário Bronze has been working in Analytical Chemistry since 1986. Her Ph.D. in 1999 contributed to the study of food products using capillary electrophoresis. The main goal of her research since 1999 has been focused on Analytical Chemistry applied mainly to the analysis of foods and by-products of food industry. She conducted research in collaboration with national and international research groups, at iBET and ITQB Technology Division. From 2017 until 2021 she was head of Food & Health Division at iBET and head of the Food Functionality and Bioactives Laboratory. MR Bronze has been an Associate Professor at the Pharmacy Faculty of Lisbon University and head of the Structural Analysis Laboratory since 2012. As a researcher, MR Bronze is a Senior Scientific Advisor at Food & Health Division at iBET and Head of Food Functionality and Bioactives Laboratory at the same Institute, Collaborator at iMED and Researcher at ITQB NOVA. Her current research is focused on quality and beneficial health effects of food components. Gas and liquid chromatography associated with mass spectrometry are used by MR Bronze in the characterization of samples. Sensory evaluation is also an important area of her research. The main food products studied by her are olive tree products (olive, olive oil, leaves), cereals such as maize, legumes (faba bean, pea, chickpea, lentils) fruits (apple, grapes, opuntia ficus), fruit juices and wine, among others. More recently her interests have also involved biodiversity, bioaccessibility, and bioavailability studies on food products and their components, mainly phytochemicals as phenolic compounds, using different analytical tools such as mass spectrometry. As a senior scientific advisor at Food & Health Division at iBET she is involved in different areas: (i) isolation, characterization and formulation of bioactive and functional compounds or extracts from natural sources and wastes from food and other related industries; (ii) pre-clinical assays to provide support to understand health claims related with the beneficial effects of food nutrients/bioactive components; (iii) establishment of analytical methodologies including mass spectrometry state-of-the-art to fully characterize different matrices, from food products, natural extracts or biological fluids (Food Functionality and Bioactives Laboratory).

Meet the Volume Editor

Dr. Salam A. Ibrahim is a Professor of Food Science in the Food and Nutritional Science Program, North Carolina Agricultural and Technical State University, USA. He has established a research program around dairy technology, yogurt starter cultures, probiotics, and bioconversion processes, in addition to food safety and the use of natural compounds to inhibit the growth of foodborne illnesses. His program is well funded by the National Institute of Food and Agriculture-United States Department of Agriculture (NIFA-USDA), the Department of Homeland Security (DHS), and other funding agencies. In addition, the food microbiology laboratory has strong connections with the food industry and often receives funds to support students and scientists around fermentation and the production of stable and functional probiotics products. Dr. Ibrahim is specifically interested in *Lactobacillus bulgaricus*, recognized in the early 1900s as a health-promoting starter culture in yogurt. Currently, Dr. Ibrahim is working on novel methods to produce stable and functional lactic acid cultures for the dairy industry.

Contents

Preface

Milk is one of the most nutritious of all foods and is widely consumed worldwide. It is a rich source of nutrients and has several biological properties that modulate the biochemical processes in the human body. Milk and milk products have not only become essential products in diets but have also become a global industry, thus, understanding the science of milk, and the biochemical, nutritional, and physical properties of its components, is important to create the unique diversity of the dairy industry. Dairy science and technology involves understanding the technical and scientific aspects of processing, packaging, distribution, and storage of dairy ingredients all the way from lactating mammals to ready-to-eat products. For example, the application of advanced science and technology in dairy processing has resulted in enhancing the shelf life of products such as yogurt, pasteurized milk, cheese and other fermented milk foods, butter, protein products, milk powders, and milk-based nutritional formulations with enhanced sensory attributes and nutrition values. Dairy science and technology are applied in optimizing processes and innovation to convert milk into value-added products, ensuring consumer well-being and food security for a growing global population. Thus, this book provides different perspectives on science and technology as they relate to dairy processing.

Chapter 1, "From Traditional Bulgarian Diary Products to Functional Foods", discusses the science of the use of specific starter cultures in the fermentation process for the manufacture of high-quality fermented foods at the industrial as well as domestic levels. The history of fermented food production begins with traditional Bulgarian dairy products. Elie Metchnikoff, a Russian-French zoologist working in Bulgaria in 1907, was encouraged by the observation that certain Bulgarians who consumed fermented dairy lived significantly longer and healthier lives. Later, it was reported that the health effects of fermented dairy products are related to the microbial biodiversity of these products. Specific microorganisms, mainly lactic acid bacteria (LAB), are used in the fermentation of milk to develop these fermented products with unique physical and biochemical properties. The chapter also evaluates the various functional characteristics of LAB microbiota in traditional fermented foods and the possibilities of implementing prospective strains in new functional formulas, following traditional recipes.

Chapter 2, "Medicinal Potential of Camel Milk Lactoferrin", examines the chemical components and medicinal value of camel milk. Camel milk is known for possessing properties similar to those of human breast milk. Moreover, camel milk differs from that of other animals such as cows, goats, and sheep by its antimicrobial, anticancer, and immunomodulatory properties that confer its medicinal potential. Camel milk consists of various bioactive peptides, minerals, mono and polyunsaturated fatty acids, and proteins. It is a rich source of the protein lactoferrin. Lactoferrin is an iron-containing glycoprotein that plays a vital role in the innate immune system of humans and possesses proven cancer-fighting and antimicrobial properties. The global market

for camel milk products is predicted to be valued at USD 53.78 million by 2027 with a compound annual growth rate of 6.8% during that period.

Chapter 3, "Milk Borne Brucellosis", is about food safety specifications related to the milk-borne infectious disease, brucellosis. Food products made with milk from domestic animals carry a high risk of contamination by harmful bacteria. Brucellosis is a common milk-borne bacterial infection that can spread from animals to people when raw or unpasteurized milk products are consumed. Brucella abortus and B. melitenses are common milk-borne pathogens of the species. The primary hosts of B. melitenses are goats and sheep, whereas cattle are associated with B. abortus. Brucella is responsible for an acute feverish illness that can eventually progress to a more serious, chronic, incapacitating disease. The control of risk factors and surveillance are thus the cornerstones of brucellosis prevention.

Chapter 4, "Innovative Approach of Cheese Making from Camel Milk: A Review", relays an innovative approach to maximizing the potential of using camel milk as a base for manufacturing cheese. The quality of the cheese product depends on the chemical composition of the milk base used and the technical characteristics of the cheese-making processes. Regarding its chemical composition, camel milk is significantly different from other kinds of milk, such as cow's milk, which is generally used in cheese production. This can lead to inefficiency in the use of the typical technology in the cheese-making process or result in low-quality end products. The chapter reviews recent advancements in making cheeses from camel milk using starter cultures and coagulants along with potential ingredients for the fortification of final products to maximize the possibility of manufacturing cheese from camel milk.

Chapter 5, "Acid-Induced Gelation of Milk: Formation Mechanism, Gel Characterization, and Influence of Different Techniques", discusses the mechanism of acid-induced gelation of milk. The techniques applied in the acid coagulation process determine the structural properties of the final gels. Thus, a better understanding of the mechanism results in desired properties in fermented dairy products. Acid-induced coagulation of milk is a complex process, and the development of the gelled structure involves the reduction of stability of casein micelles, casein aggregation, and the progressive formation of the protein network during acidification and cold storage. Other than the processing techniques of high-pressure treatment, heating, enzymatic treatment, and ultrasonication involved in acid-induced coagulation of milk, the use of polysaccharides as additives can also determine the microstructure and rheological properties of acid gels.

Chapter 6, "Milk Fat Globular Membrane: Composition, Structure, Isolation, Technological Significance and Health Benefits", discusses the details of the composition and structure of milk fat globule membranes as well as their existence and application in various types of milk and milk-based foods. The milk fat globule membrane (MFGM) is a complex and characteristic structure consisting of lipids and proteins that surround fat globules in milk. MFGM can be used in food as an emulsifier and stabilizer with excellent water-holding capacity in dairy products. MFGM has also been identified as a source of various bioactive compounds that have significant functional roles in human health. Moreover, MFGM has been proven to be a functional food and beneficial infant food due to its potential to support cognitive development and reduce risks of infection.

Chapter 7, "Volatile Aromatic Flavor Compounds in Yogurt: A Review", reviews yogurt flavor and the role of chemical compounds in defining the sensory characteristics of yogurt. The sensory quality, including flavor, texture, and other organoleptic properties of yogurt, is reliant primarily on the relative balance of volatile compounds derived from fat, protein, and carbohydrate in the milk base used during the fermentation process. The type and level of compounds derived during fermentation depend on the starter culture, lactic acid bacteria *Lactobacillus acidophilus* and *Streptococcus thermophiles*, both of which are commonly used in the yogurt industry, and the conditions of the fermentation process. It has been reported that more than 100 different volatile compounds have been identified in yogurt, including carbonyl compounds, alcohols, acids, esters, and sulfur-containing compounds. These compounds are a result of the synbiotic activities of the starter culture (*L. acidophilus* and *S.thermophiles*) in addition to the interaction of the nutrients (lipids, proteins). As with many other dairy products, yogurt is prone to deterioration due to the generation of volatile byproducts, resulting in off-flavors that make the product unsatisfactory for consumers.

Chapter 8, "Lactic Acid Bacteria: Review on the Potential Delivery System as an Effective Probiotic", reviews the importance of lactic acid bacteria (LAB) as a probiotic to human health and its metabolic fermentation and antioxidant properties. In addition, the chapter discusses biotechnological methods that improve the survival rate of probiotics during processing, storage, and gastrointestinal transit, such as microbial encapsulation, freeze drying, spray drying, and so on. LABs are essential dairy starter cultures that are used to produce several fermented dairy products, including yogurt and cheese. LABs are generally employed in food processing due to their significant contribution to enhancing the flavor, texture, and quality parameters of food products. Probiotics and postbiotics can also have antioxidant properties that help in preventing disorders linked to oxidative stress. Products with these beneficial organisms are available commercially in forms such as functional foods and beverages and dietary supplements. Since our knowledge of the relationship between diet and health has increased significantly in recent years, and consumers are now much more actively engaged in how food affects health, the market for probiotics is flourishing.

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Chapter 1

From Traditional Bulgarian Dairy Products to Functional Foods

Dilyana Nikolova, Ramize Hoxha, Nikola Atanasov, Elena Trifonova, Lili Dobreva, Veronica Nemska, Yana Evstatieva and Svetla Danova

Abstract

Production of the traditional yoghurt, white-brined cheese, and yellow cheese, named "kashkaval", in the Bulgarian region determines everyday consumption and health benefits for the local population. Artisanal dairy products and their autochthonous microbiota are a promising source for the research and creation of new minimally treated, but safe, functional and delicious food. The species from *Lactobacillaceae* are used in different fermentation technologies, improving the structure, taste, and aroma of the final products. These products possess a prolonged shelf life due to the biopreservative capabilities of the lactic acid bacteria (LAB) strains, their positive health impact, and many physiological functions in the body. This chapter examines the traditional and modern technologies for the production of typical Bulgarian dairy products. Based on the studies of artisanal products, different LAB species from non-starter microbiota are presented, which contribute to the organoleptic qualities of the products and their beneficial properties. The research focus is aimed at the evaluation of various functional characteristics of non-starter strains, such as metabolic activity and food biopreservation. The long-term goal is to study the tradition to create new functional formulas that are the desired and effective factors for health and longevity.

Keywords: Bulgarian yoghurt, white-brined cheese, lactic acid bacteria, antifungal activity, antibacterial activity, *Lactiplantibacillus plantarum*

1. Introduction

Different types of lactic acid fermentation are the base of more than 3500 traditional fermented foods found worldwide [1]. After sourdough products and vegetables, dairy products are the third most desired and consumed products in Bulgaria (**Table 1**). Yoghurt and yoghurt-like products are the most famous of all dairy products. Their consumption is the highest in the countries of the Mediterranean, Asia, and Central Europe. The consistency, taste, and aroma differ from region to region and from that of other fermented products. In some areas, yoghurt is produced in the form of a very thick liquid, while in other countries, it is in the form of a softer gel.

Dairy products	Year									
	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Pasteurized milk (L)	19.6	20.1	18.8	18.5	17.3	17.2	16.8	16.9	18.0	19.2
Yoghurt (kg)	29.0	28.1	26.9	25.8	27.5	27.6	29.3	29.2	29.6	28.9
Cheese (kg)	12.4	12.7	12.1	11.7	11.8	11.5	11.8	12.1	11.8	12.0
"Kashkaval" (kg)	3.5	3.7	3.7	3.9	3.9	4.0	4.2	4.5	4.6	5.1
Other dairy products (kg)	2.1	2.2	2.3	2.4	2.3	2.8	2.8	2.8	2.8	3.4
Overall	66.6	66.8	63.8	62.3	62.8	63.1	64.9	65.5	66.8	68.6

**Data from the National Statistical Institute (NSI) of the Republic of Bulgaria.*

Table 1.
*Average dairy product consumption per family member in Bulgaria, 2012–2021.**

Yoghurt and yoghurt-like products can also be prepared in the form of a dessert or as a drink.

The wide consumption of fermented milk products in our country is not only the result of a centuries-old tradition. They are widely accepted as a native food with a naturally balanced composition of essential nutrients (proteins, carbohydrates, fats, mineral salts, vitamins, and enzymes), which are easily digestible and necessary, especially for growing and aging organisms [2]. In addition, lactic acid fermentation with selected bacteria repeatedly increases the functional and biological significance of milk-based foods.

1.1 Traditional Bulgarian yoghurt

Bulgarian yoghurt is a traditional food produced by microbial lactic acid fermentation of pasteurized milk. Its traces date as early as 8000 BCE when the domestication of milk-producing animals like sheep, cows, and goats began [3]. Before the commercialization of dairy production in Bulgaria, yoghurt was mainly for family use and mostly made from ewe milk.

Nowadays, both industrial and artisanal technologies of yoghurt production are similar. The processes start with the filtration of the raw milk for the removal of solid particles (**Figure 1**). A common "technological" step is heating the milk for some time and subsequent storage at a suitable temperature. Regardless of the way of production (**Figure 1**), the process is carried out by inoculation with two symbiotically connected bacterial species – *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The symbiotic relationship of the starter results in the shortened fermentation process and stable characteristics of the final product [4].

The main difference between the artisanal and industrial processes is the step of homogenization (**Figure 1**). It affects the texture and body of the product by reducing whey syneresis (whey separation) and densification of the coagulum, resulting in a thicker yoghurt with improved consistency. In homemade and artisanal yoghurts, the milk fat floats to the surface, forming a creamy layer, called "kajmak". The taste and aroma of Bulgarian yoghurt are specific and also dependent on the raw milk used and the flavoring properties of the starter cultures [5, 6].

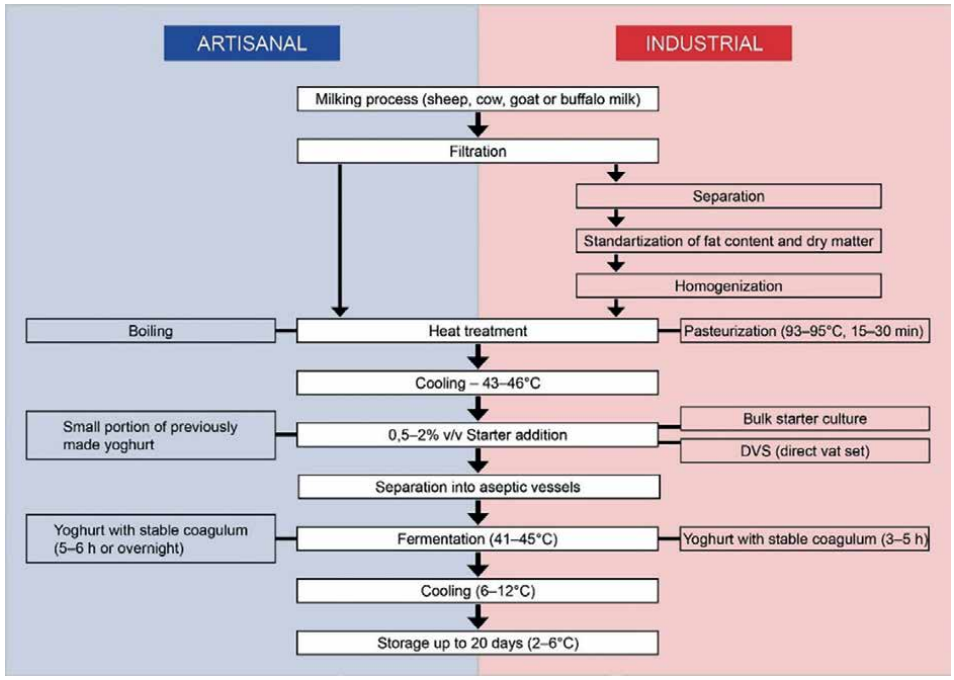

Figure 1.
Scheme of artisanal and industrial technology of Bulgarian yoghurt production.

Yoghurt is evaluated by five organoleptic qualities – appearance, color, aroma, consistency, and taste, according to the International Dairy Federation (IDF) Standard 99A (1987) [7] – and some extra qualities for Bulgarian yoghurt include consistency after stirring, body, and texture, according to the Bulgarian National Standard (12:2010) [8].

One of the rarest and almost forgotten Bulgarian dairy products is called *brano mliako*. "It is made in the region of the Rhodope Mountains by an ancient recipe that has remained unchanged for centuries. It is known to be one of the most unique organic foods in the world because of its qualities. "*Brano mliako*" is made from ewe milk and very much resembles yoghurt. The specificity is that it is made only at the end of summer. The technology starts by collecting raw milk in wooden containers, where it is filtered and thickened. The dehydrated milk then ferments spontaneously, or a starter culture can be added to it, mainly sheep yoghurt. After that, a thin layer of goat or sheep tallow is poured on top, so that the product can be "sealed". This anaerobic preservation makes "*brano mliako*" "suitable to consume for 3 to 4 months.

Gruev (1970) [9] developed a laboratory technology and obtained the same final dairy product. The raw milk is twofold concentrated at reduced pressure at 45–50°C, sterilized by the Koch method for 30 min, and cooled to 45°C. Then, the milk is inoculated with 1% yoghurt starter culture and left for the fermentation process until achieving approximately 190°T acidity. The addition of a 2% yeast-based starter culture, which has been isolated from "*brano mliako*" and cultured in grape must, continues the fermentation. At the end of the yeast fermentation, the obtained dairy product is put in glass containers, hermetically sealed, and stored at 8–10°C for 4–5 months (Gruev, 1970) [9].

1.2 Traditional cheese

- *White-brined cheese:* After yoghurt, the second most widespread and consumed dairy product in Bulgaria is white-brined cheese. It is prepared from cow, goat, sheep, and buffalo milk as well as from mixed milk. Cheese made from a mixture of different types of milk usually possesses improved nutritional value [10]. The production technology of white-brined cheese is specific and involves fermentation and storage in brine (**Figure 2**).

Traditionally at the beginning of the process, the raw milk is preheated, cooled, and fermented by using an enzyme mixture that curdles the casein in the milk, called rennet. Rennet contains the endopeptidases pepsin, lipase, and chymosin [11]. It is originally isolated from the abomasum of new-born ruminants but in industrial processes is mainly biotechnologically obtained by fermentation with recombinant microorganisms, including *Escherichia coli*, *Kluyveromyces lactis,* or *Aspergillus niger* var. *awamori* [12]. Then, the coagulate must be strained in order to separate the aqueous phase from the solid matter. The cheese is later put to ripen for at least 45 days at 15°C, a lengthy process of intensive acidification, lipolysis, and proteolysis. After that,

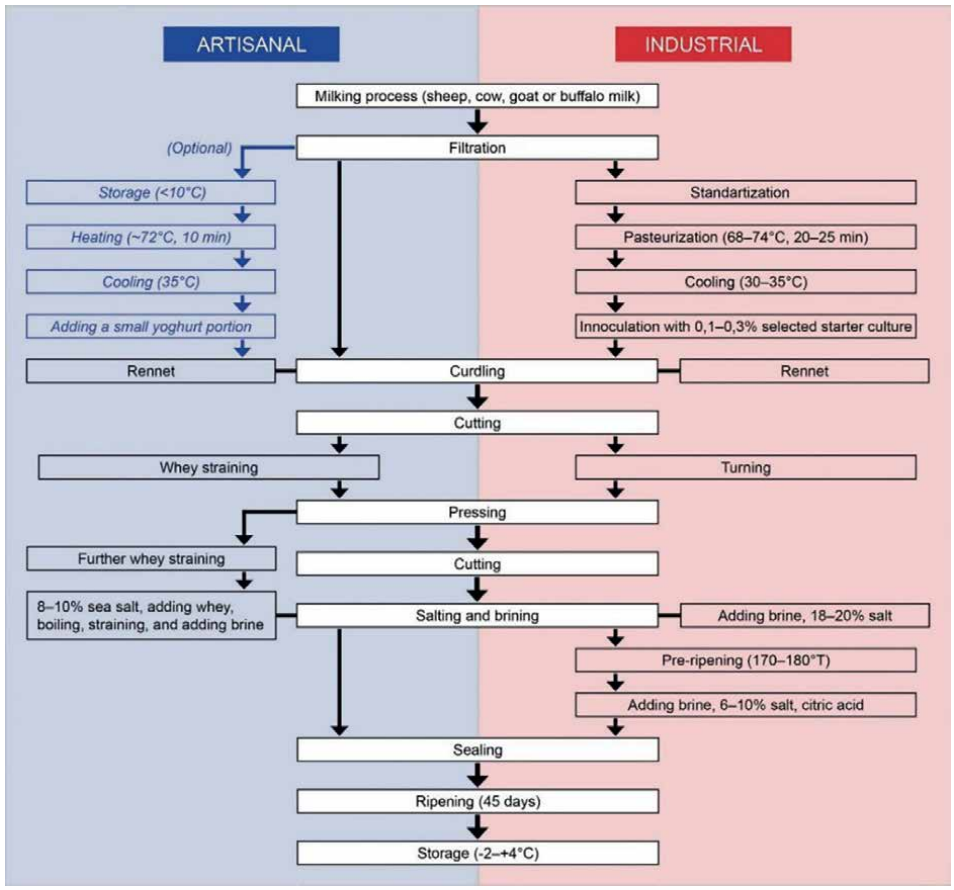

Figure 2.
Scheme of artisanal and industrial technology of Bulgarian white-brined cheese production.

the product is salted and packaged in containers with brine. The brine should comprise 6–10% sodium chloride, and the acidity of the final product must be 160–180°T. The shelf life of the white-brined cheese is 12–18 months. The final product is a hard cheese with low water and high solid content. Its quality is determined by microbiological, physical, and chemical characteristics and evaluated by its organoleptic qualities, which guarantee the safety, prolonged shelf life, and high nutritional value of the product. The high energy and nutrition of the cheese are mostly due to the high fat and protein content as well as easily digestible peptides; essential free amino acids; minerals, including zinc, magnesium, phosphorus, and calcium; and vitamins, including vitamin A, B, E, and D [13].

- "*Kashkaval*" is the most widespread yellow cheese in Bulgaria and is also from the group of semi-hard cheese. This dairy product can also be made from various kinds of milk, like cow, sheep, goat, and mixed milk, according to the National Bulgarian Standard 14:2010. The production technology of "*Kashkaval*" involves a mandatory cheddarization process, which takes a period of 2 to 6 months for the final product to develop its characteristic aroma. The technology starts with the filtering and centrifugation of raw milk. The milk is then heated to 60–63°C for 20 s to decrease the number of potential spoilage microorganisms and at the same time to preserve the LAB strains. After that, it is cooled and poured into a large vessel, then rennet is added, and the curdling process takes place for 30–40 min. The coagulated mass is cut into small pieces so that the whey can be easily released and stirred for 20 min, followed by another stirring process at 38–39°C, the so-called "cheese baking" process. In some artisanal technologies, weak acids, like acetic acid or citric acid, can be added at this stage to "boil" the cheese. This step is completed when the cheese grains become hard but elastic. The whey is then drained, and the cheddarization takes place at 35–37°C for 2–3 h. Upon this process, a rapid lactic acid fermentation starts, where LAB transform lactose to lactic acid, the pH decreases to 5.2–5.4, and calcium P-caseinate breaks down to monocalcium P-caseinate, achieving a soft and easy-to-manipulate final product. Before getting ready for the next stage, the cheese needs to harden for some time. Then, it is cut into thin slices and placed in a concentrated saline solution for water to be removed and for the cheese to become more firm. Further, the cheese is boiled to obtain a slurry consistency, which is then kneaded and placed in vessels. On the next day, the product is separated from the vessels and left to ripen at 8–12°C for around 55 days. Ultimately, to prevent it from drying out, the ripened "*Kashkaval*" can be covered with melted wax or paraffin. The LAB starter culture determines the specific taste of the final product [14].

Different "*Kashkaval*" products are being made across Bulgaria, and all are in the group of semi-hard cheese. The hardness, texture, flavor, and aroma of the final products depend on the moisture and the aging period. The quality during storage can be evaluated by physical parameters, such as water content, dry matter, total fat content, total protein content, and total salt content [15].

2. Microbiota of the unique Bulgarian dairy products

The diversity of milk, selected starter and nonstarter cultures, and technological treatments give exclusively heterogeneous products with various organoleptic,

texture, and nutritional qualities [16]. The uniqueness of presented traditional dairy products is a complex result of the climate conditions, the composition, and activities of milk microbiota [17]. With an understanding of its important role, several studies aimed to characterize LAB diversity, not only in Bulgaria. Discovery of rod- and globular-shaped bacteria (cocci), named initially *Thermobacterium bulgaricum* and *Streptococcus thermophilus,* respectively, started in 1905 with the work of Bulgarian scientist Dr. Stamen Grigorov. The cooperation between the two microorganisms was considered to be one of the most important characteristics of the typical Bulgarian yoghurt. In 1938 Dr. K. Popdimitrov considered that using only the two bacterial species is proper for the production of Bulgarian yoghurt and classified all other microorganisms as undesirable. According to the national standard, the starter cultures for Bulgarian yoghurt include strains of Bulgarian origin from species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. At least 10^7 CFU/g in a ratio of 1:2–1:5 is needed as a yoghurt starter, according to the definition of FAO/WHO (2003) [1]. LAB reach 10^7–10^8 CFU/g for *L. bulgaricus* and 10^8–10^9 CFU/g for *Str. thermophilus* in the final product. However, homemade and artisanal yoghurts contain different non-starter species that add to the different sensory qualities of the final products. The biodiversity of autochthonous microbiota and its use as starter cultures make Bulgarian dairy products exceptional. Species from the family *Lactobacillaceae* and genera *Lactococcus, Leuconostoc, Streptococcus, Enterococcus*, *Pediococcus*, and especially the new genera of previously determined genus *Lactobacillus* have been isolated throughout the years from different homemade and artisanal dairy products [18–21]. The autochthonous microbiota of "*brano mliako*" is comprised of yeast species as well, including *Kl. lactis* and *Saccharomyces cerevisiae* [2]. Our research proved that such products contain more diverse microbiota (**Figure 3**).

A special protocol with an overnight enrichment step, however, was needed. More than 90 artisanal samples from yoghurt, white-brined cheese, and "kashkaval", made at home or small farms in different rural regions of Bulgaria, have been collected. The combination of classical phenotypic and microbiological with molecular and typing methods, according to the polyphasic taxonomic approach applied, allowed us to establish the biodiversity of their LAB microbiota (**Figure 3**). The most persistent was the species *Lactiplantibacillus plantarum.*

Multiplex PCR analyses (according to Torriani et al. [22]) allowed to identify closely related *Lactiplantibacillus paraplantarum*, *Lactiplantibacillus pentosus*, and *L. plantarum* and revealed their presence in homemade yoghurt and white-brined cheese [23, 24]. By the gold standard, 16S rDNA sequencing, *L. bulgaricus* was identified in yoghurt samples. In addition, *L. plantarum* presence in yoghurt with a dominance in ripened samples of cheese was confirmed. In the early stage, however, significant number of enterococci and lactococci have to be pointed for white-brined cheese samples. Originally, *lactobacilli* have a low density in cheese (<50 CFU/g). During the ripening period, they significantly increase and become the dominating microbiota in the final product (10^7–10^8 CFU/g). Therefore, an enrichment step with pre-culturing of collected samples allowed us to establish LAB diversity.

Nemska et al. [20] isolated 74 pure cultures from different dairy products, made by traditional recipes without the addition of industrial starters. Using classical phenotypic tests, 45 out of them were identified as lactobacilli [20]. The group of lactobacilli from white-brine cheese was the most numerous (23 strains from cow, sheep, buffalo, goat, and a mixture of cow and buffalo milk), followed by yoghurt isolates (14 strains from cow, sheep, and buffalo milk), 1 strain from curd (from goat

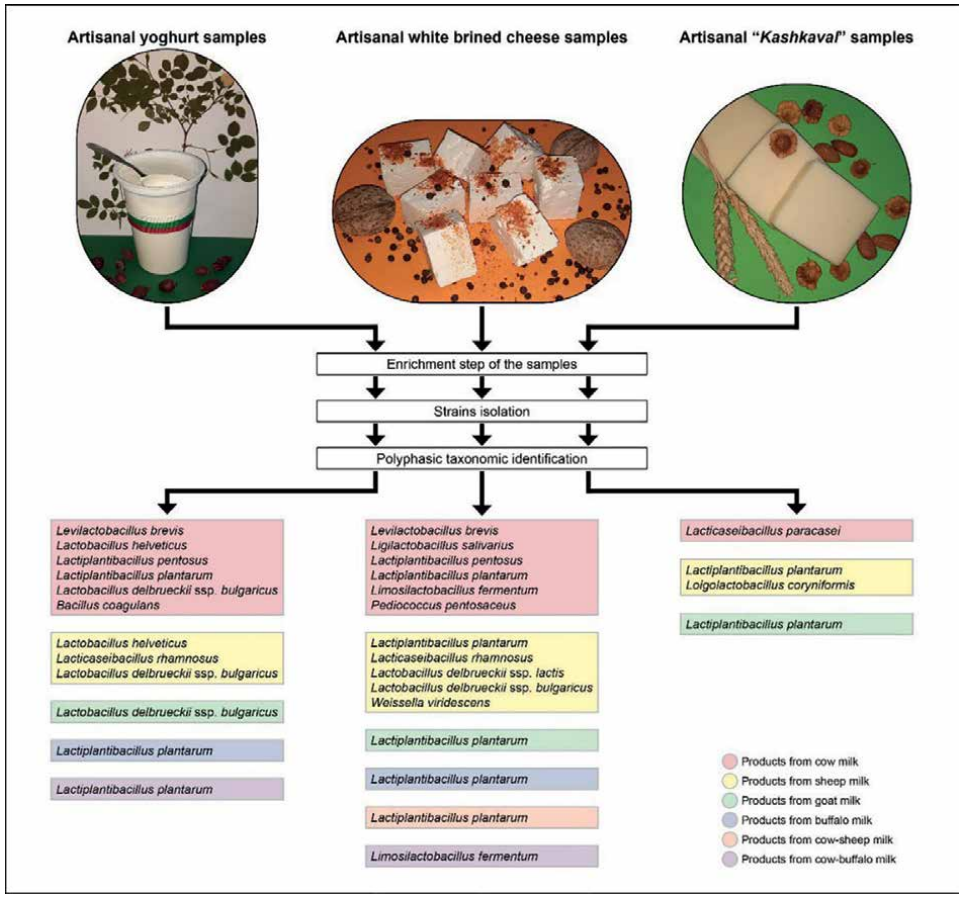

Figure 3.
Biodiversity of LAB microbiota in artisanal Bulgarian dairy products, studied during a period of 10 years.

milk), and 4 strains from yellow cheese (from goat and cow milk) [20]. Twenty-two strains were identified as *L. plantarum*. A predominance of *L. plantarum* in the non-starter microflora of Bulgarian white-brined cheese has been reported [23, 25]. This species is also reported to be present in high density in many different types of cheese [26–28]. *Ligilactobacillus acidipiscis* was also identified in a homemade ripened sample of cheese, made from raw ewe milk, in the region of nuclear power plant (Kozloduy, Bulgaria) (Danova, 2015, unpublished data). The tendency of rich LAB microbiota in samples made from non-pasteurized milk was proven in several samples from sheep, cow, and mixed milk [19, 20]. Most traditional cheese achieve their specific taste and aroma due to the natural microflora of raw milk. The raw milk that is used impacts the unique organoleptic and sensory qualities of the final product. Different cheese products can be made with rennet and natural microflora, with or without starter cultures.

Today, the wide variety of cheese with specific organoleptic properties is due to the different combinations of LAB species. It is important to maintain an optimal strain balance in the starter because of the complex microbial interactions. Even small differences in the microbial composition can lead to surprising or unwanted effects on the quality of the cheese product.

According to Bulgarian National Standard for white-brined cheese, a mesophilic and/or thermophilic starter is used in a ratio of 2:1. The mesophilic starter is predominant and contributes to the ripening processes and developing the taste and aroma of the final product. The mesophilic LAB for white-brined cheese and "*Kashkaval*" are represented by *Lactococcus lactis* subsp. *lactis* and *Lacticaseibacillus casei*. The thermophilic starter includes *L. bulgaricus* and *Str. thermophilus.* For yellow cheese "*Kashkaval*", in addition to *L. bulgaricus* and *S. thermophilus*, the thermophilic *Lactobacillus helveticus* is also present. For all Bulgarian dairy products, the strains in the starters must be isolates of Bulgarian origin.

3. Functional characteristics of the autochthonous microbiota in Bulgarian dairy products

3.1 Metabolic activity as a factor of organoleptic properties of fermented dairy products

In the manufacturing process, starters are added for lactose fermentation, lipolysis (fat degradation), and proteolysis [29]. LAB that are naturally found in milk have a major role in the fermentation processes alongside the intentionally added starter cultures. The fermentation improves the general characteristics of the end product including texture and consistency, aroma, flavor, and the development of color. The intricate biochemical processes during fermentation involve many different enzymatic reactions. With an understanding of the strain/species specificity, a large screening for metabolic activity of newly isolated Bulgarian strains was carried out. Results with identified *L. plantarum* strains from cheese (unpublished data) and [20, 23, 24, 30] are summarized (**Figure 4**).

The produced LAB enzymes responsible for lipolysis and proteolysis in milk are among the key factors for the sensory qualities of taste and texture of cheese [31]. LAB possess different degrees of lipolysis, which is important for the selection of strains that can be used for starter cultures. During lipolysis, triglycerides hydrolyze into mono- and diglycerides, free fatty acids, and glycerol. The reduced glycerides participate in coagulation processes with different components of dairy foods that lead to the texture development of the final product [32] (Esteban-Torres et al., 2014). This characteristic is related to the flavor development of fermented dairy products [33].

During proteolysis, hydrolysis of the protein peptide bonds occurs and transforms them into peptides and free amino acids. Although many LAB are considered to have weak proteolytic activity, they possess complex proteinase/peptidase systems comprising peptidases on the cell wall initiating the degradation processes, peptide transporters, and intracellular peptides that break down peptides into shorter molecules and free amino acids [34]. Thus, essential amino acids may have accumulated in fermented products, due to the high peptidase activity (**Figure 4**) estimated for *L. plantarum* strains. For 8 of tested L. plantarum strains from white-brined cheese, accumulation of free amino acids from 0.170 to 0.609 mMGly/L was shown. The accumulated free amino acids are involved in reactions of deamination, transamination, decarboxylation, and desulfurization with an impact on the flavor profile of the end product [34]. At the same time, these 8 strains were characterized by low proteolytic activity. With a sample screening protocol [35], using milk agar and calcium caseinate agar (Fluka), we differentiated with low proteolytic activity all strains generating clear zone 1–8 mm, moderate zone 8-13 mm, and high zone >13 mm.

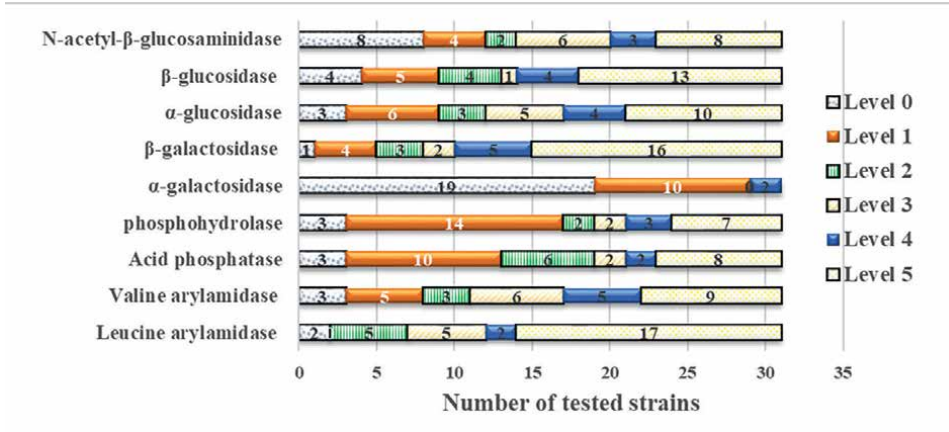

Figure 4.
Enzymatic activity of 31 L. plantarum strains isolated from artisanal Bulgarian dairy products (semi-quantitative analysis with the API ZYM test strips, bioMérieux, France).

Despite high peptidase activity (**Figure 4**), only 2 out of the 31 tested exponential *L. plantarum* cultures possessed high activity. The best results were obtained with 72 h cell-free supernatants (CFS) of *Lactobacillus* cultures. *L. bulgaricus* with yoghurt origin showed fast milk coagulation in combination with moderate to high proteolytic activity. Peptidase as well as β-galactosidase activity is promising for candidate probiotics, while other enzyme activities may contribute to organoleptic characteristics and stability of the products.

3.2 Antagonistic LAB activity and food preservation

It is well known that contamination of different types of foods with filamentous fungal microorganisms, yeast, and bacteria is the main reason for food spoilage. The presence of unwanted species in dairy products can cause different types of deterioration of organoleptic properties [36]. Also, molds such as *Aspergillus* and *Penicillium* can produce mycotoxins [37]. More than 60 yeast species have been identified as spoilage agents of dairy products, represented most frequently by a high diversity of *Candida* spp., as well as genera *Kluyveromyces*, *Geotrichum*, and *Yarrowia lipolytica* and phylum *Basidiomycota*, mainly *Cryptococcus* and *Rhodotorula* species [38]. Dairy product spoilage by yeasts results in visible alteration due to growth on the surface of the product, unpleasant changes in odor, flavor, or texture and production of different metabolites such as CO_2, alcohols, aldehydes and esters, proteolytic and lipolytic enzymes [36], and biogenic amines [39].

LAB can be used not only as starter cultures but also as protective cultures to improve the safety and/or shelf life of the product [40]. Their preservative action is due to the combined action of a number of antimicrobial metabolites produced during the fermentation process. LAB produce a large range of antimicrobial substances, including organic acids (lactic, acetic, etc.), fatty acids, antifungal peptides, reuterin, and bacteriocins [41–44]. Formally, the metabolites produced by LAB can be divided into two main groups: substances with a low molecular mass < 1000 Da and substances with a high molecular mass > 1000 Da, such as bacteriocins/bacteriocin-like inhibitory substances (BLIS). To be defined as food-grade bioprotective cultures, LAB strains are selected according to their antimicrobial properties.

As bioprotective cultures, LAB are expected to possess antibacterial/antifungal activity that is exhibited and maintained throughout the manufacturing process and storage time, to have no impact over the functions of the starter cultures, not to modify the organoleptic properties of the final product, to be used with the lowest possible inoculum that maintains the same activity to reduce the cost value, and to have easy propagation and resistance to technological processes [38]. In the later stages of ripening, *lactobacilli* are well adapted to the environment inside the cheese, withstanding the low pH, high salt concentration, absence of sugars, and anaerobic conditions, and they may produce BLIS.

Lactobacillus, as well as *Leuconostoc, Lactococcus, Pediococcus,* and *Weissella*, are the most frequently cited genera to possess antifungal properties and have been the most evaluated *in situ* in recent years as well [45]. Many *Lactobacillus* species, including *L. plantarum, Levilactobacillus brevis, L. casei,* and *Lacticaseibacillus paracasei*, were shown to exhibit antifungal activity against a large spectrum of fungal representatives, including *Penicillium, Kluyveromyces, Candida, Rhodotorula*, *Aspergillus,* etc., which are among the most common spoilage microorganisms in dairy products [46–52]. Other lactobacilli, such as *Limosilactobacillus fermentum, Lacticaseibacillus rhamnosus, Schleiferilactobacillus harbinensis, L. helveticus,* and *Lactobacillus amylovorus*, were shown to be able to extend the shelf life when added as adjunct cultures in yoghurt and cheese [49, 53–55].

The well-expressed profile of antagonistic activity of the autochthonous LAB microbiota of traditional fermented foods against a number of pathogens and food-associated contaminants is established. Our strains, isolated from yoghurt, white-brined cheese, and "Kashkaval," show a strain-specific broad spectrum of activity against Gram (+) and Gram (−) food spoilage microorganisms and clinical pathogens (**Figure 5**).

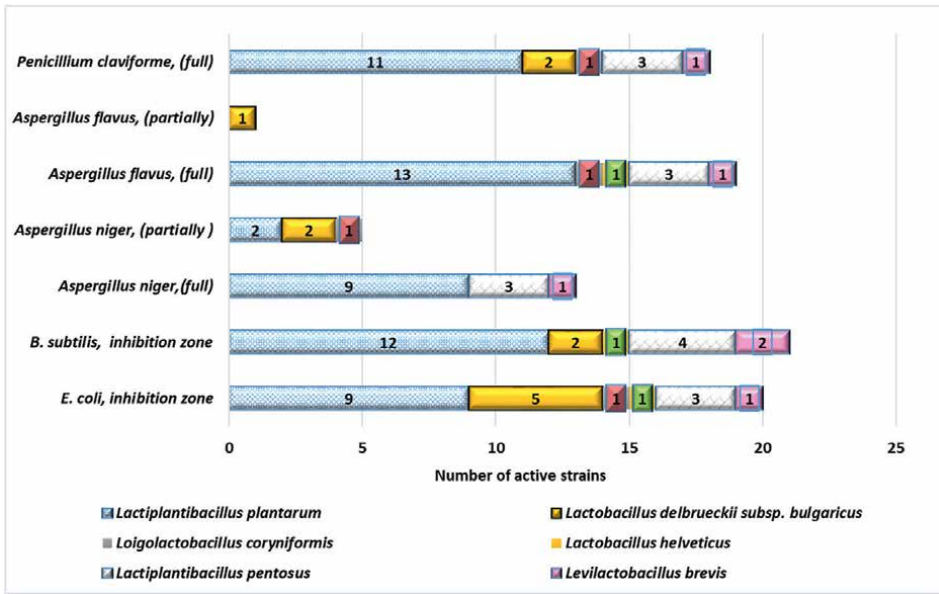

Figure 5.
Antagonistic activity of strains isolated from artisanal Bulgarian dairy products against food-associated pathogens and contaminants.

Initial results with *L. plantarum* strains from white-brined cheese were highly promising [23]. In the presence of 5% (v/v) CFS from 12 newly isolated strains from cheese and yoghurt cultures, the growth of *Pseudomonas aeruginosa* PA01 (from a patient with cystic fibrosis) was significantly inhibited up to 50–80% (unpublished data). A group of active strains belonging to the species *L. plantarum* and *L. rhamnosus* from dairy products inhibited *Staphylococcus aureus* MRSA as well as out-patient antibiotic-resistant strains [56]. In situ results of antibacterial/antifungal activity of whey fractions, skimmed milk, cow milk, and soya milk are also important [24]. For the strains of LAB, which form the microbiota of traditional fermented foods, a well-expressed profile of antagonistic activity against a number of pathogens and food-associated contaminants is established. Our isolated strains from yoghurt, white-brined cheese, and "Kashkaval" show antibacterial activity against Gram (+) and Gram (−) bacterial representatives, but their antifungal activity is also well expressed (**Figure 5**).

4. Bulgarian dairy products as functional foods

For more than a decade, the focus of nutritional science aims toward optimal nutrition, the objective of which is the optimization of the daily diet regarding nutrients, non-nutrients, and other food ingredients that favor the maintaining of good health [57]. The increased lifestyle diseases, in combination with the high healthcare costs, are the reasons for the rising research to formulate and produce foods with functions that could improve health and well-being and lower the risk of or delay ongoing major diseases [58]. Within this context, the concept of functional foods has arisen.

A functional food must have health benefits and can be classified as one if it enhances target functions or reduces the risk of specific diseases, has to provide benefits beyond the basic nutritional functions, should be or look like a traditional food, and should have a dietary pattern and be a part of the normal daily diet. From this baseline, the most complete definition of functional foods was proposed by the EC Concerted Action on Functional Food Science in Europe (FUFOSE). It states that a functional food is one that beneficially affects one or more target functions in the human body beyond normal nutritional effects, relevant to improved health state and well-being and/or reduced risk of disease, and it is consumed as a normal food pattern, not in the form of a pill, capsule, or any other dietary supplement (European Commission, 2010) [59].

Dairy products have a special place in Bulgarian diet. After Stamen Grigorov's discovery, extensive research has begun on the unique nutritional characteristics of Bulgarian yoghurt. In 1909, the Russian biologist and Nobel Prize winner Elie Metchnikoff developed a theory regarding the prolongation of life. He proposed that there is a relationship between the increased life expectancy of Bulgarians and the daily consumption of yoghurt [60]. Then, it was suggested that the consumption of yoghurt is connected with the increased number of Bulgarian centenarians. Metchnikoff's main research was on lactic acid, which is proven to reduce the number of putrefactive microorganisms [61, 62]. Then, he further proposed another hypothesis that the inhibition of pathogens and the harmful fermentation of food in the gut can slow down the process of aging.

Studies report that *L. bulgaricus* possess high antimicrobial properties and are able to colonize the human intestines, which suggests its probiotic functions [63, 64].

More studies describe that regular consumption of yoghurt that contains viable *L. delbrueckii* and *Str. thermophilus* can improve decreased lactose intolerance and the overall digestion of lactose [65, 66]. From intensive research, Bulgarian yoghurt could be considered a dairy product with functional characteristics, as scientific results state that functional probiotic foods can modulate the microbial composition in the gut, thereby improving intestinal health [67, 68].

Our achievements clearly showed that products with a century-old tradition of production and consumption in the Balkans are a promising source of beneficial LAB microbiota with the capacity to transfer them into new safety food with functional properties.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Chapter 2

Medicinal Potential of Camel Milk Lactoferrin

Neelam Mahala, Aastha Mittal and Uma S. Dubey

Abstract

Camel milk is a rich source of protein with well-recognized medicinal properties to treat various diseases. The objective of this work is to understand the role of camel milk lactoferrin in immunomodulation and in disease treatment. It has been found that camel milk lactoferrin is a very suitable nutraceutical agent by virtue of its bioactivity, immuno-compatibility, and safety. It can be used for the treatment of infectious, metabolic, and neurodegenerative diseases, besides cancer. It is a cost-effective biomolecule that also has high relative abundance and bioavailability.

Keywords: camel milk, lactoferrin, medicinal potential, commercial significance, immunomodulatory, anti-microbial, anti-cancer

1. Introduction

The medicinal properties of camel milk have long been recognized, especially in middle eastern countries. Camel Milk is a rich source of active proteins, especially enzymes that have several biological activities including antibacterial, antiviral, immunological, and antioxidant properties. Camel milk has been used to treat many diseases such as Hepatitis, Allergy, Liver, and kidney function, Diarrhea, and Diabetes. Moreover, camel milk has no allergenic properties and can be consumed by lactase-deficient people. Like human milk, camel milk has a high content of lactoferrin and α-lactalbumin but lacks β-lactoglobulin [1]. It differs from cow milk as it has lower fat, cholesterol, and lactose levels, besides this, there is an absence of beta-lactoglobulin and beta-casein. Beta casein is the allergenic component that is present in cow milk but absent in camel milk. Also, it has very low levels of lactose making it consumable by lactase deficient people [2]. It has been noted that despite the lack of refrigeration, camel's milk remains unspoiled for several days. This may be due to the antibacterial activity of certain proteins contained in camel's milk [3]. Furthermore, camel milk proteins are generally pH hydrolysis resistant and thermostable. Lactoferrin is well recognized as an adjunct to anti-cancer standard therapy by virtue of its immunomodulatory activity. It also exhibits immuno-compatibility, bioavailability, safety, relative abundance, and low-cost effectiveness. Moreover, the oral route of administration makes it very easy to be given to patients and it is usually well-tolerated [4]. Numerous studies on camel lactoferrin reported that it has anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, antioxidant, and anti-tumor properties. Camel milk lactoferrin is a molecule that not only boosts the immune system but also acts against cancer. The

objective of this work is to understand the role of camel milk lactoferrin in immunomodulation and in disease treatment [5].

2. Camel milk

Camel milk has been found to be a healthier option for people with diabetes and those with food allergies. Several studies on camel milk have found its positive impact on autism, diabetes, liver disease, jaundice, and even cancer. Camel milk is high in vitamin C, many minerals, and immunoglobulins, which boost the immune system. It is not only a very nutritious dairy beverage but it also innately includes probiotics. Camel milk helps enhance gastrointestinal health besides improving systemic immunity. The drink has a low-fat content (only 2 to 3%, compared to cow milk) and thus is likely to attract more attention of health-aware consumers. In various Middle East countries and an Africa, it is used as a suitable supplement to feed undernourished children because it's similarity with human breast milk [6].

2.1 Medicinal properties of camel milk

Naturally occurring bioactive compounds have contributed effectively to cancer therapeutics, paving a way for better disease management. Camel milk is one such dietary food with immense nutritional and medicinal value. Like human and bovine milk, camel milk also contains numerous proteins such as immunoglobulins, alpha-lactalbumin, lactoperoxidase, casein, lysozyme, lactoferrin, amylase, etc. The major proteins present in camel milk along with their clinical significance have been depicted in **Table 1**. It forms a high nutritional source with low cholesterol, low sugar, high minerals (sodium, potassium, iron, copper, zinc, and magnesium), high vitamins (vitamin C, B2, A, and E), and high concentrations of insulin compared to the ruminant milk [20–22]. Moreover, very recently camel milk casein-derived nanoparticles have been used as carriers for the delivery of sorafenib in hepatocarcinoma cells [23].

2.2 Commercial value of camel Milk

Latest reports suggest that the global camel dairy market reached 2.3 billion US$ in 2020. A total of 2.9 million tons of camel milk production has been recorded annually worldwide [24]. Camel dairy market is expected to reach USD 10.07 billion by 2027 growing at a growth rate of 8.0% in the forecast period 2020 to 2027.

Camel milk is traditionally consumed in either a raw form or in a fermented form. But to cater with preferences of urban populations now manufacturers have stated the production of novel camel milk based food products such as flavored beverages, sweets, chocolates and Ice creams. These products are becoming increasingly popular in nations such as UAE, Saudi Arabia,, Kazakhstan, Algeria, Australia, Morocco, Egypt and India. Its increasing demand has resulted in a wide opportunity for product innovation and generation of new markets. Camel milk products are relatively more expensive than other cattle dairy food items due to its high production costs. In spite of being low in fat, camel milk has a relatively high content of unsaturated fatty acids, which are beneficial for us. It's suitable for lactose-intolerant people. Camel milk is actually considered a super food because of its high mineral and vitamin content. Moreover, its benefits for joint pain and diabetes has also been well documented. It's no wonder then that it's for long been consumed by the

S. No	Clinical condition	Therapeutic molecules in camel milk	Reference
1	Diabetes	Insulin-like molecule	[7]
2	Allergy	Low levels of β-Casein & lack of β-lactoglobulin	[8]
3	Liver and kidney function	Alanine aminotransferase and aspartate aminotransferase	[9]
4	Slimming properties	Low protein content and reasonable cholesterol content	[10]
5	Antitumor activity	Lactoferrin, Lysozyme, Lactoperoxidase	[11, 12]
6	Nutritional supplements	Unsaturated fatty acids	[13]
7	Easy assimilation in Lactase deficient patients	L-lactate	[14]
8	Bone formation	High level of calcium	[15]
9	Diarrhea	High levels of sodium and potassium	[16]
10	Immuno enhancer and antimicrobial activity	Peptidoglycan recognition protein (PRP)	[17]
11	Antibacterial and antiviral activity	N-acetyl-glucosaminidase (NAGase)	[18]
12	Confers special passive immunity	Heavy chain antibodies (HCAb) or variable heavy antibodies (VHH) or nanobodies	[19]

Table 1.
Application of camel milk molecules in treatment of various diseases.

Bedouins (nomadic Arab people) and many other desert communities of the world to face their harsh living conditions.

The dairy market in India reached a value of 13,174 billion INR in 2021. In the coming times, International market analysis research and consulting (IMARC) group expects the market to reach 30,840 billion INR by 2027. This would amount to a compound annual growth rate (CAGR) of 14.98% during the time interval 2022–2027. Actually, despite its availability, India has been late in entering the market scenario. Only in the end of 2016 did the Food Safety and Standard Authority of India (FSSAI) decide the standards for commercialization of camel milk. Notably, while government dairy cooperatives have been slow to respond, some entrepreneurs, interestingly, even from the Raika community of Rajasthan, have taken multiple dynamic initiatives. India's first camel milk brand Aadvik Foods is set to disrupt the dairy and organic milk products market. This New Delhi-based company started its journey in 2016 with just one liter of camel milk. Today, it is procuring around 10,000 liters a month, having sold over 2 lakhs liters over the last three-and-half years.

3. Clinical relevance of lactoferrin

Lactoferrin is a versatile molecule that has been molded by natural selection to be amongst the first line of defense in mammals [25]. As the second most abundant protein

in colostrum, it is responsible for conferring immunity on newborns within the first few weeks of life [26]. Lactoferrin is involved in various physiological functions such as regulating homeostasis and cell proliferation, besides being a very potent antimicrobial agent. It has antibacterial, antifungal, antiviral, antioxidant, immunomodulatory, and anticancer activities [25, 27–29]. During infection and inflammation processes, the lactoferrin concentration increases through the recruitment of neutrophils. The important properties of lactoferrin have been depicted in the flow diagram in **Figure 1**.

Lactoferrin, the natural protein, is proving to be a highly promising bio-drug as an antimicrobial, immunomodulatory, and anticancer agent. According to Cragg *et al.,* over 50% of the drugs in clinical trials for anticancer activity are isolated from natural sources or their ingredients. Several drugs currently used in chemotherapy are isolated from plant species and food sources [30, 31] Lactoferrin is a multi-functional protein with many beneficial properties. It is now recognized as a functional food for several products with commercial and clinical applications [32]. It is widely distributed in all biological fluids and is also expressed by immune cells, which release it under stimulation by pathogens.

The primary function of lactoferrin has been recognized to be in the modulation of the immune responses, besides iron transport, storage, and chelation. Lactoferrin activates immune cells and enhances their proliferation and differentiation. Its potential to perform multiple activities is often attributed to its capacity to bind iron and interact with diverse molecular and cellular components of hosts and pathogens. The multiple functions ascribed to lactoferrin can either be dependent or independent of lactoferrin's iron-binding ability [33]. Furthermore, it is noteworthy that lactoferrin concentrations are locally elevated in inflammatory disorders such as neurodegenerative diseases, autoimmune diseases (e.g., arthritis), and allergic inflammation.

3.1 Lactoferrin as an Immuno-modulator

Lactoferrin is a cell-secreted mediator that bridges innate and adaptive immune responses. For immune-modulatory functions, it interacts with specific receptors of the target cells (either epithelial cells or cells of the immune system) It also can bind to bacterial cell wall LPS. Lactoferrin modulates the activation, proliferation, maturation, differentiation, and migration of immune cells. The functional modulations take place in the T and B cells, neutrophils, monocytes/macrophages, and dendritic cells belonging to the antigen-presenting class of cells. It acts via two mechanisms of intracellular signal transduction, *i.e.,* nuclear factor kappa B and MAP kinase [34–36]. Furthermore, it affects the mechanisms of the innate response, by influencing the activation of the complement system, increasing the NK cell activity, increasing the phagocytic ability of monocytes, and by enhancing their cytotoxicity [37]. There are lactoferrin receptors on many immune cells, so lactoferrin directly affects how these cells function. Its action increases levels of cytokines such as tumor necrosis factor (TNF-alpha), interleukin 8 (IL-8), and Nitric Oxide production besides limiting pathogenic growth [37, 38].

Lactoferrin modulates innate and adaptive immune response because of its ability to bind LPS and CD-14. It also interferes with the formation of the CD14–LPS complex. This results in the attenuation of the LPS/CD-14/TLR-4, a signaling pathway involved in the pathogenesis of sepsis. Lactoferrin may stimulate the immune system by binding to CD-14 and then activating the TLR-4-mediated pathway while preventing overexpression of LPS-induced inflammation [39]. Lactoferrin, which functions as a natural iron scavenger and a modulator of signaling pathways, leads to the negative feedback of the inflammatory response. This is also shown by a decrease in the production of reactive oxygen species and various pro-inflammatory cytokines [40].

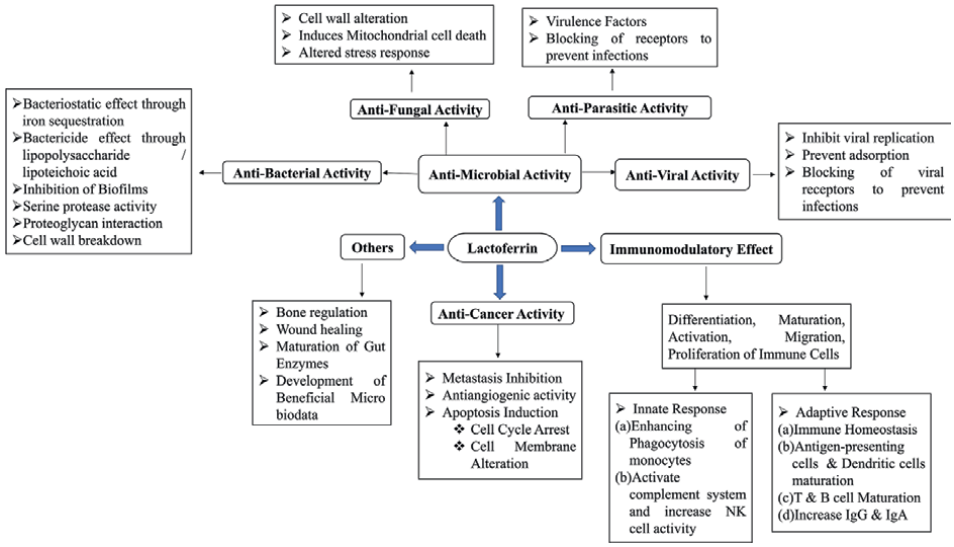

Figure 1.
Bioactivity of lactoferrin.

In addition to this, researchers have shown that in a murine model of diethyl nitrosamine-induced hepatocarcinogenesis, bovine milk lactoferrin significantly down-regulated the activity of liver antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase. It also increased the concentration of hepatic glutathione. Furthermore, bovine lactoferrin promoted the decrease of serum inflammatory markers and ameliorated in hepatic histological structures in a significant manner [41]. Furthermore, the applications of lactoferrin have been highlighted in **Table 2**.

Other than the direct modulation of the immune response, lactoferrin strategically acts as a potent anti-inflammatory agent by scavenging ROS. Pro-oxidant agents can both promote DNA damage and induce as well as sustain inflammatory disorders. Inflammation itself drastically contributes to cancer development. Lactoferrin can maintain the physiological balance of ROS levels by direct binding of free iron, one of the principal actors involved in ROS production. It can also act as a regulator of key antioxidant enzymes, thus protecting the host from ROS-mediated cell and tissue damage in an overall manner [51].

The protective character of lactoferrin against cancer has been demonstrated, on numerous occasions, including its impact on chemically induced tumors, in laboratory rodents. Lactoferrin has even been reported to inhibit the development of experimental metastases in mice [52–54]. Lactoferrin-mediated inhibition of tumor growth might be related to apoptosis of these cells, induced by the activation of the Fas signaling pathway. Nevertheless, the exact mechanism of this function has not been discovered so far [55].

3.2 Camel milk lactoferrin as an antimicrobial, anticancer, and immunomodulatory agent

Lactoferrin is a highly conserved molecule. It possesses high degree of sequence homology and exerts multiple identical functions across mammalian species. Its

S. No	Applications	Additional Information	References
1	Antihypertensive activity	Obtained from lactoferrin-derived peptides	[42]
2	Protection from anemia	Serves as an iron-containing protein useful for treatment	[43]
3	Bone regeneration	Beneficial effect	[44, 45]
4	Prevention of metabolic diseases	Eg. Obesity and diabetes	[46]
5	Acts as drug nanocarriers	Emphasis on tumor-targeted drug delivery.	[47]
6	Protection from Neurodegenerative diseases	Markedly increased expression upregulation in brain cells	[48]
7	Anti-inflammatory effect	By inhibition of the formation of hydroxyl free radicals.	[49]
8	DNA damage prevention	Prevention of tumor formation in the central nervous system	[50]
9	Activates the p53 tumor suppressor gene (TSG)	Suppression of tumor formation	[50]
10	Natural substitute for Antibiotics	Antimicrobial activity: Also, a promising candidate to help break the vicious cycle of antibiotic resistance	[49]
11	Natural food preservative	Antimicrobial activity	[49]

Table 2.
Applications of lactoferrin.

ability to act as an antibacterial, antifungal, antiviral and antiparasitic, anti-inflammatory and immunomodulatory agent is shared amongst most mammalian species and has already been discussed [56–58]. More specifically, it inhibits growth of *Escherichia coli, Klebsiella pneumonia, Clostridium, Helicobacter pylori, Staphylococcus aureus, Candida albican*s, etc.

According to studies, the most therapeutic effects of camel milk are due to lactoferrin and immunoglobulins. Redwan & Tabll, 2007 reported that lactoferrin of camel milk has anti-viral activity and inhibits the virus entry into the cells. The camel milk lactoferrin stops HCV entry and replication in infected HepG2 cells two times higher than lactoferrin in human, bovine, and sheep milk. Generally, camel milk lactoferrin may directly interact with viral molecules or receptors (heparan sulfate) on the cell surface and prevent the virus's attachment to the host cells and thus hinder infection. The virucidal mechanism of camel milk lactoferrin depends on its alpha-helical structure and cationic nature [59]. The antiviral effects of lactoferrin from camel milk have been demonstrated against many viruses. The mode of action behind this activity is the neutralization of virus particles and inhibition of their replication. Camel milk lactoferrin also has anti-pathogenic activity against human immunodeficiency virus, hepatitis B and C, cytomegalovirus as well as herpes simplex virus-1 infection. Not only this, but camel lactoferrin's immunomodulatory role is exemplified by the fact that it modulates the activation and maturation of various immune cells such as neutrophils, macrophages, and lymphocytes [60].

An earlier study on camel milk lactoferrin has demonstrated the ability to inhibit the growth of colon cancer cells line HCT-116. Camel milk lactoferrin exerted antioxidant activity through scavenging NO and the DPPH free radical. It has shown the capability to furnish reducing power as evident by total antioxidant assays. Camel milk lactoferrin also inhibited DNA damage most likely through binding catalytic iron [5].

Camel milk lactoferrin exhibits an anti-inflammatory activity against IL-1β induced activation of osteoarthritis associated chondrocytes in humans by blocking the NF-kappa B mediated signaling. Furthermore it inhibited cyclooxygenase-2 expression and PGE2 production in stimulated osteoarthritis chondrocytes. N. Rasheed et al., 2016 have reported that camel lactoferrin has cartilage protective and anti-arthritic activity. This novel mode of action of camel milk lactoferrin is very important in understanding the mechanisms behind its anti-inflammatory or anti-arthritic effects [61]. The above studies on lactoferrin derived from camel milk highlight the clinical relevance.

3.3 Anticancer potential of lactoferrin from other mammalian species

Human and Bovine lactoferrin has been suggested to be able to act in tumor prevention and treatment [62, 63]. The lactoferrin preventive effect has been demonstrated in several animal models bearing different types of malignancies, including lung, tongue, esophagus, liver, and colorectal tumors [64–67]. Whereas lactoferrin treatment, was found to be effective in inhibiting growth, metastasis, and tumor-associated angiogenesis [63, 68, 69].

Bovine lactoferrin prevents development of chemically induced tumors. This effect has been confirmed in studies conducted on laboratory rodents. Based on in vivo studies, oral administration of lactoferrin to rodents significantly decreased the chemically induced carcinogenesis in various organs such as breast, esophagus, tongue, lung, liver, colon, and bladder. It also hindered angiogenesis and decreased the incidence of metastases in experimental mice [67].

Furthermore, the combined administration of Lactoferrin and temozolomide enhances the effect of chemotherapy both in vitro and in vivo [55] Similarly, humans suffering from lung cancer undergoing chemotherapy had increased immune system response after taking human lactoferrin post-treatment [70].

Lactoferrin from a bovine source is a promising candidate as an anticancer agent [71] Although bovine milk contains lactoferrin, the human form has been found to be far more potent. Animal studies with mice or rats have shown beneficial effects of bovine lactoferrin ingestion as it can inhibit carcinogen–induced tumors in the colon, esophagus, lung, tongue, bladder, and liver [72].

The anticancer effect of lactoferrin has been extensively studied, and it has been observed that in the presence of Lactoferrin, cancer cells suffer significant damage. It is known to cause cell cycle arrest, damage to the cytoskeleton, and induction of apoptosis, in addition to decreasing cell migration [63, 73]. It decreased the viability and growth of breast cancer cell lines (HS578T and T47D). It also stopped cancer cell growth during the cell cycle and disrupted the cancer cell membrane [74]. Bovine lactoferrin efficiently inhibited the growth of breast cancer cells, suggesting that it has a potential to act as an anti-cancer agent against breast cancer [63, 75].

Lactoferrin helps to prevent the growth of cancer cells and shrinks the cancer cells. It is also known for its inhibitory action on cancer cell proliferation and its anti-inflammatory as well as antioxidant abilities against them [75]. Lactoferrin

expression levels are decreased in colorectal cancer as compared with normal tissue. Lactoferrin knockout mice demonstrated a great susceptibility to inflammation-induced colorectal dysplasia. Treatment of knockout mice with lactoferrin post-chemotherapy accelerated the reconstitution of the immune system, reducing the chances for infection, following chemotherapy treatment. Additionally, lactoferrin is significantly downregulated in specimens of nasopharyngeal carcinoma (NPC) and is negatively associated with tumor progression, metastasis, and prognosis of patients with NPC [76].

Lactoferrin was shown to have preventive effects against gastrointestinal cancers, such as cancer of the colon, stomach, liver, and pancreas, and against metastasis of such neoplasms [77, 78]. Xu *et al*. (2010) demonstrated that bovine lactoferrin induces apoptosis in stomach cancer, thereby suppressing it [79]. Oral administration of lactoferrin decreased the occurrence of colon cancer by 83%. The number of adeno-carcinoma cells in the gut of rats was reduced after the ingestion of lactoferrin.

Lactoferrin-mediated inhibition of tumor growth might be related to apoptosis of these cells, induced by the activation of the Fas signaling pathway [55]. It has been suggested that the treatment of lactoferrin knockout mice with lactoferrin (post-chemotherapy) accelerated the reconstitution of the immune system. This also reduced the chances of infection following chemotherapy treatment [76]. Lactoferrin can scavenge free iron in fluids and inflamed and infected sites, suppressing free radical-mediated damage and decreasing the availability of the metal to pathogens and cancer cells. Also, lactoferrin hinders migration in a model of human glioblastoma by reverting an epithelial-to-mesenchymal transition-like process [4, 32].

3.4 Lactoferrin in COVID-19 treatment

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection has recently become a primary global health concern, leading to the urgent development of therapeutic agents for its prevention and treatment. Iron overload is understood to have an important role in the pathogenesis of COVID-19. Actually some features (such as inflammation hyperferritinemia, hypercoagulation, and immune dysfunction) manifested a in COVID-19, are linked to iron overload. The presence of free iron, resulting from iron overload and dysregulation, is very highly reactive and toxic due to its reactive oxygen species (ROS) generation potential. The ROS produced react with very important cellular biomolecules and induce their subsequent damage. Nucleic acids, proteins as well as membranous and cellular lipids are effected by the highly activate inflammatory processes which may be either acute or chronic. The linkage of inflammation with multiple clinical conditions, such as cancer is well understood [80]. Lactoferrin has exhibited unique immunomodulatory, anti-inflammatory, and broad-spectrum antiviral activity indicating its potential for the cure of COVID-19 cases and prevention of its devastating effects on multiple target organs [81, 82]. Lactoferrin could counteract the coronavirus infection and inflammation, acting either as a natural barrier of respiratory and intestinal mucosa or reverting the iron disorders related to the viral colonization. Iron-catalyzed lipid damage is understood to exerts a direct effect on ferroptosis, the newly discovered cell death mechanism. Unlike programmed cell death (PCD), ferroptosis not only leads to amplified cell death but is also associated with inflammation. Iron chelators are generally recognized as safe and have been shown to protect patients in diseases characterized by iron overload. Research work also suggests that iron chelators exhibit antimicrobial activities. It is suggested that the naturally occurring iron chelators, such as lactoferrin, exert anti-inflammatory

as well as immunomodulatory effects. It binds to some of the same receptors used by coronaviruses and hence blocks its entry into host cells. Iron chelators may actually be of a very high therapeutic value during the present scenario of the ongoing COVID-19 pandemic [80]. Therefore, the use of lactoferrin may be of value in the prevention and management of COVID-19. The use of lactoferrin appears to be a promising approach to treating COVID-19, but further investigations are required to verify its antiviral activity *in vitro* and *in vivo* [83–85].

3.5 Lactoferrin assimilation *in vivo*

Lactoferrin shows high bioavailability after oral administration, high selectivity toward cancer cells, and a wide range of molecular targets controlling tumor proliferation, survival, migration, invasion, and metastasis. Notably, lactoferrin may either promote or inhibit cell proliferation and migration depending on whether its target cell is normal or cancerous. Significantly, its administration is well tolerated and does not exhibit any significant side effects. Furthermore, lactoferrin may prevent cancer development and growth by enhancing the adaptive immune response. Oral administration of lactoferrin has also led to promising improvement in the immune responses of antiretroviral therapy in naıve children suffering from HIV [86]. Oral administration of lactoferrin decreased the occurrence of colon cancer by 83%, while the quantity of adenocarcinoma cells was reduced in the gut of rats after ingestion of Lactoferrin, ameliorating tongue cancer.

Of particular interest is the notion that even its oral administration may be effective. This is different from many other therapeutic proteins, which typically require other invasive routes of administration [87]. Oral administration of bovine lactoferrin prevents carcinogenesis in the colon and other organs in rats. It also inhibits lung metastasis in mice. It might be mediating its anti-carcinogenesis effects is by increasing expression of relevant cytokines and inducing subsequent activation of immune cells [67]. It interacts with a wide range of molecular targets controlling tumor proliferation, survival, migration, invasion, and metastasis. It may be noted that lactoferrin can promote or inhibit cell proliferation and migration depending on whether it acts upon normal or cancerous cells, respectively. Moreover, lactoferrin can prevent the development or inhibit cancer growth by boosting adaptive immune response. Most importantly, lactoferrin administration is highly tolerated and does not present significant adverse effects.

Oral administration of lactoferrin is the most widely adopted method of its delivery into the human body. This still possesses some challenges that must be addressed before reaping the highest benefit from its intake. Since the functional domains of lactoferrin are highly dependent on its unique 3D structural conformation, the gastrointestinal breakdown of lactoferrin may cause undesirable loss of some of its functional properties. The important receptors of lactoferrin are located at the intestinal mucosa and lymphatic tissue cells in the gut [88–91]. Hence, the delivery of lactoferrin through oral administration requires that it is protected so that it passes through the stomach and is delivered to the absorption sites in a functionally active form. But the most important thing is to note that the digestive tract in infants and newborns is not mature enough (e.g., the intragastric pH and the gastric emptying rate are higher than in adults), and lactoferrin would not be completely digested under these conditions. This hypothesis has been confirmed by measuring the unhydrolyzed lactoferrin in fecal extracts of babies [92, 93]. Nevertheless, the degradation of lactoferrin during the gastrointestinal tract could also be beneficial. It has been reported that

strong antibacterial peptides such as lactoferricin and lactoferrampin are produced by its pepsin hydrolysis [94, 95]. This further benefits the utilization of lactoferrin in high value food products such as infant formula, nutritional supplements, and other formulations that aim at delivering lactoferrin through oral administration.

A commonly accepted method to protect lactoferrin during digestion is microencapsulation. In this method, a protective matrix is created around the lactoferrin core. Food grade proteins (e.g., bovine serum albumin, β- lactoglobulin) and polysaccharides (e.g., pectin, carrageenan, sodium alginate, gum Arabic) are commonly used as the shell materials. This core-shell structure excellently protects lactoferrin from the harsh environment prevailing in the human digestive system. The microencapsulation also helps achieve targeted and controlled release of lactoferrin by simply using shell materials with suitable properties.

Based on in vivo studies, oral administration of lactoferrin to rodents significantly decreased the chemically induced carcinogenesis in various organs such as the breast, esophagus, tongue, lung, liver, colon, bladder, and hindered angiogenesis [78]. During the past two decades, many animal and human studies have proved that orally administered Lactoferrin exerts many beneficial effects on the health of animals and humans [75].

3.6 Lactoferrin industries in the world

Human and bovine lactoferrin is generally recognized as a safe substance (GRAS) by the Food and Drug Administration (FDA, USA). Some pharmaceutical industries (*e.g.*, Morinaga Milk Industry Co LTD Venture LLC, Ventria Bioscience, AusBioMed, Biopharming, Max Biocare, etc.) are into commercialization of human and bovine Lactoferrin related products such as nutraceuticals and vitamin supplements for pediatric use. Also are being produced baby foods, beverages, and a cell growth promoting adjuncts for better child development.

According to Global Market Insights Inc. report, the global lactoferrin powder revenue size was US$195 million (€164 m) in 2020, which is set to surpass US$315 million (€364.1 m) by 2027 and is expected to register over 7.7% CAGR between 2021 and 2027. Owing to the anti-inflammatory attribute of lactoferrin its market is likely to surpass 70 Million USD by 2027. Its antiviral efficacy is being increasingly recognized during the COVID-19 pandemic. Furthermore, its immunomodulatory and anti-inflammatory capability is expected to raise product demand in an unprecedented manner from the pharmaceutical sector. It is estimated that the lactoferrin industry from the pharmaceutical application would actually exceed 53.78 Million USD by 2027. The global Lactoferrin Market is anticipated to attain substantial growth by the end of the forecast period (2021–2025).

Lactoferrin has also been used in different products, such as probiotics, supplemental tablets, cosmetics, and as a natural solubilizer of iron in food. It is also used in the treatment of diverse carcinomas, severe sepsis, and diabetic foot ulcers. Numerous attributes of lactoferrin, such as its iron absorption ability, antibacterial, anti-inflammatory, antioxidant, and immunity-boosting capabilities, are likely to provide promising opportunities for the lactoferrin industry. The ability of lactoferrin to prevent biofilm formation helps inhibiting the growth of bacteria. This can lead to an enhanced product demand owing to its therapeutic applications. The higher susceptibility of infections in infants and newborns due to an underdeveloped immune system can be supplemented by the lactoferrin industry. Growing demand for lactoferrin from physical fitness and sports nutrition application is likely to drive the growth of

lactoferrin capsules during the forecast period. Increasing instances of digestive and gastric disorders should boost the demand for lactoferrin as an anti-inflammatory ingredient.

4. Conclusion

Lactoferrin, a multifunctional ingredient amply found in camel milk. It has numerous applications as a natural antimicrobial food additive and pharmaceutical agent. Camel milk lactoferrin has unique antimicrobial, antioxidant, anti-infective and anti-cancer activity. It can be used as a natural alternative to chemical antibiotics. Camel milk also been suggested for weight management. Lactoferrin from the milk of different indigenous species is being increasingly used as a specialty ingredient in the dairy industry. Lactoferrin can be used for biopreservation of foods such as milk, meat, fresh-cut fruits and vegetables, and their products to increase shelf life, control diseases and enhance public health. Indeed, our feeling is that camel milk lactoferrin can be used in synergy with both, conventional therapies and recent advancements allowing many therapeutic agents with potential side effect to be administered at lower, more sustainable doses.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

Milk Borne Brucellosis

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Abstract

Milk is full of nutrients, making it an ideal environment for several infectious diseases, that come at the forefront is brucellosis. The zoonotic disease brucellosis in humans is mostly ignored, and the annual number of human cases is commonly reported as 500,000. Consumption of tainted dairy products is the most common vector for the transmission of human Brucellosis. Confirmation of disease via culture is considered the gold standard, but is not always possible. Serological tests and molecular tests are alternative methods. The milk Ring Test is considered the method of choice for the surveillance of dairy herds. The control of risk factors and surveillance are the cornerstones of brucellosis prevention. Eliminating animal infections is the most effective preventative technique. Cattle, goats, and sheep vaccination are advised in enzootic regions with high prevalence rates. The main methods of preventing human infection are public education, food safety measures, occupational hygiene, and laboratory safety. The pasteurization of milk before it is consumed directly or used to make products like cheese is a crucial step in avoiding transmission from animals to people. Both education initiatives and laws prohibiting the sale of unpasteurized milk products can be successful.

Keywords: human brucellosis, unpasteurized milk, *Brucella spp*., surveillance, food safety

1. Introduction

The issue of food safety is a serious concern in the world. World Health Organization (WHO) declares that more than 200 diseases are related to the consumption of contaminated food products, and these diseases can cause significant complications in susceptible consumers such as infants, children, the elderly, and pregnant [1].

Historically, milk and dairy products have been significant components in the nutrition of most nations [2]. Milk and dairy products are rich in nutrients, high-quality proteins, micronutrients, vitamins, and energy-containing fats [3, 4]. Therefore, milk is a good environment for the growth of various pathogens [5]. There are many factors that influence the prevalence of pathogens and spoilage microorganisms in milk and dairy products. These factors may include the dairy herd's health, hygiene status in the farm setting, milking and prestorage environment, the availability of

storage facilities and technology, the farming methods used, geographical location, and seasonal differences [6].

Brucellosis is among the most investigated zoonosis. The consumption of contaminated milk or milk products, in addition to direct or indirect contact with infected mammals, leads to the transmission of brucellosis to humans [7]. The proper thermal process of milk can reduce the population of *Brucella spp*.; however, post-contamination of proceeding products or failure in the pasteurization process of the milk could provide an ideal condition for the growth of *Brucella spp*. For instance, *Brucella melitensis* can live in infected unpasteurized milk kept at 4°C for five days, and for nine days at −20°C. In addition, *Brucella*'s life rate improves up to 18 days at the ambient condition in cheese, which is produced by infected unpasteurized milk [8].

Even though brucellosis seldom poses a threat to human life, it is widely seen as a burden for the economy and public health, notably in the Middle East, Mediterranean region, North and East Africa, Southern and Central Asia, India, Central, and South America. According to the European Food Safety Authority, brucellosis incidence rates in developed countries range from 0.3 cases per million per year to over 1000 cases per million in endemic regions [9, 10].

In the dairy food chain, from production through handling and processing to consumption, productivity and safety are inextricably intertwined. Therefore, a continuous system of preventive measures is required to reduce the risk of brucellosis associated with milk and dairy products, starting with the safety of mammal feed and continuing through best farming practices and on-farm control systems, good production and sanitary processes, customer safety awareness, and appropriate implementation of food safety management systems across the dairy chain [11, 12].

Here comes the importance of this chapter to spotlight the complexity of neglected brucellosis. It clarifies the etiology, epidemiology, sources, transmission, clinical features, diagnosis, prevention, and control of this infection.

2. Etiology of human brucellosis

Numerous names for human brucellosis exist, such as undulant fever, Gibraltar fever, gastric fever, Malta fever, Mediterranean fever, Maltese fever, intermittent fever, and rock fever of Gibraltar. It is a zoonotic disease that has been around for a long time; fresh evidence from ancient Egyptian skeletons suggests it is been around since, at least, 750 BC. The Mediterranean, the southern and central United States, Africa, Asia, the Arab peninsula, the Indian subcontinent, and the Middle East all have a higher prevalence of this infection.

Many different kinds of animals are susceptible to the bacterial zoonotic disease known as brucellosis, which can be passed on to people through infected food or by casual contact. The zoonotic disease brucellosis in humans is mostly ignored. In humans, brucellosis begins as a devastating acute infection that can become chronic and have numerous problems; it is a disease of poverty. Infections in animals have a large socioeconomic impact. The annual number of human cases is commonly reported as 500,000, but this is likely an underestimate due to the fact that many of the worst-affected countries lack the resources to properly diagnose the disease, and because the flu-like symptoms are also present in a number of other febrile illnesses [13].

As a bacterial illness with a global reach, brucellosis affects not just individuals but also the livelihoods of entire communities and economies.

Several host-specific *Brucella* species are responsible for this disease. In order to spread throughout the body, only need just 10–100 infective organisms. Brucella species are gram-negative cocci bacilli (GNCB) that are nonmotile, do not produce spores, and are encapsulated. They are named after Sir David Bruce (1855–1931), the man responsible for discovering the brucellosis-causing bacteria. Although aerobic, certain bacteria can only be successfully isolated in the presence of 5–10% carbon dioxide. In mammals, *Brucella* organisms concentrate on the reproductive organs, where they cause abortions and infertility. The animal's fluids, such as urine, milk, and placental fluid, are rich in them [13, 14].

So far, twelve distinct *Brucella* species have been identified; while they can spread to a wide variety of hosts, each species has a preferred host. These twelve species consist of six traditional species and six new species. The traditional species are *B. abortus*, *B. melitensis*, *Br. suis*, *Brucella neotomae*, *Brucella ovis*, and *B. canisand*, while the new species are *B. canisand*, *Brucella ceti*, *B.pinnipedialis*, *B.microti*, *B.inopinata*, *B. Papionis*, and *B. vulpis*. Three of the *Brucella* species are known to be endemic in most countries, and they are highly virulent to both their natural hosts and humans. They include *Brucella abortus*, which mostly infects cattle; *B. melitensis*, which primarily infects sheep and goats; and B. suis, which has a tropism for domestic, feral, and wild pig populations [15, 16].

3. Transmission of human brucellosis

The following is a rundown of the most prevalent ways that humans contract *Brucella* species infections.

3.1 Food borne transmission

3.1.1 Unpasteurized milk and dairy products

Consumption of tainted dairy products is the most common vector for the transmission of brucellosis in humans. Consumption of unpasteurized milk and dairy products is a major route for the transmission of *Brucella* species to humans, as these bacteria are spread from diseased animals to humans through the milk they produce. Unpasteurized cheeses, also known as "village cheeses," are very probably the foods that cause human brucellosis. This is especially true of goat and ewe cheeses. The usual lifespan of Brucella in this cheese is one month, but it can last for as long as three months [17, 18].

3.1.2 Raw or undercooked meat products

In societies where raw or undercooked meat or meat products are highly valued, meat may also be a major source of infection [19].

3.2 Environmental contact with infected animals or their products

Direct inoculation occurs most frequently through cuts and abrasions in the skin. Veterinarians and those working in red meat processing factories or slaughterhouses run the risk of skin wound contamination. Animal tissue, fetal remains, birth

products, blood, urine, and vaginal discharges are all potential vectors for the transmission of disease [20].

Hunters can become infected with *Brucella* by cuts in the skin or from ingestion of the bacteria while cleaning wild pigs, moose, elk, and deer that they have entrapped.

Newly aborted animals or rains running off of contaminated land might contaminate water supplies like wells.

Brucellosis can also be contracted through the inhalation of infected dust, dried dung, or other noxious excrements. *Brucella* species can persist for extended durations in a wide variety of environmental conditions, including dairy, meat, aborted fetuses, dung, dust, soil, slurry, and water, making the problem complicated. A lot of factors, including the type of substrate, the quantity of Brucella, the temperature, the pH, the amount of sunshine, and the presence of other microbial contaminants, all play a role in determining an accurate duration of survival [21].

3.3 Occupational exposure

The risk of brucellosis infection is higher in certain professions. Infection can happen through inhalation, contamination of the conjunctiva, ingestion, contamination of the mucous membranes, contamination of the skin, particularly through cuts or abrasions, and unintentional self-inoculation with live vaccines [22]. These professions involve:

A. Veterinarians and those working in the lab who are culturing *Brucella*.

B. Individuals who deal with animals raised for food.

C. Individuals who are employed in the meat sector.

D. Individuals who are employed by the dairy business.

E. Personnel who work in the upkeep of farms, industries, or facilities that process animal products.

F. Farmers, farm workers, caretakers of animals, and pig keepers.

G. Shepherds, sheep shearers, goatherds, and artificial insemination.

H. Families who raise livestock and are farmers; families who burn dried dung as fuel.

I. Meat packers, butchers, and slaughtermen.

J. Fetal calf serum collectors, wool processors, and hide and skin processors.

3.4 Infection by the inhalation

It is uncommon for people to become infected with *Brucella* bacteria through inhalation, but it can be a serious risk for those who work in particular occupations, such as medical labs, where staff members may be exposed to the aerosol while preparing samples for isolation, in addition to abattoir workers, and those who work with animals used in food production or in the meat business [23].

3.5 Infection through conjunctiva

Vaccines for *B. abortus* include strain 19 and RB-51, and those for *B. melitensis* include Rev-1. Any contact with infected tissue or body fluids, such as a splash onto the conjunctiva of the eye or a needle stick from a vaccine administered when working with animals, can lead to self-inoculation. Vaccines administered via conjunctive splash are likely to be more potent than those administered via injection. That is why it is possible to catch brucellosis through the eyes' conjunctival sac [24].

3.6 Blood and bone marrow transfusion

Contaminated blood and bone marrow transfusions are other potential routes of transmission. This transfusion has a high risk of *Brucella* transmission [25].

3.7 Tissue transfer (transplantation)

A tissue transfer is a form of transplantation. Organs, tissues, or groups of cells are transplanted when they are physically removed from a donor and placed into a recipient, or when they are relocated inside the same body. It is probable that these actions will spread disease [26].

3.8 Person to person transmission

Although cases of brucellosis being transmitted from person to person are extremely unusual, they do occur [25].

3.9 Venereal transmission

Rare reports have also suggested sexual transfer as a possible transmission pathway [26].

3.10 Vertical transmission

Babies of infected moms who are breastfed have a higher chance of acquiring the illness themselves. Birth-related exposure to the mother's blood, urine, or feces can also cause congenital illnesses in infants [27].

4. Clinical features of human brucellosis

Although brucellosis in humans can affect people of any age, it most commonly affects men in their twenties and forties because of the more exposure to occupational risk [28].

Each species of *Brucella* is a facultative intracellular pathogen, meaning that it can live and reproduce within the host cell's phagocytic immune system. Still the ways by which Brucella evades intracellular death are poorly understood. But in the end, *Brucella* organisms are contained within RES monocytes and macrophages in organs including the lymph nodes, liver, spleen, and bone marrow [29]. Brucellosis is a systemic disease that can infect all the organs or tissues of the body. After Brucella enters the body, it goes through three distinct phases: the incubation period, the acute phase, and the chronic phase. The incubation period can vary from five days to five months and is not always straightforward to estimate. However, it is typically two to

four weeks. About half of all cases of the disease have an abrupt beginning within days to weeks after infection, whereas the other half have a more gradual onset within weeks to months following the initial infection. Clinical symptoms might be rather general and diverse. Symptoms may include high body temperature, sweating, weariness, general malaise, lack of appetite, weight, headache, aching joints, and pain in the back. Patients typically improve first thing in the morning and then experience a worsening of their symptoms throughout the day [30].

Fatigue, fever, sweating, splenomegaly, and hepatomegaly are all symptoms and signs that may appear during the acute phase.

Brucella possesses a wide variety of virulence characteristics that allow it to establish persistent infection by hiding within host cells and evading the immune system. Because of the high degree of clinical polymorphism, brucellosis is often missed at the first point of care [31].

Additionally, brucellosis is known to induce serious clinical problems involving the internal organs such as meningitis, encephalitis, arthritis, spondylitis, endocarditis, prostatitis, and orchitis [31].

The need for sleep might be overwhelming and sadness is common. The fever will fluctuate in intensity over the course of several days ("intermittent fever") if it is left untreated.

5. Diagnosis of human brucellosis

Given the large variety of clinical symptoms, most of which are nonspecific, it is impossible to make an accurate clinical diagnosis of human brucellosis; instead, it is required to achieve serological and bacteriological testing, in order to decrease diagnostic errors.

5.1 Culture

Confirmation of disease via culture is considered the gold standard but is not always possible. Clinical samples from sites of the disease's focus, such as bone marrow, spleen, synovial fluid, and abscesses, can be tested for the presence of *Brucella*. Whole blood is typically the biological material of choice for isolating *Brucella*. The following conditions are necessary for cultural isolation to be successful [32]:

A. Disease phase: Isolation rates as high as 40–90% are seen during the acute phase of an illness, whereas rates as low as 20% are seen during the chronic or decline phase.

B. Bacteremia level: Blood is completely sterile in healthy people. In the medical community, blood cultures are the gold standard for diagnosing bacteremia since only living bacteria can survive in the blood.

C. Species of *Brucella*: While *melitensis* species have a high rate of isolation, *non-melitensis* species have a far lower rate of isolation.

D. Culture methodology: *Brucella* can be isolated, purified, and identified using a variety of techniques. The most reliable methods for obtaining sterile cultures are the solid-media, streak-plate, and pour-plate approaches.

5.2 Serological tests

Antibodies against *Brucella* were assessed using a series of serological tests to confirm the presence of infection with this bacterium [32–34].

5.2.1 Rose Bengal test (RBT)

Although this test was developed for veterinary screening, it is now routinely used to diagnose human brucellosis. The Rose Bengal test is a rapid (5–10 minute), simple to conduct, and highly sensitive diagnostic tool for acute brucellosis; nevertheless, it has a high rate of false-negative results in chronic and severe cases, and false-positive results emerge due to cross-reaction with *Yersinia enterocolitica*.

5.2.2 Serum agglutination test (SAT)

Although this test was first introduced in 1897, it still relies upon today for the serodiagnosis of brucellosis. In endemic areas, SAT is the most widely employed serological test. In non-endemic locations, an agglutination titer of 1:160 or higher is regarded as noteworthy, but in endemic areas, a titer of 1:320 or higher is required.

5.2.3 Microagglutination test (MAT)

This exam is a condensed version of the SAT. In comparison to SAT, it can test many samples simultaneously and requires less serum and reagents.

5.2.4 Indirect coombs test

Incomplete, blocking, or non-agglutinating IgG can be detected with the help of the Coombs test, which is an extension of the SAT.

5.2.5 Brucella capt test

When it comes to diagnosing human brucellosis, the *Brucella* capt test has been proposed as an alternative to the Coombs test. This is because it is an immune capture agglutination test. The BRUCAPT test is an immunocapture assay that can detect total anti-*Brucella* antibodies in a single step. The Coombs test was previously the only method for measuring agglutinating and incomplete antibodies; this assay allows the detection of both. To diagnose brucellosis, especially in its more advanced stages, and to monitor its treatment, this test is the gold standard of serological methods; quick and easy, the results will be available in 24 hours. The best possible sensitivity and specificity are achieved without subjecting samples to any kind of washing or dilution.

5.2.6 Enzyme-linked immunosorbent assay (ELISA)

When other tests are negative and there is significant clinical doubt, ELISA is the test of choice for focused, difficult, and chronic patients. This test has high sensitivity and specificity. Moreover, it may detect immunoglobulins (IgG, IgM, and IgA) during 4 to 6 hours. According to the research done in this area, ELISA is a highly effective tool for both the detection of *Brucella* antibodies and the discrimination between the

acute and chronic stages of the disease in large populations. It has been proven by the research community that ELISA assays are quick, accurate, sensitive, and specific. This technique complements agglutination assay in clinical laboratories for the detection of brucellosis and provides an alternative for large-scale screening.

5.2.7 Indirect fluorescent antibody (IFA) test

This test is quick, with findings available in just a couple of hours in the same illustration of ELISA. However, the interpretation of results is subjective, and it might not differentiate between IgA, and research findings have shown that different manufacturers produce varying levels of antibody responses to the same antigens.

5.2.8 Immunochromatographic lateral flow assay

The test is easy to perform and read, simple, and rapid, with high sensitivity and specificity (> 90%).

5.3 Molecular assays

The traditional methods of *Brucella* typing are gradually being replaced by newer molecular technologies. The laboratory diagnosis of human brucellosis has benefited from advancements in molecular-based technology. Pure *Brucella* culture DNA and DNA from clinical specimens can both be amplified and detected using PCR tests. Extracting *Brucella* DNA from whole blood, serum, and tissue samples is now possible with the help of a wide variety of commercial kits.

5.3.1 Standard PCR

A single-pair PCR technique was devised to amplify the target genomic sequence of *Brucella* species for the diagnosis of human brucellosis. It has been shown through research that conventional PCR is a more sensitive approach than culture methods, both for the initial diagnosis of infection and for the early detection of relapses [35].

5.3.2 Real-time PCR

Real-time polymerase chain reaction is an effective method for determining the concentration of nucleic acids in isolated blood specimens. In addition to being quick, sensitive, and specific, it has a high rate of reproducibility [36].

5.3.3 Nested and semi-nested PCR

In nested PCR, two sets of PCR primers are utilized to study the same genomic region. Currently, nested PCR and semi-nested PCR techniques are being developed to detect *Brucella* in human blood samples and are being tested in clinical settings for the diagnosis of human brucellosis. Lin et al. reported a nested PCR for the laboratory diagnosis of human brucellosis. During semi-nested PCR, two sets of PCR primers are used, but one of the primers in the second set is the same as that in the first [37].

5.3.4 Other PCR-based assays

Polymerase chain reaction-enzyme immunoassay (PCR-EIA) was used by Vrioni et al. (2004) for the quick laboratory diagnosis of human brucellosis from peripheral blood. According to the findings, the PCR-EIA assay is a sensitive and specific technique that could help in the quick and precise diagnosis of acute human brucellosis [38].

6. Detection of *Brucella* antibodies in livestock milk

The milk ring test (MRT) was first introduced by Fleischhauer in Germany in 1937; it is the best test for screening the milk of suspected cases of animal brucellosis. MRT is characterized by simplicity, ease, accuracy, and inexpensive method, also MRT is not consumed time. Therefore, MRT is considered the method of choice for the surveillance of dairy herds. This test mainly detects the *Brucella* antibodies IgM and IgA in fresh milk. The sensitivity and specificity of MRT are 85% and 95%, respectively [39–41].

7. Prevention and control of human brucellosis

Milk and dairy products play an important role in the transmission of *Brucella* to humans, and the risks are increased because an infectious dose of just 10–100 organisms is sufficient to cause systemic infection. The genus *Brucella* is composed primarily of mammalian pathogens, and the key species are food-producing animals, especially milk-producing animals [42].

Additionally, Brucellosis' zoonotic status is now established. Therefore, the only way to effectively prevent sickness in humans is to remove the animal reservoir.

Medical, public health and veterinary authorities must often work together to eradicate brucellosis and ensure its prevention and control. The beginning of a good control program begins with this cooperation.

The concept of "One Health" (OH) is a catch-all phrase referring to the commonalities between people, animals, plants, and the environment. Integrative health practices are encouraged by increasing cross-disciplinary communication and coordination.

The following guidelines should be followed to reduce the risk of contracting brucellosis [43]:

7.1 Prevention of food-borne brucellosis

7.1.1 Milk and dairy products

Milk from affected bovines (i.e., cattle, buffalo, camels, sheep, goats, yaks, and reindeer) is a major contributor to the disease of milk-borne brucellosis.

There is a particularly high danger from consuming milk and dairy products like cheese, cream, and ice cream [44]. All milk and dairy products destined for human consumption, whether directly ingested or utilized in the production of other foods, must undergo proper heat treatment in one of the following ways [45]:

- The process of pasteurization.

- Boiling
- Ultra heat treatment (UHT)

Be wary of various kinds of cheese, especially soft cheeses made from raw milk, as they may harbor high numbers of *Brucella* species if they have not been adequately heated. Making cheese in this style should be highly discouraged. We found that the acidity of hard cheese mitigates some of the risks associated with eating it, allowing us to infer that it is safer than softer varieties. The acidification methods used to make sour milk, sour cream, yogurt, and butter; all drastically lower the *Brucella* concentration.

It is also important to keep in mind that the rennet enzyme, if it is made from the stomachs of *Brucella*-infected ruminants, can potentially be a source of infection [46]. Rennet enzyme is a complex set of enzymes, such as chymosin, pepsin, and lipase, naturally present in the fourth stomach or abomasum of an unweaned calf, kid, or lamb. Chymosin essentially turns milk into a soft cheese in the stomach of these young animals so that digestion occurs more slowly and nutrients can be absorbed. Cheesemakers mimic nature by using the coagulating capability of chymosin to separate milk into the solid curds needed for cheesemaking and the liquid whey that is left over. *Brucella* can be transmitted to humans and animals through the watery whey layer that remains after cheese manufacturing. Additionally, if shipping containers are not cleaned properly before use, it could contaminate the contents.

Also, be cautious of ice cream made with milk that has been infected with *Brucella*, as milk can be extremely dangerous, especially when it comes from a variety of sources that may be combined to form a single serving. Heat treatment is required for all milk used in this formulation.

7.1.2 Meat and meat products

Estimates vary but as little as 10–100 *Brucellae* are sufficient to infect a human [42]. Therefore, contaminated meat and meat products may be a vector for spreading the disease, especially if they are sourced from animals slaughtered during the acute phase of the disease and are eaten raw or undercooked. There is arising in a real possibility of transmission of human brucellosis from the consumption of meat and meat products. Household meat and meat product processing procedures can be a source of widespread pathogens exposure risk to the family and the community [47].

Not only the hunters and butchers but also other family members may have contact with the meat. Brucellosis is a disease that can be passed from animals to humans through contact with their flesh, organs (liver, kidney, spleen, viscera, udder, testicles), blood, or even saliva. The killing of an infected animal or the preparation of infected meat can also spread the disease. Most people are not in danger of contracting brucellosis but those who work in the veterinary industry, a slaughterhouse, a meat processing plant, a farm, and a hunting camp or who eat raw or undercooked meat are [47].

The high concentration of *Brucella* species in the liver, spleen, lymph nodes, mammary glands, testes, and bone marrow of the carcasses warrants special attention [48]. In this situation, these tissues, if not appropriately cooked, can cause infection by contact or ingestion.

Contamination of other foods and cooking utensils is possible during the handling and preparation of contaminated meat and offal.

All meat products must be fully cooked prior to consumption because meat borne brucellosis cannot be prevented by using most common meat preservation methods such as salting, drying, smoking, refrigeration, or freezing [47].

7.1.3 Marine mammal brucellosis

Roughly 120 species of marine animals either exclusively inhabit maritime environments or are heavily reliant on marine resources for survival. These animals include pinnipeds (true seals, eared seals, and walruses) [49]; cetaceans (which contain two suborders: Mysticeti (baleen whales) [50] and Odontoceti (toothed whales, which includes dolphins and porpoises) [51]; polar bear (Ursusmaritimus) [52]; sirenians (manatees and dugong) [53]; and several species of otters [10].

Veterinary meat inspectors, researchers, occasional consumers of marine mammal meat, those working with stranded marine mammals, those working with products derived from marine mammals, whale and seal hunters, those working with raw marine products, and people in traditional communities where products from whales and seals are still an important part of the diet are all at risk of zoonotic transmission of marine mammal brucellosis.

In light of the wide variety of human brucellosis symptoms and the relatively recent realization that marine mammals play a role in the transmission of the disease from marine mammals to humans, it is important to pay attention to this type of food and conduct additional research on the virulence of recent *Brucella* species in humans.

7.2 Personal hygiene

Veterinarians, laboratory employees, meat inspectors, abattoir workers, farm laborers, farmers, inseminators, stockmen, and anyone involved in the processing of animal products are at a high risk of occupational exposure to *Brucella* infection. Procedures involving aborting animals, aborted materials, or those in the process of parturition, as well as clinical examination, inspection, shearing, dipping, insemination, treatment, vaccination, and the disinfection and cleaning of contaminated premises pose a particularly high risk of spreading brucellosis. Therefore, the following procedures should be followed by those who are at a high risk of contracting *Brucella* due to their place of employment [54].

A. Everyone working in potentially hazardous environments should dress appropriately and use Personal Protective Equipment (PPE). The uniforms should be kept on the premises and used only for this function. Personal protective equipment (PPE) consists of an overall or coat, a rubber or plastic apron, rubber gloves, boots, a face shield or goggles, and a respirator (a mask) if the mode of transmission is airborne.

B. Handle fetuses, animal placentas, and animal discharges with care.

C. Immediately after coming into contact with the animal, fetus, placenta, or animal secretions, wash hands thoroughly with liquid soap and water.

D. Apply antiseptic (tincture of iodine, for example) and plaster or self-adhering bandage to any wounds or scrapes that you find.

E. If symptoms worsen throughout the incubation period, prompt medical attention should be requested.

F. Work garments should be used only for the work and kept on the Premises; after each use, they should be boiled, steamed, fumigated with formaldehyde, or soaked in a disinfectant solution of suitable concentration, such as phenolic soap, iodophor, hypochlorite, or chloramine.

G. Shoe disinfection is essential to prevent the spread of disease from outside the home or tent to the living space.

H. Sanitize workwear and contaminated spaces following usage.

I. Eye protection is essential because of the high risk of infection from conjunctival contamination, and any infectious material that enters the eye must be removed under clean or aseptic conditions away from the work area.

J. Respiratory contamination is also a high risk in heavily infected environments. Protect yourself from breathing in dust or aerosols created by dried excreta or tissues discharged after abortion, parturition, or slaughter by donning a mask. It is important to frequently replace the bacteria-catching filters and sanitize the apparatus using chemicals or steam.

K. Serological testing at regular intervals is a great way to keep an eye on the health of your workforce. Before commencing work, new hires are encouraged to submit a blood sample as a baseline.

L. Pregnant women and children under the age of 18 should not be allowed to work in hazardous environments.

7.3 Occupational hygiene

Occupational hygiene is the study of how to prevent, detect, evaluate, and remediate risks to workers' health and safety on the job, while also considering the potential effects on bystanders and the larger community. This field of study improves working conditions and practices by raising awareness of potential dangers among employers and workers. Occupational exposure to human brucellosis can occur in the following groups [55]:

Teams whose duties need them to come into contact with diseased animals or animal byproducts. Farmers, stockmen, shepherds, dairymen, goatherds, abattoir workers, butchers, and those who do artificial insemination are among these professionals.

Groups whose work involves the processing of hides, viscera, wool, and skins, as well as individuals involved in the servicing of buildings or machinery used for these purposes.

Another crucial category comprises laboratory workers who may come into contact with infected materials and *Brucella* cultures during diagnostic procedures or vaccine manufacture; the creation and administration of live vaccines also offer some danger.

7.4 Laboratory biosafety

One of the most common and simple laboratory illnesses is brucellosis. *Brucellae* are classified as a high-risk pathogen by the World Health Organization (WHO), placing them in risk group 3. Actually, one of the most common and simple laboratory illnesses is brucellosis.

Although *Brucellae* are rarely present in sufficient numbers to provide a substantial risk to people handling blood samples and biopsy material for either serological or bacteriological diagnosis, these specimens should nonetheless be treated with caution at biosafety level 2. When *Brucellae* have been cultured, however, they multiply to serious levels, necessitating special safety measures. It is also necessary to use biosafety level 3 equipment, methods, and practices [56].

7.5 Farm hygiene

The following are the main points in this context.

A. When working with animals, especially those suspected of having brucellosis, farmhands and animal caretakers should take precautions by wearing protective gear or using other forms of personal protective equipment (PPE).

B. Use an approved disinfectant, such as iodophor, hypochlorite, or phenolic disinfectant, at the prescribed working strength to thoroughly clean any location where an abortion or infected parturition took place.

C. After handling potentially hazardous materials, agricultural equipment should be disinfected by submerging it in a solution of diluted caustic soda, iodophor, or phenolic soap.

D. Liquid manure, which can remain infectious for long periods of time, especially at low temperatures requires special attention, including daily removal, burning, or disinfecting before disposal.

E. Disinfectants should be used in a shallow trough for vehicles entering or exiting contaminated facilities.

F. After cleaning and disinfecting a building that has previously housed animals infected with *Brucella*, the building should not be restocked for at least four weeks.

G. Buildings that have not been decontaminated should not have maintenance employees (such as builders, plumbers, or electricians) present.

H. Preventing rodent and insect entry to buildings should be a top priority for building maintenance.

I. Fly screens, light traps, and insecticides should be used to reduce the number of flying insects and keep rodent populations under control.

J. Making available the best possible living conditions in terms of things like clean water, clean air, and clean storage sheds.

7.6 Hygienic precautions among slaughterhouse

The term "slaughterhouse" or "abattoir" refers to a facility that has been licensed by the appropriate government agency to perform sanitary animal slaughtering and inspection, including pre and postmortem examinations, as well as processing, preservation, and storage of meat products for human consumption. Slaughterhouse operations necessitate the consideration of specific qualification programs, which provide the fundamental environment and operating conditions needed to produce safe meat and meat products. Good manufacturing practices (GMPs), good hygiene practices (GHPs), and standard operating procedures (SOPs) are all examples of such necessary programs (SOPs). Slaughterhouse personnel must adhere to the rules of the expert authority for how to deal with brucellosis to ensure that the postmortem inspection is conducted under proper conditions, and in particular that killed animals may be inspected accurately [57].

At the time of slaughter, *B. melitensis* and *B. abortus* infections in cattle, buffalo, camels, sheep, and goats and *Brucella suis* infection in pigs pose a serious health risk. During the bacteremic stage of the illness, *Brucella* is distributed throughout the body; however, the uterus, mammary glands, and testes may be particularly severely afflicted. Aborted or newly born animals may also have widespread exterior contamination.

Brucellosis-infected animals must be butchered in a separate area of the main slaughterhouse called the "emergency slaughterhouse," where the workforce has received specialized training and has access to specialized equipment [22]. Those are involved in the slaughtering process should dress in personal protective equipment (PPE).

In the workplace, no one should be allowed to eat, drink, or smoke. Required facilities for the disinfection of protective materials and personal washing should be available.

7.7 Precautions when animals under nomadic or migratory conditions

In arid or semiarid regions, livestock management becomes much more complicated when it is performed on a strictly nomadic basis. In these settings, it is difficult at best to practice the level of hygiene recommended to avoid spreading disease [58]. Brucellosis can have a significant impact on society, but it can be mitigated by public awareness campaigns that highlight the disease's key characteristics and its mode of transmission.

Most of the adult population in these areas will have had prior exposure to *Brucella* species infection and hence will have developed some level of immunity. Children are particularly vulnerable to the effects of the sickness and should be kept away from recently born or aborted animals. Raw milk, raw or undercooked meat, and raw or undercooked carcasses should also be avoided. Vaccination is sometimes the only effective intervention for these communities, yet effective vaccinations are currently unavailable.

7.8 Control of reemergence brucellosis

The World Health Organization/Food and Agriculture Organization/World Organization for Animal Health joint consultation on emerging zoonotic diseases, held in Geneva in 2004, defined an emerging zoonotic as "a pathogen that is newly

recognized or newly evolved, or that has occurred previously but shows an increase in incidence or expansion in geographical host or vector range" [59].

Brucellosis is a re-emerging zoonotic infection that transmits in all mentioned routes.

Brucellosis and other zoonotic diseases have emerged as a result of different causes, including global travel and commerce; climate change and weather; changing ecosystems; human demographics and behavior; poverty and social inequality; breakdown in public health measures; the industrialization of food production; globalization; microbial adaptation, in addition, to change in technology and industry; and economic development and land use [59].

Human brucellosis control relies heavily on animal brucellosis control. Effective veterinary and health control of animals (during trading, transport, and slaughter) and animal products (especially meat and meat products, milk and dairy products), education of the community, continuous state financial support, institutional cooperation, and regional cooperation are central to preventative measures.

7.9 Vaccines

Good vaccinations are essential to vaccination efforts. Over the past few decades, small ruminant and bovine brucellosis control programs have had resounding success because of the use of two live vaccines: *B. melitensis* Rev. 1 and *B. abortus* S19 [60]. *B. abortus* RB51 is suggested as a vaccine for bovine brucellosis to be applied in the last stages of protective programs in addition to tests and slaughter [61]. Vaccines can induce brucellosis in humans and can cause abortion in both the animals they are intended for and those that are not. Rifampicin is commonly used to treat human brucellosis, although RB51 is resistant to it. More effective vaccines that are also human and animal-effective are urgently required. New vaccines are being developed, and there are many ongoing efforts to increase the effectiveness and safety of the ones already on the market [62, 63]. The first novel brucellosis vaccine to be licensed will get a sizable prize, and there is now an international call for the creation of such a vaccine. (https://brucellosisvaccine.org/).

7.10 Public health aspects

From a public health perspective, the two most common ways people contract human brucellosis are through their diet or through unprotected, nonmedical contact with animals.

Public health must be focusing on the following aspects:

A. Public health agency collaboration: For these goals to be achieved, it is essential that public health organizations work closely with veterinary services and other relevant agencies.

B. Public health education: Humans are infected by *Brucella spp*. essentially by animal-origin food that is incorrectly prepared and/or preserved. There is a big gap in knowledge among the different sections of the community on the significance of food safety and foodborne diseases.

C. Involvement of local residents is crucial to the success of any health promotion or disease prevention initiative.

D. In order to effectively combat zoonotic and foodborne diseases, health educators should include school teachers in their training and incorporate them into disease prevention and control initiatives (FBDs).

8. Conclusion

One of the most significant and pervasive zoonotic diseases in the world is brucellosis. People who have contact with animals and consume animal products, especially dairy products, are at risk of contracting this illness. To develop effective preventative measures and control programs to enhance the milk production process in endemic regions, all possible risk factors must be thoroughly identified, and their individual and combined influence on milk production must be studied. Additionally, the disease must be managed properly, and both humans and animals should be screened for brucellosis using a combination of specific tests.

The management of brucellosis has been greatly aided by a "one health" plan that includes the extension of health education and the improvement of veterinary capabilities and services. As a result, we suggest control programs based on the principles of "one health" to significantly lower the incidence of brucellosis in dairy farms through national immunization, brucellosis testing, and prevention education, while enhancing public health capabilities and global partnerships across endemic regions.

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Chapter 4

Innovative Approach of Cheese Making from Camel Milk: A Review

Birhanu Bekele

Abstract

Camel milk contains all essential important components of human diet and generates cash, ensures food security, and provides health benefits. Compared to cow milk, camel milk has higher levels of whey protein, lower levels of αs1-casein, larger size of κ-casein, and a very low κ- to β-casein ratio. As a result, the technical characteristic of the acidic or enzymatic coagulation process of camel milk for cheese making is affected by all these factors. Camel milk cheese is a recent product that enters into both the domestic and global milk product markets. Cheese made from camel milk can have processing issues and be of lower quality if it is produced using the same technology as dairy products made from bovine milk. To maximize the possibility of manufacturing cheese from camel milk, various trials were conducted over time utilizing different methods. This chapter reviews the advancements in making cheeses from camel milk using starter cultures and coagulants. Furthermore, the relevant studies describing the fortification of camel milk with ingredients for cheese making are included.

Keywords: innovates, camel milk, starter culture, chymosin, cheese making

1. Introduction

Milk is believed to be the most essential product obtained from dairy animals including camel (Dromedary), being a complete food, helps to provide a nutritious and balanced diet to nomadic desert people under harsh conditions [1, 2]. Many people give a great value to camel milk because it is essential to their nutrition in the Gulf Cooperation Council, Middle East, Middle Asian, and African nations [3]. Additionally, it has been claimed that camel milk helps households in pastoral areas to generate cash, ensure food security, and provide health benefits [4]. A significant source of protein, fat, lactose, vitamins, and minerals can be found in camel milk. All of the essential amino acids are also present in camel milk's protein, while unsaturated aliphatic fatty acids are present in the fat. Furthermore, compared to cow milk, camel milk has higher levels of whey protein, lower levels of αs1-casein, and a very low kappa- to beta-casein ratio. The technological characteristics of the acidic or enzymatic coagulation process of milk are affected by all these factors, which cause the final curd to almost be weak and brittle, and have an open body and texture [5–7].

Even though the gross composition of camel milk is similar to bovine milk, the relative composition, distribution, and the molecular structure of the milk components are reported to be different. Consequently, manufacturing camel dairy products such as cheese, yogurt, or butter using the same technology as dairy products from bovine milk can result in processing difficulties and products of inferior quality [5]. As a result, camel milk is consumed, usually in a raw form by the people living in remote areas where camels are reared. On some occasions, to extend its shelf-life, this milk is consumed in a fermented form [4, 8]. However, scientific evidence points to the possibility of transforming camel milk into products by optimization of the processing parameters [5].

Camel milk is a newcomer to domestic markets and especially to the international milk market. This recent emergence has been accompanied by a diversification of processed products, based on the technologies developed for milk from other dairy species. However, technical innovations had to be adapted to a product with specific behavior and composition. The transformation of camel milk into dairy products such as fermented milk, cheese, powder, or other products was supported, under the pressure of commercial development, by technological innovations made possible by a basic and applied research position. Through time differences, trials were done using different methods or parameters, fortification with ingredients, starter cultures, and milk coagulants to optimize possibility of cheese making from camel milk [9, 10].

The coagulation of bovine milk is faster than camel milk since the casein micelles of the former milk are very smaller in size and coagulated within a short period of time. However, the processing of camel milk into cheese is technically more difficult than the milk of the other domestic dairy animals under the same conditions. This is mainly due to the lower contents of total solids content, αs1- casein, and κ-casein as well as the large casein micelles, which may relate to the poor rennet ability of camel milk [8, 9]. Trials on cheese prepared from camel milk by direct acidification adding starter culture of lactic acid bacteria [11], and soft white cheese from camel milk [12] were evaluated. Recently, researches have been done on fortification of camel milk to improve soft cheese using milky component and sweet potato powder [7]. Moreover, mixing of camel milk with other dairy animal milk have been researched for cheese making. Even the process of developing camel milk into cheese alone is a novel; additionally, scientific innovations have been made to enhance the production of cheese from camel milk, including the use of starter cultures, coagulants, and fortification of camel milk. Therefore, the aim of this chapter is to review technological advances for cheese making from camel milk.

2. Effect of starter cultures on camel milk cheese properties

The diversity of the camel milk microflora is vital for both the antibacterial activity and acidity of the milk, both of which are necessary for the fermentation of milk products and the making of cheese. However, because camel milk proteins have stronger antibacterial characteristics than those in cow milk and, in certain circumstances, because camel milk samples have poorer sanitary conditions, the acidification process appears to be slower for camel milk than for cow milk. In order to increase the shelf life of liquids that have been consumed since the dawn of time, technical advancements involving fermented camel milk have been made [9]. According to studies, in order to produce camel milk cheese, the pH must first be acidified in order to reduce it to about 6.4 before enzymes are added to shorten the clotting time. Some studies

reported that reducing the pH of camel milk to 5.6 at temperatures up to 42^0C further reduces the coagulation time. It is possible to directly acidify milk by adding acid or glucono-6-lactone, although it is more customary to do so indirectly by using cultures that may create lactic acid [13]. It is vital to highlight that starter cultures have a considerable impact on the yield, nutritional content, textures, and sensory quality of camel milk cheeses. Mesophilic, thermophilic, or a combination of these starters were employed to treat camel milk for cheese making or fermentation, and they resulted in an acidification rate that was between 33% and 79% lower at 37°C than for cow milk [5, 9, 12]. Thus, starter cultures are added to cheese milk for acidification affecting several aspects of the cheese manufacturing process and finally cheese composition through the production of lactic acid with the resulting in pH reduction.

2.1 Acidification process of starter culture

The study conducted the effects of five different commercial starter cultures as shown in **Table 1** [starter cultures; i.e., 1 thermophilic (STI-12), 2 blended (RST-743 and XPL- 2), and 2 mesophilic (R-707 and CHN-22) cultures; starter cultures STI-12 and RST-743 were inoculated at 37°C, whereas XPL-2, R-707, and CHN-22 were inoculated at 30°C] on physicochemical properties of soft white cheese (SWC) revealed that camel milk inoculated using STI-12 and RST-743 cultures resulted in faster acidification than XPL-2, R-707, and CHN-22 cultures [12].

It has been noted that starter cultures with slower rates of acidification (**Table 2**) produced cheese curds that were less vigorous. As a result, it was challenging to transfer camel milk curds inoculated with the XPL-2 and CHN-22 cultures from the cheese vat to the mold, and the majority of the fine grains were lost in the whey [12]. The variations in acidification time and pH values may be attributed to differences among starter cultures in rate and intensity of acidification.

2.2 Effect of starter cultures on camel milk cheese composition

It has been stated that a higher cheese yield (13.44 %) was obtained for cheese made using R-707 culture [12]. Khan et al. [11], who reported a 13.2% yield from fresh SWC made from camel milk using a starter culture, discovered the same outcome. SWC manufactured using the CHN-22 culture, on the other hand, was

Culture	Type	Composition
STI-12	Thermophilic	*Streptococcus thermophilus*
RST-743	Blended	*Lactococcus lactis* and *Streptococcus thermophilus*
R-707	Mesophilic	*Lactococcus lactis* without biovar *diacetylactis*
XPL-2	Blended	*Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, *Leuconostoc* species, and *Streptococcus thermophilus*
CHN-22	Mesophilic	*Lactococcus lactis* ssp. *cremoris*, *Leuconostoc pseudomesenteroides*, *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, *Lactococcus lactis* ssp. *lactis*, and *Leuconostoc mesenteroides*

Table 1.
Composition of commercial starter cultures; Blended = mixture of mesophilic and thermophilic cultures.

	Starter culture					
Time (min)	**STI-12**	**RST-743**	**R-707**	**XPL-2**	**CHN-22**	***P*-value**
0	6.45 ± 0.01^{b}	6.47 ± 0.01^{c}	6.52 ± 0.00^{a}	6.51 ± 0.00^{a}	6.52 ± 0.01^{a}	*
10	6.41 ± 0.00^{c}	6.39 ± 0.00^{c}	6.47 ± 0.01^{b}	6.49 ± 0.01^{ab}	6.50 ±0.00^{a}	**
20	6.32 ± 0.01^{d}	6.34 ± 0.01^{c}	6.44 ± 0.01^{b}	6.47 ± 0.01^{ab}	6.49 ±0.00^{a}	**
30	6.24 ± 0.01^{c}	6.24 ± 0.02^{c}	6.40 ± 0.01^{b}	6.43 ± 0.01^{ab}	6.45 ±0.00^{a}	**
40	6.24 ± 0.01^{c}	6.24 ± 0.02^{c}	6.35 ± 0.01^{b}	6.38 ± 0.01^{b}	6.42 ±0.00^{a}	**
50	6.24 ± 0.01^{c}	6.24 ± 0.02^{c}	6.25 ± 0.02^{b}	6.32 ± 0.01^{ab}	6.36 ± 0.01^{a}	**
60	6.24 ± 0.01^{b}	6.24 ± 0.02^{b}	6.24 ± 0.01^{b}	6.27 ± 0.01^{a}	6.27 ± 0.00^{a}	***
70	6.24 ± 0.01	6.24 ± 0.02	6.24 ± 0.01	6.24 ± 0.00	6.24 ± 0.00	NS

$^{a–d}$Means with different superscripts within the same row are significantly ($P < 0.05$) different.[1]pH values in the table are mean ± SD of n = 2. STI-12 and RST-743 were inoculated at 37°C, whereas R-707, XPL-2, and CHN-22 cultures were inoculated at 30°C for manufacturing of soft white cheese from camel milk.
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$ [12].

Table 2.
Acidification rate of camel milk using different commercial starter cultures.

noted to have poor cheese production and greater moisture content. It is possible that this is a result of the extremely fragile cheese curd produced by this slowly acidifying culture, which causes a larger loss of tiny curd particles through the pores of the cheesecloth during whey drainage. The study found that by employing starter cultures, fresh soft white cheese could be produced. The most palatable cheese was discovered to be fresh, soft, and white, made from camel milk and starter culture. Without starting cultures, camel milk cheese had a very high pH and high moisture content, which could encourage the growth of harmful bacteria and result in major health issues [11].

When compared to other cultures, cheese prepared with RST-743 was shown to contain more fat. The strength of gels' rheological and microstructural features, as well as the increased curd loss from the cheese vat, may all be contributing factors to these variances in cheese fat [13]. Additionally, it was discovered that the starter culture employed to make the cheese had a substantial impact on the protein, ash, and total solids contents of SWC [12]. The original milk composition and cheese-making processing conditions may be to blame for the fluctuation of TS seen in the cheese. Depending on how the cheese is processed and how the whey is drained, the majority of the TS constituents, such as protein and fat, are gradually concentrated into the cheese curd. Additionally, throughout the cheese-making process, the type, ash level, and salt addition can all have an impact on the minerals present in the cheese. The acidification process is crucial for the elimination of colloidal minerals from casein micelles, coagulant retention in the curd, syneresis of the gel, coagulum strength, and cheese yield, in addition to its effect on milk clotting.

2.3 Effect of starter culture on cheese texture

It was reported that compared to cheese made with the STI-12, R-707, XPL-2, or CHN-22 cultures, camel milk SWC made with the RST-743 culture had a stronger

resistance to deformation. However, compared to SWC prepared from camel milk using the STI-12, RST-743, and R-707 cultures, camel milk SWC made using the XPL-2 and CHN-22 cultures showed lower deformation values. The enhanced resistance to deformation compared to camel milk soft cheese made with cultures R-707, XPL-2, and CHN-22 may be due to the lower moisture and higher TS levels of camel milk SWC manufactured with cultures STI-12 and RST-743 [12].

This study has notified that the moisture content and protein content of camel milk SWC produced with the XPL-2 culture were comparable to those of cheese produced with the RST-743 and STI-12 cultures. The SWC produced with XPL-2 suffered considerable syneresis during storage, in contrast to other cheeses, which may account for its low resistance to deformation [12]. The features of a cheese's texture have been demonstrated to be influenced by its moisture content in the past [14, 15]. Cheese samples with lower moisture concentrations exhibit resistance to deformation. Acidification, which affects the cheese's pH, and casein matrix hydration, which results in an increase in the curd's stiffness with a pH decrease, both have an impact on the cheese-making process's curd formation (**Figure 1**).

The study on SWC prepared from camel milk using several cultures found that the cheese's textural attributes varied, see Ref. [12]. The report showed that compared to camel milk SWC prepared using other cultures, RST-743 SWC had significantly higher firmness and brittleness characteristics. A decrease in pH caused by the acidification of cheese milk during cheese production has an impact on the moisture content and, as a result, the mineral content of the cheese curd [16]. This has been explained by the degree of casein sub-micelle swelling brought on by the rise in the casein-to-moisture ratio. As a result, even minor changes in moisture content can have a big impact on how fresh cheese feels [17]. In addition to these, Ref. [18] examined how cheese's microstructure and texture are affected by its fat level. They explained that increase in fat content result in smoother and softer cheese, and increase in casein content result in firmer cheese. It was also revealed that higher fat and water contents tend to weaken the protein structure of the cheese, as well as its texture [19].

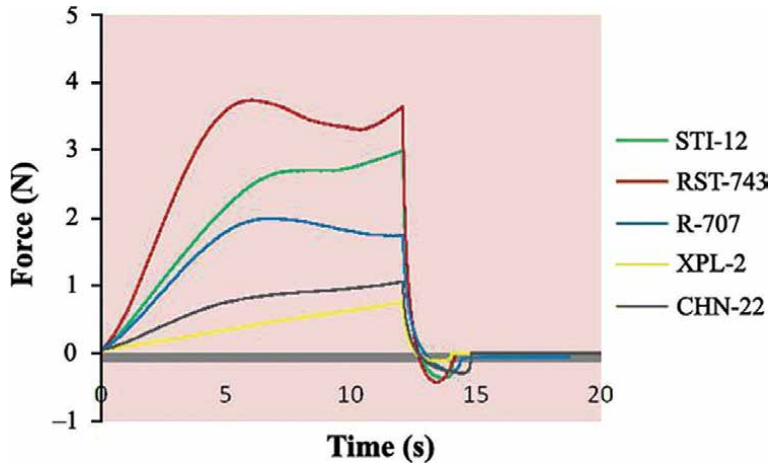

Figure 1.
Compression curves of soft white cheese (SWC), made from camel milk, using different starter cultures: Compression values from a single replication are shown from the texture analyzer software. The STI-12 and RST-743 cultures were inoculated at 37°C, whereas R-707, XPL-2, and CHN-22 cultures were inoculated at 30°C.

2.4 Effect of starter cultures on sensory characteristics of camel milk cheese

Cheese's sensory qualities are regarded as one of the key factors influencing consumer preference. The customer can detect a variety of cheese sensory qualities, which are commonly categorized under look, flavor, and texture. All of these characteristics influence cheeses' acceptability and eating quality. There are many different kinds of cheese around the globe, each having a different sensory character. It reflects the properties of the milk used to make the cheese, the cheese-making environment, and the physical and chemical alterations that take place during ripening [20]. Since cheese can come in a wide variety, numerous trials are required in order to offer consumers a wide range of products. Different cheeses based on the techniques for producing feta and halloumi [8], soft unripened [21], gruyere [22], and mozzarella [18, 23] were examined, but the finished product's texture, flavor, and taste did not match those of the bovine equivalent. In fact, when making cheese, the "proteinic-lipidic matrix" of camels behaves differently from milk to cattle. To comprehend the changes that occur during the various steps of acidification, coagulation, draining, brining, and refining as well as the impact of different starters and thermal treatments, such discrepancies between milk from different dairy species require more fundamental investigations of rheological properties.

With the exception of color preferences, it was reported, using a different starter culture affects test results for consumer preferences. In comparison with camel milk SWC made using STI-12, RST-743, and R-707 cultures, camel milk soft cheese made using XPL-2 and CHN-22 cultures received higher scores for flavor (aroma and taste). The similar outcome was discovered, showing that starter culture-prepared cheese samples were preferred over cheeses made through direct acidification with citric acid in terms of look, flavor, and texture [24]. The starting cultures XPL-2 and CHN-22's natural abilities to produce aroma molecules like diacetyl may be the cause of the flavor variances. When citrate is co-fermented with lactose to make various kinds of cheese, lactic acid bacteria, particularly *Lactococcus lactis biovar diacetylactis*, naturally generate diacetyl [25]. The characteristics of the various commercial starter cultures utilized may be to blame for the variances in consumer preference test ratings in this study, particularly in appearance, scent, taste, and overall acceptability of the cheese samples. Additionally, substances including CO_2, diacetyl, and acetaldehyde may have contributed to the cheese developing unique texture and flavor characteristics [25, 26].

3. Use of coagulant in camel milk cheese

The difficulties in clotting found in this method should be explained by the different casein proportions between cow and camel milk, particularly the lower concentration of κ-casein: 3–4% of casein, compared to 13–15% in cow milk. Furthermore, camel milk's casein micelles cannot coagulate well with the bovine chymosin utilized in the dairy industry, resulting in a weak curd. Therefore, the first difficulty addressed by researchers studying camels and dairy plants processing camel milk was obtaining a hard coagulum [9]. Animal rennins, such as pepsin and chymosin, plant-based proteases, starter cultures, or organic acids, for acidification are all utilized as coagulants while making cheese. After neutralizing the negative charges of the κ-casein, hydrolysis by enzymes or precipitation by acids lead to the instability and precipitation of the casein micelles, which is how milk coagulation with proteolytic enzymes proceeds [27, 28].

3.1 Animal source rennet enzymes

The animal source rennet enzymes are aspartic peptidase, and the most used are the combinations of chymosin A, B, C, and pepsin extracted from the stomach of calves and other ruminants [29]. When utilizing bovine chymosin, numerous investigations have consistently indicated that the coagulation of camel milk proceeds at significantly lower rates and results in a more fragile coagulum than that of bovine milk [30]. It was discovered that camel chymosin, which does not effectively coagulate camel milk, had 70% greater clotting activity for bovine milk than bovine chymosin [31]. Due to a lack of coagulation enzymes for camel milk, different researches have been conducted for substitute proteolytic enzymes that worked similarly. This led to the development of many microbial recombinant chymosin products as an alternative to animal rennet in the cheese-making process. Camel chymosin is a desirable alternative for both small- and large-scale cheese productions due to its high clotting activity [32].

Comparatively to the Phe105-Met106 bond in bovine κ-casein, camel and bovine chymosins preferentially cleave the Phe97-Ile98 bond in camel κ-casein. This results in the hydrophilic C terminal of κ-casein dissociating from it, destabilizing the casein micelles, and causing the milk to aggregate and coagulate as a result. Better substrate binding, made possible by camel chymosin's surface charge, is thought to be the cause of its increased milk-clotting activity. By expressing the camel chymosin gene in a strain of Aspergillus niger, recombinant camel chymosin is created [33]. Studies that recently assessed the usage of camel chymosin to produce soft white cheese from camel milk discovered that chymosin when combined with other ingredients improves cheese yield [34, 35].

The effect of camel chymosin on coagulation and preparation of soft unripened cheese made from camel milk has been studied by employing three levels of camel chymosin concentrations (40, 70, and 100 IMCU/L) and two levels of cooking (cooked and uncooked curd) [35]. The shortest gelation time was reported for camel chymosin concentration of 100 IMCU/L and 70 IMCU/L, whereas the highest maximum gel firmness was observed for camel chymosin level of 40 IMCU/L. In this study, it was found that highest cheese yield was observed for uncooked cheese at 100 IMCU/L coagulant level. In this investigation, it was discovered that raw cheese with a coagulant level of 100 IMCU/L produced the largest amount of cheese. Protein, total solids, ash, and hardness were considerably higher in cooked cheese prepared with 100 IMCU/L. Protein, total solids, ash, and hardness were considerably higher in cooked cheese prepared with 100 IMCU/L. On the other hand, 40 IMCU/L cooked cheese received higher ratings for color, texture, and aesthetics. However, the cooked cheese prepared with 70 IMCU/L received the greatest rating for taste, scent, and acceptability. It was determined that heating camel milk curd and employing medium-level chymosin concentration (70 IMCU/L) could be effective methods for producing soft and unripened cheese from camel milk [35].

Another study examined the protein degradation, rheological characteristics, sensory characteristics, and aroma profile of soft brined cheese made from camel milk over a ripening period of 60 days using two levels of brine (2% or 5% NaCl, w/w) and two levels of coagulant (camel chymosin) [55 and 85 International Milk Clotting Units (IMCU)/L] [6]. The finding showed that when cheese ripened and coagulant levels rose, casein degradation in soft brined camel milk cheese increased. With rising levels of salt and moisture in the cheese during ripening, Young's modulus and stress at fracture rose. However, cheese prepared with 85 (IMCU)/L coagulant had a softer texture and absorbed more salt. The experimental cheeses were described as salty,

sour, and hard using descriptive sensory analysis. The amount of coagulant, NaCl content, and ripening duration all have an impact on the volatile fragrance molecules produced in soft-ripened camel milk cheese [6].

3.2 Plant source rennet enzymes

Recombinant enzymes are unpopular in some countries due to religious matters and diets. Additionally, the decreasing supply and rising cost of calf rennet, along with the rising demand for cheese on a global scale, have prompted researchers to look at alternative clotting enzymes that could take the place of traditional rennet in the cheese-making process. Furthermore, different rennet alternatives have emerged as a result of religious considerations and those connected to the vegetarianism of some consumers [36, 37]. Due to the difficulty in producing cheese of a high enough quantity and quality from camel milk, research on plant-based coagulants has been conducted recently to investigate potential substitutes for rennet enzymes. Several milk-clotting enzymes produced from plants are now used in cheese making. Many attempts have been made to contrast their effects with those brought on by animal rennet in terms of the rheological and sensory characteristics of cheese. However, due to their strong proteolytic activity, which aids in the formation of a bitter flavor, vegetable coagulation enzymes are still only partially suited for cheese making [37].

Plant proteases have been divided into groups based on the hydrolytic process mechanism: aspartate, serine, and cysteine proteases [38]. Studies conducted on plant source coagulants such as *Zingiber officinale* extracts [39], cysteine proteases isolated from *Ficus carica* [40], and aspartic proteases from *Withania coagulans* [41] have been used in camel milk cheese production and the resultant cheeses were found acceptable.

The study carried out on the clotting activity of camel milk using ginger rhizome (*Zingiber officinale*) crude extracts (GCE) reported that GCE would result in strong coagulation of camel milk [39]. According to the finding of this study, the camel milk's clotting activity (MCA) was highest at pH 5.0, 65°C, and 10% crude extract by volume of milk, while pH 4.5, 55°C, and 40% GCE by volume of camel milk produced the lowest value. According to a study on the clotting activity of camel milk using crude extracts of ginger (*Zingiber officinale*), camel milk will strongly coagulate when using GCE [39]. According to the report, the highest camel milk clotting activity (MCA) was noted at pH 5.0, temperature 65°C, and crude extract concentration of 10% by volume of milk, while the lowest value was noted at pH 4.5, temperature 55°C, and GCE concentration of 40% by volume of camel milk.

The experiment conducted by fractions of latex protease from *Ficus carica* on camel milk-clotting properties for use as rennet alternatives revealed that latex fractions, extracted from the fig tree, have a proteolytic activity of 23491.24 IU LG1 (*Ficus carica*), showed proteolytic and milk-clotting activity. *Ficus carica* latex protease, which may coagulate milk after production, can be utilized as an alternative to commercial animal chymosin in the cheese-making process. The amount of cheese produced at various enzyme doses was evaluated, and it was discovered that 1mL of the enzyme extract in 100 mL of camel milk produced 15% of the cheese [40].

The effects of camel chymosin and *Withania coagulans* extract on camel and bovine milk cheeses were performed on cheese's yield and hardness [41]. The result showed that pure Withania extract exhibited the lower coagulating effect resulting in cheeses with low yield, hardness, fat, protein, and total solids compared to camel chymosin. It was concluded that *Withania coagulans* extract protease alone is not

sufficient to produce good quality cheese, especially camel milk cheese but a mixture of *W. coagulans* and camel chymosin produced better quality camel and bovine milk cheeses than chymosin alone [41].

In a comparison of the effects of camel chymosin and *Withania coagulans* extracts on the yield and textural quality of camel milk and bovine milk cheeses, it was discovered that camel milk had a longer gelation time and softer cheese than bovine milk [41]. This study demonstrated that higher moisture entrapment, which lowered cheese hardness, consistently resulted in better yields of unripened camel milk cheese generated by chymosin or the Withania extracts than that of bovine milk cheeses. This study also showed that optimal camel milk as well as bovine milk cheese hardness was obtained by clotting the milk with mixtures of Withania extracts and chymosin suggesting some synergistic interactions, an effect that deserves further investigations.

3.3 Effect of acid coagulants on camel milk cheese making

Acidification is an established process commonly used in combination with heat treatment or rennet addition to prepare fermented milk products (yogurt) and acid-fresh cheeses [42]. The foundation for a huge variety of cultured dairy products is the acid coagulation of milk. By lowering their charge, dissolving part of the insoluble calcium phosphate crosslinks, and altering internal protein bonds, acidification has a direct impact on the stability of casein micelles. At some crucial point, when electrostatic repulsion is diminished and is unable to repel attractive forces, such as hydrophobic contacts and aggregates, eventually gels begin to develop. Acid-induced milk gels become more rigid over time as a result of continuing casein particle-to-casein link formation inside the network.

Cheese prepared from camel milk by direct acidification of milk and by adding starter culture of lactic acid bacteria was evaluated. When starter culture was added to milk to coagulate it, a larger cheese yield was obtained than when cheese was made directly by acidification. Additionally, the starting culture-made cheese contained more total solids, protein, and fat. It was suggested that camel milk can be used to make cheese by coagulating it with starting culture [11]. Another study conducted on the effect of starter cultures on camel milk cheese properties revealed that camel milk treated with nonaromatic cultures such as STI-12, RST-743, and R-707 (see **Table 1**) for SWC manufacture showed a rapid acidification rate and formation of appreciable fine curd properties. As a result, camel milk SWC made using nonaromatic cultures gave better curd firmness, cheese compositional quality, and texture [12].

4. Effect of camel milk fortification on cheese making

Camel milk presents a high nutritional value and plays a key role in providing milk of superior quality (e.g., more vitamin C and minerals (e.g., K^{+}, Cu^{2+}, and Mn^{2+})) and essential and polyunsaturated FAs than CM [43]. It is also believed to possess abilities to treat chronic illnesses [44]. The demand for dairy products made with camel milk has grown over the past ten years, and large-scale commercial production of camel milk from contemporary camel farms is expanding. Many milk and dairy products are now produced and sold in Mauritania and the United Arab Emirates, including pasteurized milk, milk powder, fermented liquid milk, and cheese. Due to its coagulation qualities, camel milk is only occasionally used in processed food items and does

so with certain challenges [44–46]. As discussed above, under natural circumstances, making cheese from camel milk is a challenging process because of two primary aspects: the low concentration of κ-casein and the larger micelle sizes compared to cow milk cheese. Cheeses made from camel milk typically have a weak curd and a fragile diverse structure following coagulation [5, 9, 13]. As a result, various methods for strengthening the cheese structure were investigated by combining camel milk with bovine milk.

Shahein et al. [47] were likely the first group to investigate the feasibility of producing soft pickled cheese by combining camel milk with bovine milk in various ratios. According to the scientists, increasing the amount of bovine milk added to camel milk led to higher total solids, fat, and protein levels, whereas moisture and ash content decreased. When camel and cow milk were combined to make a white Sudanese cheese called Jibna-beida (1 camel milk: 1 cow milk, v/v), Siddig et al. [48] discovered identical results. They also identified variations in the Ca^{2+}, Na^{+}, and K^{+} mineral contents of cheeses. Furthermore, the examination of the ash content in mixtures demonstrated an increase when compared to pure camel milk cheese. However, protein and lactose levels decreased in cheese containing cow milk compared to cheese produced with pure camel milk.

According to the research by Siddig et al. [48], the method used to coagulate milk (either 10% citric acid or 5% starter culture) had an impact on the finished cheese's composition. When faced with pure camel milk cheese, starter cultures coagulation resulted in an increase in fat and total solid contents, whereas citric acid caused a decrease. The mixture (1 camel milk: 1 cow milk cheese) formed following starter cultures coagulation had a relatively higher Ca^{2+} concentration than pure camel milk and the milk mixture (1 camel milk: 1 cow milk cheese) coagulated with citric acid, according to the data regarding mineral content. In contrast, a relative decrease in K+ in pure camel milk was noted when it was compared with cheeses containing both camel milk and cow milk.

Derar and El-Zubeir [45] investigated how soft cheeses would react if camel milk and EM were combined. Prior to manufacturing cheese, they observed that camel milk had a lower compositional level than milk that had been fortified with ewe milk. Additionally, they noticed variations in the whey made from camel milk, ewe milk, and their milk blends, as well as between various milk kinds. They discovered variations in the total solid contents of the cheeses between the samples of 1 camel milk and 3 ewe milk (v/v). However, when compared to normal cheeses, the protein level was the same. Additionally, during storage, variations in the fat content of cheeses manufactured from camel milk, ewe milk, and their mixes were discovered.

When camel milk and cow milk were combined to make cheese, there were discrepancies in the final cheese composition that can be attributed to variations in the initial combination, composition, and coagulation characteristics of camel and cow milk. According to report, casein in particular has a lower total solid content in camel milk coagulum than CM. Additionally, casein micelles from camel milk had greater average sizes (200–500 nm) than those from cow milk (220–300 nm), although fat globule sizes were the opposite. It is known that cheese yield is influenced by the size of the fat globules and the network created within the milk fat globule membrane. The finding of the aforementioned experiments demonstrated that (i) the species origin of the milk and (ii) the manufacturing procedure of the cheese both affect the proximate composition of the finished cheese [43]. Recently, other scholars notified cheese making from cows, buffaloes, goats, sheep, dromedary camels, and donkey's milk. They found that camel milk, when compared to the milk from the other species,

has a similar coagulation time but a less favorable curd-firming process, with lower nutrient recovery and cheese yield. They also explained camel milk requires specific cheese-making conditions and the use of camel chymosin [49].

Technically, making cheese from camel milk is more challenging than making cheese from milk from other domestic dairy animals under the same circumstances. However, success can be obtained by lowering the pH of the milk, adding calcium chloride, and increasing the renneting temperature. In order to standardize camel milk before making cheese, it has been suggested in several studies to use milk that has been ultrafiltrated (UF) retentate. This supplementation has a number of potential advantages, such as increasing the total solids, thereby increasing the yield, facilitating the coagulation process, and improving the organoleptic and rheological properties as well as the nutritional value of the finished cheese. Mehaia [50] has reported on the use of ultrafiltration technology to standardize the total solids of camel milk used to make soft white cheese. It has been demonstrated that milk concentrated by UF produces cheese of high quality (smooth and creamy body), enhances curd stiffness, and has a higher nutritional value due to the end product's higher protein, fat, calcium, and phosphorus contents.

Desouky et al. [51] have done a research on the impact of fortifying processed cheese sauce with camel milk powder (CMP) on its stability and quality attributes. In this study, the effects of replacing the cheese foundation in the production of processed cheese sauces with highly acceptable quality and sensory qualities with camel milk powder at various percentages ranging from 5 to 15% were examined. Depending on the amount of CMP applied and the storage period at 6 0.5°C for 30 days, all treatments had statistically different characteristics. When compared to the other treatments, whether they were used fresh or during storage, the cheese sauce containing 15% CMP was distinguished by greater viscosity values during the studied time of shearing and displayed larger upward shifting of the flow curve. All cheese treatments had higher ratings and were considered above average by the participants, especially after a 10% increase in the percentage of CMP added. It was recommended that the addition of CMP enhanced the quality characteristics of cheese sauces and might be taken into consideration as a new source to replace cheeses used in processed cheese base blends [51].

To solve the issue that arose when preparing soft white cheese, research was done on the effects of substituting 20 or 30 percent of the camel milk with a milky component, having (BMR) secret code, and supplementing with 1, 2, and 3 percent sweet potato powder (SPP) [7]. According to this study, camel milk fortification with BMR and SPP enhanced the physic-chemical qualities of cheese by lowering the pH value, whey syneresis, and pepsin coagulation time when compared to control cheese. With higher levels of additive usage, yield, titratable acidity, and curd tension all rose. After 30 days of storage, these additions additionally enhanced the total solids, fat, protein, ash, and salt contents as well as the cheese ripening indices and total volatile fatty acid values in treated cheeses. Pure camel milk cheese (the control) and the ones that had been processed had quite different microstructures in terms of the form, homogeneity, compact or open body, and texture of the casein micelles network. Due to variations in the chemical composition, manufacturing processes, and added agents utilized, variations in the size and number of voids or vacuoles and fat globules were also documented. This finding was back in the control cheese's body and texture, which weakened, loosened, and opened. Moreover, it was suggested addition of BMR and SPP improved greatly the texture profile of cheeses and their technological aspects [7].

5. Conclusions

The gross composition of camel milk is similar to bovine milk; however, the relative composition, distribution, and the molecular structure of the milk components are different. Consequently, manufacturing cheese from camel milk is difficult. The processing of camel milk into cheese through technological innovations was made possible by applied research. Cheeses prepared from camel milk using starter cultures, utilization of camel chymosin, and fortifications of camel milk with ingredients have considerable improvement in some parameters of cheeses. Starter cultures are added to cheese milk for acidification affecting several aspects of the cheese manufacturing process and finally cheese composition through the production of lactic acid, while reducing pH of curds. Employing medium level of recommended chymosin concentration could be effective method for producing cheeses from camel milk. Furthermore, camel milk mixed with other milk types and fortified with milk powder, milk component, and sweet potato powder have substantial acceptance in some properties of cheeses. The suitability of camel milk processing and its associations with nutritional quality have attracted many studies, but more researches are needed to improve the processing parameters and functional properties of camel milk cheeses.

Conflict of interest

Author has no conflicts of interest to declare.

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Chapter 5

Acid-Induced Gelation of Milk: Formation Mechanism, Gel Characterization, and Influence of Different Techniques

Xiuju Wang and Zhengtao Zhao

Abstract

Understanding the acid coagulation of milk is the core of producing different fermented dairy products. The formation of the gelled structure includes the decreased stability of casein micelles, aggregation, and the gradual development of the bonding between proteins during acidification and cold storage. The coagulation behavior of casein micelles and the physical properties of the final gels can be modified by processing techniques. Exopolysaccharides (EPS) produced by starter culture during fermentation also contribute significantly to the microstructure and texture of acid gels. This chapter discusses the mechanisms of acid-induced gelation of milk based on the modified nanocluster model of casein micelles. The recent findings of heating, high-pressure treatment, ultrasonication, and enzymatic modification on the acid gelation behavior of milk are described. The influence of different ingredients such as polysaccharides (endogenous and exogenous) and phenolic compounds on the physical properties of acid gels are also summarized.

Keywords: casein micelle, acid gelation, yogurt, Exopolysaccharride, whey proteins

1. Introduction

Acid-induced milk gel products are one of the most traditional and widely consumed foods, which have a variety of health claims and curative benefits [1]. The acid coagulation of milk proteins is an irreversible and complicated process accompanied by demineralization, reduction of electrostatic interactions between protein molecules, and aggregation of caseins through hydrophobic interaction and calcium bridging [2–4]. The formation, structure, and physicochemical properties of acid gels have been reviewed recently [5]. Much-related research has been done to understand the gelation mechanism and the role of different components in the final gel texture. The influence mechanism of exopolysaccharides (EPS), produced by starter culture during the acidification process, on gel formation is under debate and has become a hot research topic in recent years.

In general, there are two types of acid-gel dairy products: fresh acid-coagulated cheese products (cream cheese, cottage cheese, quarg, tvorog, and frais) and yogurt products. In fresh acid cheeses, acid and heat are usually combined to coagulate the milk, cream, or whey. The acidification can be achieved by adding acids (such as HCl) or fermentation through culture. Utilization of EPS producing culture can improve the functionality (such as stiffness, serum retention, and creaminess) of fresh acid cheese [6]. In real production, a small amount of rennet, which specifically works on the surface κ-casein layer and thus decreases the steric repulsion between casein micelles, is usually combined with acidification to increase the gel properties, such as decreased coagulation time, increased gel strength, and decreased syneresis [3, 7]. Unlike fresh acid cheese products, yogurt is acidified by the thermophilic starter bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*), which ferment the lactose to lactic acid and produce EPS during acidification [8]. There are two types of yogurt products: set-style yogurt that is fermented (undisturbed) in the retail pot, and stirred-type yogurt produced by breaking the set gel before mixing with fruit or other ingredients and filling it into containers [9]. The effect of different starter cultures and processing conditions on the physicochemical properties of yogurt and fermented milk has been reviewed [10, 11].

The production of yogurts and fresh acid cheeses usually involves the pretreatment of milk like heat treatment and homogenization. Those processing show significant influences on the structure of milk proteins and their gelation behavior [3]. For instance, the heat-induced whey protein denaturation and the attachment of denatured whey protein aggregates on the surface of casein micelles are known to increase the gelation pH (from 4.6 to 5.3) and the gel strength [12]. Homogenization increases the protein hydration and the density of network strands, resulting in increased gel rigidity and resistance to syneresis [13]. In addition, other techniques such as ultrasonication and enzymatic treatment (rennet and transglutaminase (TG)), and the addition of prebiotic or bioactive compounds, can also improve the gel texture and the gelation properties of milk [4, 11, 14]. This chapter summarizes the formation mechanism of acid-induced gels, methods to characterize the physicochemical properties of gels, and updates recent progress in using different strategies to improve the texture and microstructural properties of acid gels.

2. Acidification of milk

2.1 Method of acidification

There are different methods to acidify milk, including the direct addition of acids, such as lactic acid [15], citric acid [16], citric acid, and sulfuric acid [17], and indirect fermentation by bacterial culture or glucono-delta-lactone (GDL) which hydrolyses into gluconic acid in solution [3]. The addition of inorganic acid can decrease the pH of the milk rapidly, while indirect fermentation decreases the pH slowly. The hydrolysis of GDL is temperature-dependent, where the pH reduction is more rapid at a higher temperature. GDL decreases the pH much faster initially than culture but then stabilizes. By contrast, the pH of milk added with starter bacteria continues to decrease slowly with time. The final pH of GDL-induced gel is determined by the amount of GDL added to the milk, while the pH of culture-induced gel can reach a very low pH (e.g., 4.1) until the bacterial activity is inhibited [5]. The differences in

the acidification rate lead to different changes in the physicochemical properties of casein micelles and the aggregation behavior of casein particles, which further influence the rheological and physical properties of the final acid gels.

2.2 Structure of casein micelle and its changes during acidification

The formation of a gel structure of milk is mainly from the changes in milk proteins, particularly for the caseins, which constitute approximately 80% of total milk protein. There are four main caseins: α_{S1}, α_{S2}, β, and κ-caseins with a ratio of 4:1:3.5:1.5 [18]. Caseins cannot form a globular structure due to the presence of a high amount of proline [3]. Alternatively, caseins can combine with calcium and assemble into a particular spherical micellar structure, named casein micelles, which have a diameter range of 50–500 nm (average 150 nm), containing 94% protein and 6% minerals (calcium, phosphate, magnesium, and citrate) [19]. The structure of casein micelle has been developed over the past decades. Among all the proposed models, the nanocluster model proposed by Holt et al. [3] can best characterize all phenomena that occurred to milk during processing. This nanocluster model was improved by Dalgleish and Corredig [18], considering the location of a large amount of water in the micellar interior, as shown in **Figure 1**.

In the nanocluster model, α_S- and β-caseins (blue coils), which are rich in phosphoserine in their structure, are considered to interact with and surround the colloidal calcium phosphate nanoclusters (black spheres), forming the internal structure of casein micelles through hydrophobic interaction and hydrogen bond [4]. κ-caseins lack phosphate centers and are present on the surface providing strong steric repulsion to maintain their colloidal stability [20, 21]. Casein micelles have an isoelectric point of 4.6.

Acidification influences both the surface and internal structure of casein micelles. The colloidal calcium phosphate gradually dissociates from casein micelles [22], the surface charge of casein micelles decreases, and caseins are released into the serum phase [23]. The dissociation of caseins is temperature-dependent. At low temperatures (4°C), around 40% of caseins dissociated from micelles at pH 5.5, while no virtual dissociation of caseins occurred at 30°C [24, 25]. On the contrary, mineral solubilization is independent of acidification temperature. The extent of mineral solubilization

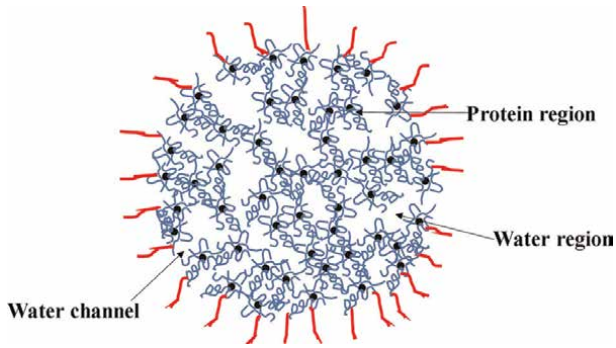

Figure 1.
The structure of casein micelle: Black spheres represent the calcium phosphate nanoclusters that solubilize during the acidification process. Blue coils represent αS and β-caseins; red lines on the outermost part of the surface represent κ-caseins. (Source: Wang and Zhao [4]).

increases markedly below pH 5.6 and is almost complete at around pH 5.0 [3, 26]. No changes in the hydrodynamic diameter of casein micelles occur during the acidification to pH 5.0 [22], although the charge of κ-casein decreases with acidification, resulting in the collapse of the κ-casein layer and the reduction in the stability of the micelles, as the intra- and inter-chain interactions are insufficient to keep the protein fraction extended in solution [27]. Further decrease to pH around 4.8 results in the aggregation of caseins and the formation of gel structure in unheated milk [2].

3. Characterization of acid milk gels

3.1 Mechanism of gel formation

Acid milk gels are typical particle gels where aggregated protein particle forms continuous network structures throughout the entire volume. The mechanism of the gel structure formation is still controversial. Different theoretical models have been proposed to characterize the gel-forming process, including the adhesive hard-sphere model, fractal model, and percolation model [5]. The adhesive hard-sphere model focuses on the surface κ-casein layer of casein micelles. In this model, the highly charged glycomacropeptide (GMP) part of the κ-casein sterically stabilizes the casein micelles [27]. The strong steric repulsion provided by GMP prevented the aggregation of casein micelles against pH and ionic modifications [22, 23, 28]. The rheological behavior of casein micelles fits well with the hard-sphere model [22]. The radius of casein micelles also remains stable at different concentrations. But once the pH approaches the pKa of the charged GMP brush, the surface of casein micelles collapses and aggregates. This model well defined the aggregation behavior of casein micelles. However, it fails to explain the development of gel structure and its mechanical properties [29].

The fractal aggregation model describes that the spherical particles of casein micelles can encounter each other through Brownian motion and form aggregates. The aggregates can then also aggregate with each other. Once no further changes happen among the particles in the aggregate and they are incorporated, this cluster-cluster aggregation process directs to the aggregates that obey the following scaling Eq. (1):

$$N_p/N_0 = (R/\alpha_{eff})D - 3 \quad (1)$$

where N_p is the number of particles in an aggregate of radius R, N_0 is the total number of initial particles that could form the floc, D is the fractal dimensionality constant ($D < 3$), and α_{eff} is the radius of the effective building blocks forming the fractal clusters. One limitation of this model is that it assumes all the aggregates have the same size, which is not the case in reality [5]. It also fails to explain the aggregates' rearrangement (before, during, and after gelation), which can influence the D value.

The percolation model combines the concept of fractal aggregate formation and the hard-sphere model. It assumes that percolation clusters form random bonds between adjacent micelles in a lattice, which are random and their sizes increase with the increasing number of bonds. A larger cluster appears above a certain threshold which extends throughout the lattice. Analogies between percolation and gelation can be drawn with particles establishing an increasing number of links as they aggregate until, at a certain threshold, a cluster is created and spans the container/system [2].

Only a small portion of bonds are joined, and not all individual fractions are incorporated into the system-spanning cluster. This model successfully explained the continuous increase of elastic modulus (G′) after gelation [29]. However, it is hard to use this theory to model the mechanical properties of acid gels.

In addition to modeling the acid gelation process, the physical–chemical changes of milk during acidification have been well studied. When the pH of milk is decreased from 6.7 to 6.0, there is a decrease in the net negative charge and reduced electrostatic repulsions. But the solubilization of CCP is minimal, and the micelle integrity is preserved [22]. Decrease the pH from 6.0 to 5.0 leads to further neutralization of the surface charge and shrinkage/collapse of the hairy layer. CCP is fully dissolved, while the internal structure is more homogeneous [3]. At pH lower than 5.0, the destabilized caseins come closer to each other and form the gel structure [4]. Subsequent cooling/refrigeration causes the gels to swell, increasing the contact area of particles and the gel firmness/strength. As the hydrophobic interaction are lower at low temperatures, the increased gel firmness during cooling storage indicates that other forces, such as electrostatic and van der Waals' interaction, also contribute to the gel integrity [1].

3.2 Rheological properties

Acid gels are viscoelastic materials. In the dairy industry, the rheometer is the most widely used technique to characterize acid gels. There are two main test methods: small-amplitude oscillatory rheology and large-amplitude oscillatory shear. Large deformation studies can provide information on properties related to the consistency during shearing (a step in the production of stirred-style yogurt) and consumption.

Small-amplitude oscillatory rheology (dynamic testing) is a nondestructive method, involving an applied oscillatory strain or stress that provides very useful information about the gelation process [2, 30]. The main parameters determined during this test include the elastic or storage modulus G′, which indicates the energy stored per oscillation cycle, the viscous or loss modulus G″, which indicates the energy dissipated per cycle, and the loss tangent (tan δ), which is the ratio between the viscous modulus and elastic modulus. The definition of these parameters is shown in the following equations:

$$G' = (\tau 0/\gamma 0)\ \cos\ \delta; G'' = (\tau 0/\gamma 0)\ \sin\ \delta; \mathrm{Tan}\,\delta = G''/G' \qquad (2)$$

where τ0 is the shear stress, γ0 is the shear strain, and δ is the phase angle.

In reality, the majority of preceding rheological measurements of milk gelation were performed under low strains (<1%) and oscillating strain rates (<0.1 Hz) to avoid gel destruction [7]. The gelation point is where the elastic and viscous modulus cross over (Tan δ = 1) [28]. The rheological properties of acid gel made from unheated milk at 30°C have been well studied, as summarized in previous reviews [1, 2]. After passing the gelation point, the G′ increased rapidly and plateaued during the aging of the gel. Loss tangent (Tan δ) decreased to <0.4 quickly after gelation and then to around 0.25 during aging. Heat treatment significantly increased the G′, and the gelation pH increased from 4.8 to 5.2. [3]. Renan et al. [31] compared the gelation profiles of acid gels produced with culture fermentation and GDL. As shown in **Figure 2**, the elastic modulus of acid gels fermented by culture increased much faster than the GDL. The resulted gels were firmer with a more heterogeneous structure. Both methods produced gels with a similar final loss tangent value of about 0.22. Moreover, acidification methods also influence the rheological properties of acid-

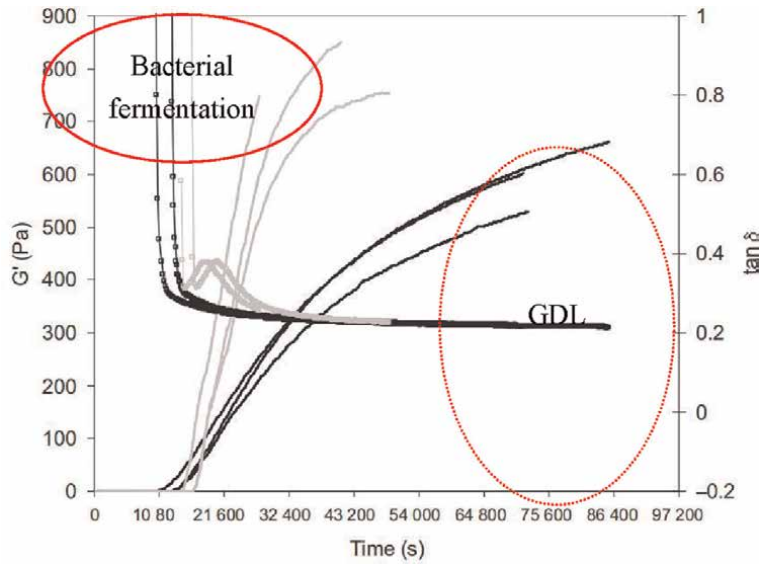

Figure 2.
Rheological properties of heat-treated milk during acidification with glucono-delta-lactone at 20°C (black line) or a bacterial culture at 38°C (gray line) in coaxial cylinders versus time. Zero time for bacterial acidification was taken at the time when the temperature reached 38°C. three repetitions for each procedure. (Source: Renan et al. [31]).

induced gels. The presence of EPS, which are produced by starter culture during fermentation, enhances the protein distribution and viscoelastic properties of acid gels [8, 32].

3.3 Microstructure of acid milk gels

The microstructure of the gels is directly correlated with their texture, appearance, and organoleptic properties. In the dairy industry, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CSLM) are the most commonly used techniques to observe the microstructure of acid gels. Accordingly, the acid gels consist of a coarse particulate network of casein particles linked together in clusters, chains, and strands [5]. Gastaldi et al. [33] monitored the pH-induced changes of casein micelles during the acidification process. As shown in **Figure 3a**, casein micelles started to aggregate forming clusters when the pH was decreased from 6.7 to 5.8. The initial shape was still discernible. At pH 5.5 to 5.3, most casein particles lost their original structure and were deformed, stretched, and extensively coalesced, forming a pseudo-network with an open structure (**Figure 3b** and **c**). After decreasing the pH to between 4.8 and 4.7, the protein network appeared denser, and the pore size between casein aggregate particles became smaller. At this stage, the formation of acidified milk gels is completed, where the casein particles are aggregated into a three-dimensional network (**Figure 3e** and **f**). Much more related research using SEM to investigate the changes in the microstructure of acid gel has been done recently [34, 35]. One shortcoming of SEM is that many preparation steps are required, including dehydration, fixation, embedding, sectioning, and staining, which may disrupt the native structure of gel products and result in the formation of artifacts.

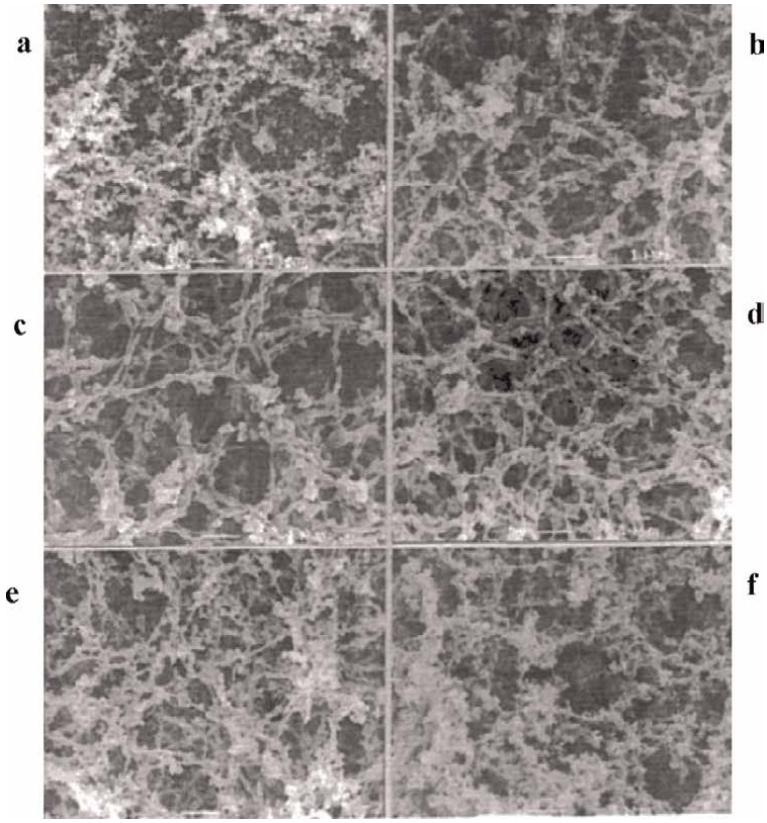

Figure 3.
SEM micrographs of acidified milk critical-point dried samples at different pH: pH 5.8 (a), pH 5.5 (b), pH 5.3 (c), pH 5.0 (d), pH 4.8 (e), pH 4.7 (f). The scale bar represents 1 μm. (Source: Gastaldi et al. [33]).

Compared to SEM, CLSM is a relatively new technique. It allows observing the overall microstructure of milk gels with minimal preparation steps due to its unique optical sectioning abilities and high spatial resolution [2]. **Figure 4** shows the CLSM images of acid gels produced by GDL or yogurt culture, GDL-produced gel exhibited a denser and more homogeneous structure compared to the gel fermented by culture [31]. Another advantage of CLSM is that it can identify different components in the gel by using specific fluorescence labels. The protein network has been stained with Congo red (0.01% in water) and fluorescein isothiocyanate (FITC, 0.025% in dimethyl sulfoxide) [36]. In another study, the microstructure of low-fat yogurt was observed with CLSM using fast green FCF fluorescent stain to label protein and lectin wheat germ agglutinin Alexafluor 55 conjugate to label EPS produced by starter culture [37]. In reality, the combination of SEM and CLSM can provide more thorough information about the overall and detailed microstructure.

3.4 Syneresis/whey separation

Syneresis is defined as the spontaneous contraction of a gel, leading to the expulsion of liquid from the pores. In acid milk gel, syneresis is also called whey separation, which refers to the occurrence of whey on the surface of a milk gel. Syneresis relates to the instability of the protein network, which causes a loss of the capacity to entrap

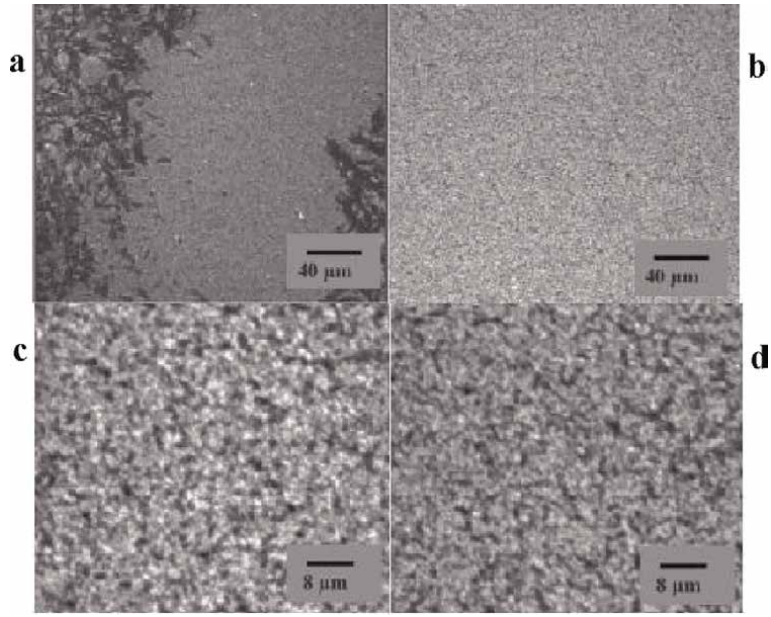

Figure 4.
A comparison of the microstructure of acid milk gels produced by yogurt culture (a and c) and GDL (b and d). (Source: Renan et al. [31]).

the whey in the network [38]. Rapid fermentation, proteolysis, and high incubation temperatures are the main factors that lead to the whey separation of acid gels [39]. Proteolysis during fermentation causes the reduction of interconnections within the protein network and the rearrangement of the intra-network. On the other hand, the acid curds are more prone to syneresis at increased temperatures due to higher rearrangements causing contractions in the gel network, which creates pressure for the whey to move [40].

Whey separation can be simply quantified by determining the quantity of whey expelled from yogurt after centrifugation or drainage through a screen [38, 41, 42]. Both methods are not related to the spontaneous separation of whey from set-style yogurt. The centrifugation method determines the water-holding capacity of the gels under different forces. The drainage of whey from a disrupted gel distributed over a screen measures the whey separation over a large surface area, which is more relevant to the products such as cottage or casein than to set yogurt [2]. Lucey et al. [43] proposed a new method that produces the gels directly in a container and determines the quantity of expelled whey on the surface. During the manufacture of acid-induced gel products, heat treatment is used to sterilize the milk, and gelation is done at a high temperature, which increases whey separation in acid gels. Dairy scientists have used different ways to increase the gel properties, such as the use of high-EPS yield culture [44], enzymatic treatment to strengthen the protein network [3], and increase the protein concentration or adding different exogenous polysaccharides [42, 44, 45].

3.5 Texture properties

The textural properties of acid milk gels can be measured by different instrumental methods, such as dynamic-amplitude oscillation, large-amplitude oscillatory, texture

analyzer (penetration), and rotational viscometry [2]. The main challenge for the acid milk gels is the "lumpiness" or "granular" body texture, which is against consumers' expectation of a smooth, fine-bodied product. This textural defect is due to forming large protein aggregates that often range in size from 1 to 5 mm [46]. Many factors contribute to the formation of dense protein clusters, including incubation at a high temperature, rennet, and adding excessive starters [2, 47]. A recent publication indicated that the vibration during fermentation resulted in the formation of bigger aggregates, which caused the graininess of set-style yogurt [48]. In addition, other factors such as a very high amount of total solid and adding excess whey protein concentrate to the milk also increased the "lumpy" or granular defect [2, 49]. Stabilizers, both exogenous and endogenous, have been proved to provide smooth body texture to the acid gel products [44, 50]. Optimizing parameters such as heat treatment, total solid level, amount of additives, amount/variety of starter added, and incubation temperature are necessary to produce acid milk gels with desired texture.

4. Strategies to improve the acid-induced milk gels

4.1 Heating

Heat treatment is a standard procedure before further processing of the dairy product. In the production of fermented dairy products such as yogurt, the heat treatment is usually performed at high temperatures (such as 90°C, 5 min). Heating can destroy the raw milk flora and decrease the dissolved oxygen level which can prevent the growth of the starter cultures. More importantly, heat treatment can denature the whey proteins in milk, increasing the firmness and texture of acid milk gels [3, 51].

The structure of casein micelle is relatively heat-stable as they lack tertiary structure. On the contrary, the main globular whey proteins such as α-lactoalbumin (α-la) and β-lactoglobulin (β-LG) undergo irreversible denaturation at temperatures higher than 70°C [52]. The denatured whey proteins can aggregate with themselves or free caseins (mainly dissociated κ-casein) through disulfide bonds and hydrophobic interaction [53]. Denatured β-LG can also attach to the surface of casein micelles by interacting with the surface κ-casein layer. The coating of denatured whey proteins is pH-dependent. At neutral pH 6.7, heating resulted in around 30% of denatured whey proteins associated with the surface of casein micelles, and this number increased to 75% when heating at pH 6.3 [54]. Whey protein denaturation significantly altered the acid gelation behavior of milk, particularly at a higher denaturation degree (>40%). The gelation pH increased from 4.9 to values between 5.1 and 5.3, and the elastic increased drastically [55].

The distribution of denatured whey proteins between serum and the surface of casein micelles has a significant influence on the gelation process. At lower pH 6.3, most denatured whey proteins are present on the surface of casein micelles, and they gel first entrapping the casein micelles and triggering gelation at pH 5.3. In contrast, at pH 7.0, most denatured whey proteins are present in the serum phase as soluble aggregates, which contribute to the formation of stiff gels by associating with casein micelles during acidification [51]. Both heating at lower pH (<6.7) and higher pH (>6.7) resulted in slightly weaker acid gels than at neutral pH (6.7) [56–58].

There are still some divergent opinions regarding the role of soluble whey protein complexes and micelle bind complexes in the acid milk gels. Some researchers think that the small number of denatured whey proteins associated with casein micelles

during heating is responsible for the increased gel properties [43, 58]. In contrast, other researchers think that the soluble denatured whey proteins play a more crucial role than the denatured whey proteins associated with casein micelle [56, 59]. In recent research, glutaraldehyde was added to milk to reduce micellar kappa-casein dissociation, which decreased the formation of soluble protein complexes. This reduction in soluble complexes resulted in the forming of weaker gels [60]. In addition to the gelation process, high heat treatment increases the brittleness of acid gels prepared by microbial fermentation, while it decreases the brittleness of gels prepared via GDL [51, 61]. The differences are due to different acidification rates between these two methods.

4.2 High-pressure treatment

High-pressure treatment can reduce milk fat globule size, disintegrate/re-associate casein micelles, and denature whey proteins [4]. The size of casein micelles is stable under pressures lower than 200 MPa [62]. Increasing pressure to 250 MPa increased micelle size by 25%, whereas a further increase of pressure (300–800 MPa) decreased casein micelle radius by about 50% [63]. The soluble caseins and soluble calcium increased after high-pressure treatment [64].

High-pressure treatments significantly improve the acid coagulation behavior of milk. The rigidity, strength, and resistance to syneresis of acid gels were improved [13, 65, 66], which are a result of the increases in protein hydration and density of network strands, resulting from the incorporation of denatured whey proteins in the acid gel [13, 67]. The elastic modulus and yield stress of acid milk gels increased with decreasing fat globule size as the adsorption of proteins onto the newly created surface of fat globules after high-pressure treatment, resulting in the formation of a more porous protein network with thick strands [66]. Homogenization performed prior to heating resulted in higher adsorption of proteins to the fat globules than homogenization after heating, which further led to the formation of acid gels with higher elastic modulus and yield stress [68].

4.3 Ultrasonication

Ultrasound refers to sound waves with a frequency higher than 20 kHz, which modifies the structure functionality of protein molecules through the cavitation effect, based on the implosion of bubbles that produce shock waves surrounding the probe and jets of high velocity. It is a relatively new technique used in dairy processing to improve the acid gelation properties of milk. The influence of ultrasound on the lactic fermentation, growth and cell viability of lactic acid bacteria, lactose metabolism, texture, and sensory attributes of fermented dairy products has been reviewed recently [69, 70].

Pretreatment of caseins with ultrasound postponed the gelation point to lower pH, decreased the syneresis, and enhanced the elasticity of acid gels, which have a more interconnected structure [71, 72]. The increased acid gelation properties are related to the increased surface hydrophobicity [73]. Whey protein denaturation and increased association of casein with the milk fat globule membrane during ultrasonication also contribute to the increased gel strength [74]. In contrast, ultrasound treatment during the lag phase of lactic acid bacteria reduced the fermentation time, promoted the speed of lactose hydrolysis, and increased the storage modulus of the final gels [75]. For the yogurt products with high protein concentration, ultrasonication during

fermentation (for instance from pH 5.8 to 5.1) decreased the firmness and provided a smooth texture for yogurt products, which solved the difficulty of further processing issues [76]. The influence of ultrasonication on acid gelation properties is temperature-dependent. Ultrasonication at temperatures lower than 60°C produced acid gels with higher firmness than those produced at temperatures higher than 60°C [77].

4.4 Enzymatic treatment

Rennet is a complex of enzymes with the active enzyme chymosin, which works on the Phe105-Met106 bond of surface κ-caseins. It cuts κ-casein into hydrophobic para-κ-casein, which remains on the surface of casein micelle, and hydrophilic GMP, which is cleaved from casein micelle [3]. Partial hydrolysis of κ-caseins reduces the negative charge of casein micelles, promoting aggregation during acidification [78]. The gelation pH and elastic modulus increased with increasing hydrolysis degree [79, 80]. Inactivation of enzymes at a lower temperature (60°C) resulted in firmer acid gels than at a higher temperature (85°C). However, gels produced after partial κ-casein hydrolysis exhibited higher syneresis [80].

Transglutaminase (TG) is another enzyme used to improve the acid gelation behavior of milk. It crosslinks peptides and proteins through an acyl transfer mechanism between glutamine and lysine residues [81]. At neutral pH, TG predominately works on the κ-casein surface layer of casein micelles, which prevents the dissociation of κ-caseins, and increases the colloidal stability of casein micelles [82]. TG treatment positively influences the physical properties and microstructure of the yogurt gels [83]. It prevented the release of the caseins into the serum phase which further decreased the formation of soluble complexes during heating [84, 85]. The rearrangements within the protein network were also limited by TG during the gelation, which produced acid gels with a more homogeneous network consisting of smaller aggregates and better WHC [83].

4.5 Endogenous and exogenous polysaccharides

Exopolysaccharide (EPS)-producing starter cultures are preferred in the manufacture of fermented products. The production of EPS in situ has been shown to improve the texture and rheological properties of the yogurt [46, 86]. EPS can improve the structure of milk gels by attaching to the protein network and the bacteria and forming a web-like structure [87]. The influence of EPS on the physical properties of acid gels are affected by EPS location, its structure (molecular mass, side chains, stiffness, and charge), and the interactions of EPS with other components (proteins and minerals) [88]. Depending on the location, EPS can be divided into ropy-EPS (free EPS in the medium) and capsular EPS (located on the surface of the bacterial cells) [89]. Ropy-EPS can produce a stringy and slimy appearance and affect the rheological properties and microstructure of milk gels. Most EPS-producing strains can increase the firmness and WHC of acid gels compared with non-EPS strains. However, slightly weaker gels produced from EPS-producing strains have been reported [90]. Charged EPS can interact with milk proteins through electrostatic attractions during fermentation which increases the gel texture, whereas uncharged EPS can induce depletion flocculation in casein systems [91].

A wide variety of endogenous polysaccharides have been used as additives in the production of acid milk gels in recent years. They can combine with water in the gel

and interact with milk proteins during fermentation and storage, resulting in the formation of gels with improved texture and sensory properties. When selecting polysaccharide additives, many factors need to be considered, such as structure, charge properties, and adding amount. Pang et al. [92] found that anionic polysaccharides enhanced the acid gelation properties of yogurt. In contrast, neutral polysaccharides inhibited milk gelation from the beginning [92]. Apple pomace (pectin and soluble fibers) improved the firmness and cohesiveness of set yogurt. At the highest adding amount of 1%, gelation happened at a much higher point (pH 5.9) [93]. Many other polysaccharides, such as okra polysaccharide, dietary fiber, salecan, and oat β-glucan, have been used to improve the structure and rheological properties of acid milk gels recently [44, 94–96].

4.6 Functional bioactive compounds

Phenolic compounds can combine with milk proteins through hydrophobic interactions [4, 97]. Polyphenol addition does not influence the fermentation process or the lactic acid bacteria viability during the storage of yogurt [98]. Because of the capacity of polyphenols to interact with milk proteins, the acid gels incorporated with phenolic compounds had a higher firmness value and a stronger water-binding capacity within the gel matrix [99–101]. The influence of phenolic compounds on gelation and the physical properties of acid milk gels depends on the source and addition timing. Phenolic compounds extracted from different herbs (thistle, hawthorn, marjoram, and sage) prevented the syneresis and improved the water-holding capacity of yogurt [102]. Polyphenols extracted from the honeysuckle berries did not influence the rheological properties of yogurt but decreased the viscosity during storage [103]. In contrast, EI-Said et al. noticed that adding pomegranate peel extracts to milk decreased the viscosity of stirred yogurt [104]. The addition of gallic acid before heat treatment resulted in a longer gelation time and decreased final storage modulus (G') and fracture stress. On the other hand, no influence was found when adding gallic acid after heat treatment [105].

5. Conclusion

Acid-induced coagulation of milk is a complicated process. It involves the demineralization of casein micelles, dissociation of caseins, the collapse of the surface κ-casein layer, casein aggregation, and the development of the protein network. The acid-induced coagulation behavior of milk can be influenced by different processing techniques such as heating, high-pressure treatment, ultrasonication, and enzymatic treatment. Utilizing appropriate processing parameters and polysaccharide additives can improve the rheological properties and microstructure of acid gels. Although much work aims to improve the texture and physical properties of acid gel products has been performed, many “unknowns” need to be resolved. The role of the distribution of heat-induced whey protein/κ-casein complexes and their influence on the acid coagulation behavior of milk needs to be clarified. The effect of released caseins during acidification on the gel structure development during storage requires further investigation. The secretion of EPS by starter culture, the interaction between EPS with milk proteins, and its improvement mechanism of the final gel structure is undoubtedly one of the primary scientific challenges to be solved.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Milk Fat Globular Membrane: Composition, Structure, Isolation, Technological Significance and Health Benefits

Dharani Muthusamy

Abstract

Milk Fat Globular Membrane comprises less than 1% of the total milk lipids, but the technological significance and health benefits of MFGM are immeasurable. MFGM as a bioactive compound present in milk, constitutes the majority of indigenous enzymes and plays vital role in stability of fat globules while processing. Due to its benefits, MFGM and its fractions became a hot topic in functional food especially in the infant food formula category. MFGM contributes several health benefits such as anticancer, anticholesterolemic and improves physical and dermal health. Food application of the MFGM can be highlighted as an emulsifier and stabilizer with excellent water holding capacity in dairy products. Beyond its technological significance, MFGM is also used in food emulsion and lactic acid bacteria encapsulation techniques. MFGM is considered to be a nutraceutical ingredient which gives more opportunity for exploration of milk lipids.

Keywords: milk fat globular membrane, phospholipids, cream, buttermilk, membrane separation, infant foods

1. Introduction

Milk is a wholesome food that contains several health providing nutrients ranging from carbohydrates, fat, protein, minerals and up to some bioactive substance. In recent years, active research has been done on the underlying or unrevealed part of milk components such as oligosaccharides, metal binding proteins, fatty acids, lactoferrin and milk fat globular membrane (MFGM). Several studies were carried out in the finding the bioactive components. Due to its favorable outcomes, milk bioactive substances were being commercialized and consumed in day to day life for short term benefits to treating ailments. Milk is an emulsified solution that comprises fat as dispersed phase, protein as colloidal particle, and minerals as the true solution. The average size of fat globule present in the milk ranges from 0.1–15 μm and 95% of micro fat globules are concealed into 8-10 nm thick globular membrane which is the Milk Fat Globular Membrane [1]. In the 17th century, Van Leeuwenhoek discovered

the fat globules with the aid of a light microscope through a thin glass capillary tube. Later in the 19th century, Ascherson revealed that fat globules had a membrane that was the structure comprising condensed form of protein and accumulation of small fat globules at the surface. Followed by several studies were carried out in finding the structure and composition of MFGM along with their properties in stabilizing milk fat.

In this chapter, structure, composition, technological significance and health benefits of MFGM will be discussed.

2. Structure of MFGM

Understanding the origin and mechanism of membrane formation is crucial for the better knowledge in case of MFGM, since its complex structure has effect on stabilizing and sensory application on dairy products. The origin of MFGM was found during lipid secretion along with formation of fat globules in the mammary gland. MFGM has three different origins, primarily from apical plasma membrane, endoplasmic reticulum (ER) and certain post- golgi apparatus of mammary gland cells. Fat globules of diameter < 0.5 μm accumulates and reaches ER at centre and gets trapped between the outer and inner lipid bilayer of ER, which is later expelled into cytosol as cytoplasmic covered lipid droplets. The monolayer protein present in ER is responsible for the growth and fusion of lipid droplets before reaching the apical plasma membrane.

In budding stages of lipid droplets, there occurs a static distance of 10–20 nm between lipid globules and apical plasma membrane, that gap gets covered with electron clouded inner face of apical plasma membrane and phospholipids (PLs) that forms as primary lipid bilayer of MFGM (pathway A in **Figure 1**). Few micro lipids resist the

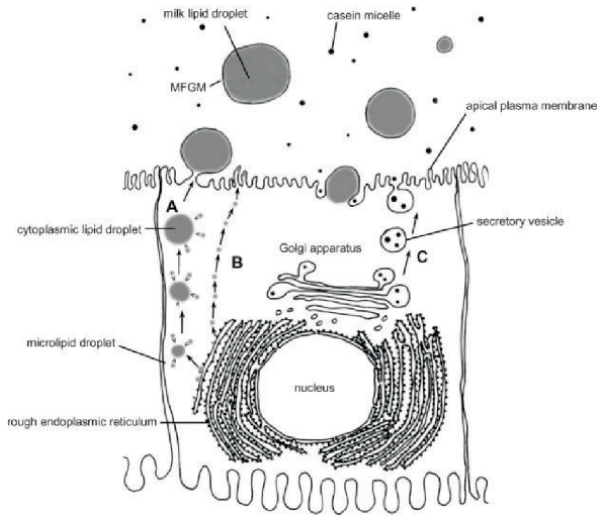

Figure 1.
A = cytoplasmic lipid droplet pathway; B = microlipid droplet pathway; C = secretory pathway for milk plasma constituents.

change in the size that constitutes microlipid pathway (pathway B in **Figure 1**). Milk plasma components like casein, whey protein, lactose and other substances condense in the golgi apparatus and get compacted within the secretory vesicles membrane through a process called exocytosis (pathway C in **Figure 1**). Similar patterns of micro lipid fusion have been reported in cell free systems with the aid of gangliosides in the presence of calcium [2, 3].

Understanding the fusion mechanism in the growth of micro lipids and sealing into MFGM would be useful in separation of milk, based on the size of fat globules that help to avoid the use of centrifugation. Manipulation of expression level of fat from the mammary cell by means of genetic engineering would be able to produce low fat milk naturally from udder. Since MFGM anchors several lipolytic enzymes this manipulation will be useful in the elongation of storage stability of the fat rich dairy products. Historical review of MFGM with their physico-chemical properties was reviewed briefly by Leroy S. Palmer, refer [4].

2.1 Composition of MFGM

2.1.1 Protein fraction of MFGM

On isolation and characterization of MFGM from fresh cream of jersey cow, it was concluded that the membrane is mostly protein in nature [5]. The composition of MFGM itself can be divided into lipid rich MFGM and protein rich MFGM [6] since, protein and lipid constitute 90% of MFGM. Protein content of MFGM ranges from 26 to 60% as its concentration is greatly affected by the method of isolation. The highly sialylated part of MFGM is mucins, it can be further classified into MUC 1 and MUC 15 with molecular weight of 160–200. MFGM also consists of Butyrophilin (BTN), Adipophilin (ADPH), lactadherin, Proteose peptone 3, some fatty acid binding protein [7] and RNA [3]. Molecular weight of native proteins present in MFGM was tabulated in **Table 1**.

Protein name	Molecular weight
Breast cancer protein (BRCA1 and BRCA2)	210
Mucin I (MUC1)	160–200
Xanthine oxidase (XO)	146–155
PAS III	94–100
CD36	76–78
Butyrophilin (BTN)	52
Adipophilin (ADPH)	47–59
PAS 6/7 (lactadherin)	47–59
Proteose peptone 3	18–34
Fatty Acid Binding Protein.	13–15

Table 1.
Protein fractions of naturally extracted MFGM isolates, from [7].

2.1.2 Lipid fraction of MFGM

MFGM contains 35% of high melting point unsaturated fatty acids constituting 3% of total triglyceride composition [5], but these fatty acids was not originated from MFGM, rather it comes from fat globules attached to the membrane during processing operation [6]. The major lipid present in the MFGM was found to be PLs (26–31% of total lipids) in the form of protein –phospholipid complex [8] that exhibits emulsion stabilizing property along with other phosphatides proteins lecithin, cephalin and sphingomyelin. Among these phosphatides lecithin was notably prominent in creaming stability and emulsion of cow milk [9]. Triacylglycerols constitutes 62% of total lipids and other minor constitutes are mono, di-acylglycerols (responsible for the lipolysis in dairy products), sterols and their esters, non-esterified fatty acids and hydrocarbons [5, 10]. Next to protein and lipids, enzymes are highly concentrated in MFGM that are significantly crucible for lipolytic activity in dairy products. About 28 enzymes have been found in MFGM, but their physiological activities are undiscovered. The major enzyme is xanthine oxidase (XDH), responsible for the development of fat globules in plasma membrane and purine metabolism. The origins of these enzymes are predominantly from plasma membrane [11, 12] and cytosol. Composition and their proportions are detailed by Keenan and Mather [3].

3. Industrial applications of MFGM

3.1 Isolation and production of MFGM

Several isolation studies on MFGM have been done due to the presence of nutraceutical proteins and their nourishing effect towards the infant. In spite of its uses, these isolates are produced on commercial scale and it have been supplemented in various nutritive formula and functional foods. Since casein and MFGM are same in size and share almost similar isoelectric points, it is a tedious isolation process. The separation techniques used for MFGM, involve many physical processing methods with repeated washings using chemicals to remove milk proteins, lactose and salts which is suitable for lab purposes, but not optimal for commercial production [13]. The molecular size of milk protein casein and MFGM are same, this makes it even more difficult to isolate during membrane separation [14].

The natural extraction of MFGM can be obtained from the by-products such as buttermilk, cream serum and cheese whey while processing butter, cream and cheese respectively (**Figure 2**). These by-products are the raw materials for the production of MFGM. Processing conditions like cooling, heating and physical separation techniques such as churning and phase inversion affects the migration and association of MFGM fragments in dairy products. Chilling or cooling shifts MFGM towards whey and heating causes complex association between whey protein and exterior layer of MFGM. Same pattern takes place in cream serum that MFGM gets concentrated at water-in-oil emulsion (AMF) during cooling. In cheese preparation, the disruption of MFGM during processing of cheese curd results in migration towards the milk serum portion. Condensation of defatted fluid whey into whey protein concentrates and isolates (WPC and WPI) is used for MFGM extraction. Among the by-products, cream serum and buttermilk provides a great source of MFGM. Dehydration and membrane separation of the above described final ingredients are used to produce MFGM enriched powder [7, 15].

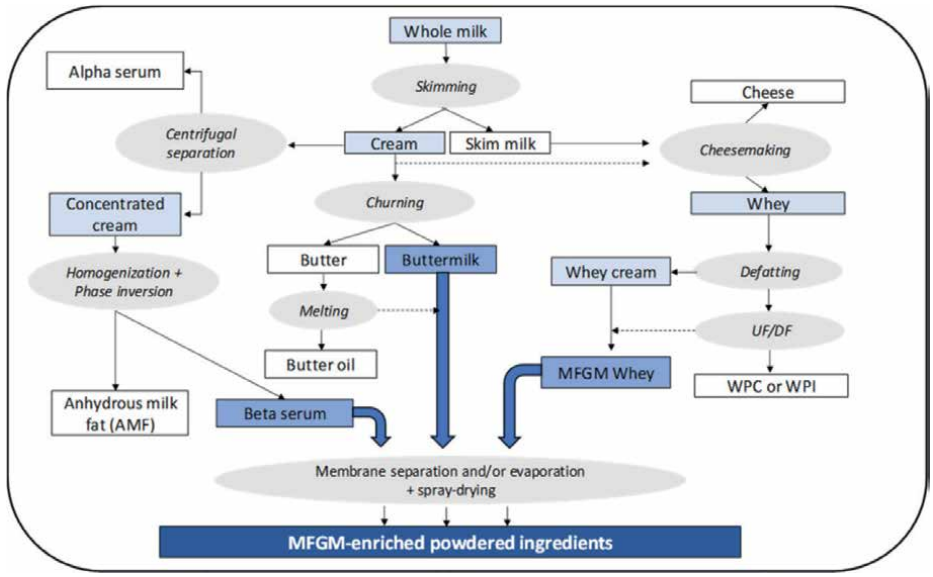

Figure 2.
Production of MFGM enriched powdered product from various processing methods. Adapted from [7].

During manufacturing of butter, phase inversion occurs by churning that involves in conversion of oil-in-water to water-in-oil, MFGM associated with TG gets drifted into buttermilk due to coalescence. MFGM isolates from buttermilk at lab scale was studied by [16], commercialized buttermilk powders was used to characterize lipids present in the MFGM isolates. The study showed that MFGM fractions had high cholesterol and PUFA content and serves as the best source of bioactive lipids. The lipid profile proved that MFGM had higher concentration of medium molecular weight TGs mainly due to linoleic acid.

Most successfully used method for production of MFGM from buttermilk was reported [13], using microfiltration and multiple diafiltration. Each batch contains 8 to 16 liters of reconstituted buttermilk with total solids content of 8% w/v in water was used. 2% sodium citrate at 1.4%w/w was added to reconstituted buttermilk to disrupt the casein micelles and to increase the PLs content in the buttermilk whey (pH 7.2), then stored for overnight at 6°C. The first step in separation involved in membrane filtration polyvinyl-dilfluoride (PVDF) membrane with 250,000 and 500,000 Da cut-off at 50°C. Then retentate was feed into 2-HP centrifugal pump with the pressure of 1.2 MPa at 50°C by circulating in shell and tube heat exchanger until favorable concentration is reached. Again the retentate goes to multiple diafiltration at 50°C, followed by high speed centrifugation to isolate MFGM fragments.

The authors noticed that, intact of small amount of β- lactoglobulin (30%) in MFGM even after 2 steps of diafiltration, this is due to the complex formation of whey protein and kappa casein with MFGM during heat processing [17]. Absence of sodium citrate resulted in increased level of skim milk proteins in the retentate and caused contamination of non- MFGM material in final retentate. To obtain MFGM isolate in powdered form, the retentate was freeze dried. During SDS-PAGE electrophoresis analysis, the final isolate contained 60% and 35% of protein and lipid (w/w) respectively, among the protein composition was 70%, 24% and 6% of MFGM, whey protein and casein respectively.

Another method of isolation was studied by [18], coagulation of native casein protein to obtain specific MFGM isolate cannot be obtained through membrane separation due to skim milk protein contaminants. 40% raw cream was cream separated and skim milk was subjected to continuous butter making process and the resulted buttermilk was coagulated by addition of rennet at 0.03% to hydrolyse the casein micelles at 45°C for 30 min to reach pH of 6.8. The obtained buttermilk whey was passed through microfiltration with average pore size of 80 nm at the constant pressure of 0.1 MPa at 50°C to remove whey protein. To remove further residues of whey protein, the retentate was diafiltered with deionized water (6 diafiltration steps). Interestingly, after 6 diafiltration steps complete absence of whey protein was detected during SDS-PAGE protein analysis.

High amount of protein loss was encountered in this isolation method, however the loss was considerably lesser when compared to the cream washing method [19]. The final isolate contained 70%, 30% and 20% of peripheral protein – periodic Schiff acid (PAS 6/7), XO and butyrophilin (BTN) respectively. Compared to the previously mentioned method of isolation [18], the coagulation method [19] showed neither whey protein nor casein in the final isolate and this method can be used industrially.

Effect of addition of cationic salts like calcium acetate and zinc acetate on selective isolation of MFGM in cheese whey was studied by [20–23]. The studies showed that removal of Ca^{2+}/Mg^{2+} from cheese whey through diafiltration and addition of 25 m*m* of zinc acetate at pH 4.2 at 30–35°C causes the maximum precipitation of MFGM.

Ethanolic extraction method (90% ethanol at 70°C) was used to solubilize the calcium chloride and acetate in dairy by-products to maximize the extraction of MFGM and phospholipids to produce dairy lecithin [22]. Composition and physico-chemical properties of spray-dried and freeze- dried MFGM isolate from cheese whey showed that freeze dried MFGM has higher retention of bioactive components and better oxidative stability than spray dried MFGM [24]. This study supports the findings of [25], concentrated buttermilks from raw milk (RCB) and pasteurized creams from buttermilk (PCB) were produced by condensing cheese buttermilk in falling film evaporator to reach 20% total solids, and then the spray-dried concentrate was used in the study. Unexpectedly, the amount of lipids was higher in PCB at the level of 19.7% vs. RCB 8.29% under the same skimming level. Double the amount of lipid concentration in PCB was stated due to attachment of milk protein to the exterior of MFGM, inhibiting the coalescence of fat globules [26].

Spray drying of buttermilk resulted in major loss of all classes of phospholipids and found that the inner portion of MFGM was exposed to interaction with other components. As already discussed in previous studies, serum protein contaminants were higher in PCB due to interaction with β- lactoglobulin and formation of complex systems. The color of RCB was reddish brown and PCB was yellowish white due to the presence of iron in RCB. Micrographs of MFGM in RCB and PCB revealed that casein is entrapped in the MFGM rather than its attachment to the exterior layer of MFGM. The study does not explain the storage stability and loss of phospholipids after spray drying.

From above stated studies of MFGM, it was clear that membrane separation techniques like microfiltration, diafiltration and ultrafiltration, addition of sodium citrate and cationic salts plays a major role in isolation of MFGM. Heat treatments like pasteurization of cream and spray drying mainly affected the phospholipids concentration and increased the serum protein contaminants in final MFGM isolates. Optimization of separation techniques and processing conditions should focus on minimal damage on functional bioactive components in MFGM fragments to ensure

the delivery of clinical benefits to the consumers. Most of the studies were conducted only on the characterization of bovine MFGM isolates rather than other species. Characterization of other species's MFGM uncovers the underlying potential health benefits and commercialization of novel MFGM fractions especially in the neonatal nutrition.

4. MFGM as key strategy in infant food (IF) formulation

In recent decades, formulations of infant and neonatal foods have introduced several new components and modifications to enhance its functional health performance to mimic human milk. Major changes in the IF that has been successfully introduced are supplementation of prebiotics (FOS and GOS) [27, 28], probiotics [29–31], docosahexaenoic acid (DHA) and arachidonic acid (ARA) [32], meat protein [33], plant protein (pea and soy protein) [34], taurine [35], MFGM [7, 36], polyamines [37, 38], folates [39] and osteopontin [40]. Even though many of these modifications are being carried out, very little knowledge is known to us on human milk's minor bioactive compounds that are essential for the neonatal development on long and short term studies.

Sometimes, supplementation of IF with bioactive compounds as same in human milk can cause adverse effects on infant growth and nutrition. Example, addition of opioid protein (beta-casomorphin) in IF can cause life threatening events due to its exogenous nature. Adaptation of infants and biosimulation of metabolic activities in infant digestion can be responsible for this condition [41]. Still now the regulation on fortification or supplementation of bioactive compounds in IF and nutrition was not standardized globally. However, fortification of MFGM was profitably done and studied for their effect on health benefits in many infants. Animal and human studies of commercially available MFGM formula and their outcomes are tabulated in **Table 2**.

5. Health benefits of MFGM

5.1 Anticancerous effect

Anticancerous activity of bovine MFGM was well detailed by Spitsberg and Gorewit, especially they play an important role in prevention of breast and ovarian cancer. A notable protein called Fatty Acid Binding Protein (FABP 1) present in bovine MFGM has effect on cancer cell proliferation on the epithelial part of mammary gland during lactation period [60, 61]. The presence of genes like BRCA1 and BRCA2 that have a capability to suppress breast cancer was also found in human milk. The origin of these genes was through exocytosis from the epithelial cells and usually covered by plasma membrane similar to the origin of milk fat globules in bovine milk. The study also addressed that presence of BRCA1 and BRCA2 in human milk fat globule (HMFG) has a vital role in neonatal nutrition [62].

The structure of bovine and human milk BRCA1 was quietly identical (72.5% similarity rate) and also had the same type of reaction towards DNA repairment and cell nuclear expression pattern in vitro study [63]. The major functions of BRCA1 and BRCA2 are repairing the damaged DNA and regulation of cytokinesis [64]. The principle behind the suppression process of cancerous cells involves, after MFGM is

Source	Formulation	Model	Brand	Dosage	Experimental finding	Reference
Whey	MFGM-10 Lacprodan® with phospholipids and sialic acid	Rat pups	Arla Food ingredients	100 mg/kg of body weight	Improved cognition and object recognition performance	[42]
Whey	MFGM-10 Lacprodan®	Rat pups	Arla Food ingredients	45 mg/day for 30 g pup	Increased brain lipid composition, ARA, improved functional maturity and reflex response	[43]
Whey	MFGM-10 Lacprodan® with prebiotics (PD polydextrose and GOS)	Rat pups	Arla Food ingredients	15.9 g/Kg MFGM with GOS 20 .86 g/Kg and PD 6.44 g/Kg	Effect on microbiota and beta diversity on genus level, reduced early stress level	[44]
Whey	MFGM-10 Lacprodan® with lactoferrin and prebiotic (PD&GOS)	Piglets	Arla Food ingredients	MFGM (5 g/l), DHA (182 mg/l), ARA (364 mg/l), PD (2.4G/l) and GOS (7 g/l) 285–300 ml/day based on weight	Increased concentration of gray matter in brain which is related to brain signaling to sensory organs	[45]
Whey	MFGM-10 Lacprodan®	Pigs	Arla Food ingredients	2.5 and 5 g/ l	Increased serum high density lipoprotein in 2.5 g/l MFGM and no difference in weight	[46]
Whey	MFGM-10 Lacprodan® Neonatal mouse	Neonatal mouse with low birth weight	Arla Food ingredients	100 mg and 200 mg as per body weight	Improvement in body weight, increased anti-oxidative activity and inhibition of inflammation	[47]
Whey	MFGM-10 Lacprodan®	Rats	Arla Food ingredients	1.5 g/kg/day	Lowers the body weight and elevates gut barrier against infections	[48]
Whey	MFGM-10 Lacprodan® with prebiotics and bovine lactoferrin	Neonatal piglets	Arla Food ingredients	MFGM (2.5 g), lactoferrin (0.3 g), GOS (3.5G) and PD (1.2 g)/ 100 g diet for 30 days.	Weight gain, modified gut microbes, exclusion of gut pathogens through feces	[49]
Whey	MFGM-10 Lacprodan® with prebiotics and bovine lactoferrin	Neonatal rats	Arla Food ingredients	MFGM (15.9 g), lactoferrin (1.86 g), GOS (21.23 g) and PD (6.58 g)/ kg of feed.	Improved sleeping quality associated with gut microbiota and reduced stress	[50]
Whey	MFGM-10 Lacprodan®	Rat pups	Arla Food ingredients	MFGM 6 g/l	Increased villus length in intestine, increased secretory cell which improves immune functions	[51]

Cream	PL-20 phospholipid concentrate MFGM	mice	Arla Food ingredients	MFGM as emulsion and replaced with drinking water	Slower lipid absorption and increased growth of *Lactobacillus* and *Bifidobacterium*	[52]
Whey	MFGM-10 Lacprodan®	Human infant-6 to 11 months	Arla Food ingredients	complementary food with MFGM 40 g/day	Reduction in prevelance in diarrhea and bloody diarrhea	[53]
Whey	MFGM-10 Lacprodan®	Human infant- < 2 to 6 months	Arla Food ingredients	4% MFGM of total protein content	Improved cognition capacity and functions	[36]
Whey	MFGM-10 Lacprodan®	Full term infants <14 years	Arla Food ingredients	3 g/day	Increased weight gain, sign of eczema and decreased immune response to polio virus type 1	[54]
Cream	Lipid rich MFGM fraction	Full term infants <14 years	Fonterra Co-operative Groups	3 g/day	Increased weight gain	[54]
Milk lipid	Complex milk lipid (CML)	2–8 week infant during 24 weeks	Fonterra Co-operative Groups	2–3 mg/100 g of infant formula	Increased hand and eye co-ordination, higher serum ganglioside for brain development and higher IQ	[55]
Milk lipid	Complex milk lipid (CML)	8–24 months	Fonterra Co-operative Groups	2 g CML/day for 12 week	No adverse on long term consumption	[56]
Milk lipid	MFGM supplemented infant formula	2.5–6 years old	INPLUSE®, Büllinger SA	0.5 g phospholipids and 2.5% INPLUSE®	Improved behavioral regulation and less febrile episodes	[57]
Milk lipid	Complex milk lipid (CML) with SureStart™ MFGM Lipid 100	Pregnant mothers – 11 to 14 weeks	Fonterra Co-operative Groups	8 mg/day for first trimester	No adverse effect on mothers and the fetus	[58]
Whey	MFGM-10 Lacprodan®	< 2 to 6 months human infants	Arla Food ingredients	4% MFGM of total protein content	Less prevalence of otitis and positive effect of plasma lipids	[59]

Table 2.
Animal and human studies of commercially available MFGM formula.

ingested, the protein would be degraded into several inhibitory peptides in the stomach and reaches the bloodstream and starts its action on cancerous cells in particular tissue or organ.

The role of MFGM against intestinal cancer was studied [65] that 0.88 g/day of trysin derivatives of MFGM significantly reduced 90% of β- Glucuronidase activity and kappa casein reduced 35% of activity in the mice. β- Glucuronidase acts as a catalyst in conversion of onco-precursors to carcinogens. Less than 20% of MFGM such as 5% and 10% had only 15–20% inhibitory effect on β- Glucuronidase activity. The possible science behind the scene was due to release of inhibitory peptidase of ingested MFGM. However, the exact mechanism was not clarified by the authors, these studies proved that supplementation of MFGM particularly in solubilized state has potent effect on intestinal cancer, which can be effectively used in geriatric functional foods. The action of MFGM-PLs and sphingolipids towards colon tumors was reported by several authors [66–71].

5.2 Anticholesterolemic effect

The positive impact of MFGM on serum cholesterol was studied [71], the study involved in supplementation of cream and butter into the diet of subjects. The results revealed that volunteers who had butter showed increased levels of serum cholesterol than those who were fed with cream. The obvious reason behind the relation is that MFGM is the principle compound responsible for reducing the level of serum cholesterol by its association with cholesterol binding in the intestine.

Similarly, consumption of 4 liters of whole milk per day reduced the uptake of cholesterol in the body [72]. A study [73] compared egg sphingomyelin (SM) and milk SM for their effective control on cholesterol absorption. Undoubtedly, the milk SM inhibited the absorption of cholesterol due to the presence of long chain and saturated fatty acids in their complex structure which makes them difficult to solubilize and unfold.

Cohort study by direct supplementation of milk fat 40 g/day showed markedly decreased level of total cholesterol and LDL lipoprotein in adults during 8 week observation [74]. Micellar insolubilization and transfer of cholesterol molecule in micellar cells to enterocytes are the major roleplay of MFGM in anticholesterolemic effect.

5.3 MFGM in physical health

Muscle strength, mass and function are important factors in physical performance of sports nutrition. Milk products especially whey protein plays a major role in muscle protein function and became an undeniable product in sports nutrition. Nutritional significance of milk on muscle function was mainly due to its nutrient rich constituents and its ability for specific gene expression [75]. In that way, the effect of uptake of milk during leg resistance exercise was studied in 3 groups of young volunteers who were fed with free-fat milk, whole milk and free-fat milk isocaloric with whole milk. The results showed increased levels of two aminoacids such as phenylalanine and threonine which indicates net muscle protein synthesis in the group fed with whole milk. The balance of net muscle protein shifted from negative to positive in same group. Hence the study concluded that intake of milk serves a reservoir for amino acids (phenylalanine and threonine) for muscle protein synthesis [76]. In an animal study with senescence-accelerated mice, intake of MFGM along with regular

exercise improved the muscle contractile force and lipid fraction of MFGM (PL and SM) had a beneficial effect on mechanical strength of muscle (quadriceps muscle) [77]. Long term effect on supplementation of MFGM diet on endurance capacity on swimmers was studied [78]. The MFGM regulated the gene expression for energy metabolism, increased oxygen intake, lipid oxidation, energy recharge and fat catabolism in 12 a week study. Similar studies on physical performance in human were studied [79–81]. Most of the studies are focused only on the supplemented MFGM fraction, study shall also be focused on natural MFGM rich dairy products intake and their effect on weight gain and loss in athletes.

6. MFGM in dairy based functional foods

As supplementation of MFGM is popularized in IFs, it also gained interest in incorporating dairy products like yoghurt, cheese and dairy beverages. Incorporation of MFGM in skim milk yoghurt from 1 to 4% (w/w) total solids concentration increased the firmness, water holding capacity and adhesiveness. Addition of MFGM also improved the concentration of polar lipids and protein content (including casein) (5.3% MFGM yoghurt *vs* 4.08% control). Below 3% of MFGM solids the gel strength was slightly weaker but increased when added above 3%. Similarly, firmness also decreased when yoghurt has 1% MFGM solid due to prevention of contraction of casein during curd formation.

Overall quality of the MFGM supplemented yoghurt was superior and shows the technological applications like water holding capacity [82]. Cohort study in yoghurt was done by [83], homogenized and unhomogenized set yoghurt were prepared with 4% Lacprodan®PL-20 with addition 0f 0.5% yoghurt culture with final total solids content of 15%. During homogenization, the surface area of the MFGM gets decreased and due to its polarity the affinity between PL and milk protein increases resulting in improved body and texture of the set yoghurt. The authors commented that addition of MFGM in yoghurt prevents the physical defects and improves the health benefits of the product.

Since MFGM is an amphiphilic molecule, it can be used as a stabilizer in dairy products to avoid the usage of artificial stabilizers to improve the quality of the product. Beyond the use of MFGM as a technological ingredient, MFGM was also used to encapsulate the lactic acid bacteria (LAB). Study on interaction between MFGM and LAB revealed that most of the bacteria are located at whey protein-fat interface or trapped in MFGM in the food matrix. Even after ripening, the bacteria are found to be trapped in MFGM [84–86]. This interaction was probably due to the reaction between bacteria and mucin factor of MFGM [87]. These studies clearly depict the encapsulation efficacy of natural ingredient MFGM as an emulsifier for LAB culture, especially in the location of bacteria on the surface of the cheese matrix. Further investigation on microstructure of MFGM material in cheese could be advantageous in improving flavor and color (in blue veined cheese) in cheese for the higher consumer preference.

Most recently, development of oil-in-water food emulsions are being prevalent due to their emulsion stability and protection against the oxidation of lipids. Likewise, MFGM coated lipid systems are studied to mimic the human fat globule to deliver the health benefits notably in IFs. The process involved in coating of MFGM around the triacylglycerol (TAG) through higher pressure homogenization methods [88]. Not only in IFs, these types of emulsions can be used in various products ranging from cheese, cream and dairy beverages irrespective of the consumer age. This concept

of emulsion leads to the production of commercial MFGM –PL (from beta serum or butter serum) coated vegetable oil in IMF called Nuturis®. This novel formula was prepared by mixing bovine PL (0.5 g PL in one liter) from beta serum in the aqueous phase of formula containing other ingredients and heating for 85°C/6 min, followed by homogenization with lipid phase (vegetable oil mixture) in inline mixer to form larger fat droplets coated with phospholipid. The microscopic examination showed that the fat droplet size and the location of cholesterol exposure were more or less similar to the human milk. This regulates the uptake of cholesterol in early childhood that maintains the weight gain and loss similar to the human milk intake [89]. This proves that MFGM and its fractions are important in emulsion formation because of their emulsifying capacity and physical stability due to their complex formation while processing.

In conclusion, deeper knowledge on human milk morphology and characterization of distribution of lipid will lead to develop novel and more innovative technologically significant functional food for the infant nutrition to reduce the incidence of overweight and obesity in early stages of childhood which was considered to be most prominent problem encountered while ingestion of other infant formula with imbalanced concept of nutrition.

7. Conclusion

In this chapter, we can summarize that MFGM prepared from dairy by-products such as buttermilk, a major by-product in butter processing and whey by-products in cheese and paneer processing, can be effectively used rather than discarded. However, almost all the minor components in MFGM were well established. But still some of the minor proteins present in MFGM resulting from processing were not well defined. Several studies on health benefits in infants and adults are mannered in various populations with excellent outcomes. Still now no adverse effect on intake of MFGM was reported even when taken at a higher level than the recommended level. Promising effect of MFGM on tumor inhibiting, altering gene expression and modulation of cholesterol absorption will have a positive impact on weight management and obesity control while consumption of fat rich dairy products. MFGM has excellent bioactive compounds, production of MFGM on larger scale and supplementing in commercially available foods will be upgrading the dairy foods and industry to the next level.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

Volatile Aromatic Flavor Compounds in Yogurt: A Review

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Abstract

Lactic acid bacteria are of great importance in the production of yogurt worldwide, yet very little is still known about the mechanisms of aroma formation in foods subjected to lactic acid fermentation. However, advances in the Adolfsson development of instrument methods have made it possible to avoid some of the difficulties in extracting flavoring substances from the otherwise complex matrix of lactic acid products. In this chapter, we present recent developments related to the impact of yogurt starter cultures on the production of the aromatic components in yogurts. In addition, we examine and characterize the aromatic compounds based on the chemical structures and discuss modern analytical techniques for yogurt analysis. As described in this chapter, a large number of flavoring substances can be studied, isolated, and identified with the help of advanced instrument analysis such as synthetic fibers for solid-phase extraction (SPME) and gas chromatography combined with mass spectrometry (GC-MS). These techniques can help us reach a more advanced level of understanding of the importance of specific strains for obtaining the desired sensory qualities of fermented, lactic acid products. At a more advanced stage, these analyses could allow scientists to develop rapid methods for determining the quality and authenticity of lactic acid products based on the aromatic-metabolic profile of starter cultures in the final product.

Keywords: yogurt, volatile, aromatic flavor, *Lactobacillus bulgaricus*, *acetaldehyde*

1. Introduction

Yogurt is defined as the product of fermented milk by *L. bulgaricus* and *Streptococcus thermophilus*. Yogurt was first discovered in the Middle East and has been a part of the human diet for thousands of years. However, it was not until the twentieth century that scientists started to provide scientific evidence for the health benefits associated with yogurt consumption. In 1905, a Bulgarian scientist, Stamen Grigorov, was the first to report on *Bacillus bulgaricus* (now *L. bulgaricus*), a lactic acid bacterial strain found in Bulgarian yogurt. Then, in 1908, the Russian scientist Elie Metchnikoff theorized that one's health could be improved, and senility delayed by colonizing the gut with the host-friendly bacteria found in yogurt. The popularity of yogurt is attributed to its various health benefits as well as the flavors and sensory characteristics [1]. Yogurt is described as having a smooth, viscous gel-like texture

with Sharpe acid associated with green apple aroma. These characteristics play a significant role in the consumer acceptability to yogurt products (**Table 1**). The traditional yogurt flavor is a combination of aroma and taste that are typically produced during lactic acid fermentation by the yogurt starter cultures. During fermentation, the yogurt starter culture coverts lactose and other nutrients in milk to several chemicals that lead to the production of various flavor and aromatic compounds. More than 100 different volatile compounds have been reported and produced by various yogurt cultures. Several advanced techniques and instrumentations have been applied to determine the volatile compounds in yogurt products. **Table 2** lists some of the common analytical instrument used for flavor analysis with advantages and limitations of each instrument.

The selection of suitable strain combinations in yogurt starter culture is important for achieving the best technological performance and desirable sensory characteristics. However, the use of genetically modified lactic acid bacteria with encoded and targeted flavoring [7] is not an acceptable solution primarily due to the lack of consumer acceptance of this technology, and restricted regulations for the use of such bacterial strains in food products; this is especially true for the European market [7]. In this regard,

Volatile compounds	Odor descriptor	Volatile compounds	Odor descriptor
Acetic acid	Vinegar like	Pentan-2-one-4-ol	Cucumber, lettuce
Propanoic acid	Cheesy	Ethanol	Alcohol
Methional	Cooked vegetables	2,3-Butanediol	Creamy
Methyl benzoate	Vanilla-like	Butyl acrylate	Tropical fruity
Benzaldehyde	Almond-like	1-Octen-3-one	Mushroom earthy
Nonanoic acid	Earthy	2-Pentanone	Wine-like
Ethyl nitrite	Sweet	Acetone	Fruity
Hexanoic acid	Sweaty	Pentanoic acid	Disgusted
Acetaldehyde	Fresh, green apple-like	Hexanal	Grass-like
Butanal	Cocoa-like	Ethyl acetate	Mild
Octanoic acid	Cheesy	Decanal	Floral
1-Hexanol	Greasy	Isobutyric acid	Buttery
Decanoic acid	Rot-like	1-Nonen-3-ol	Mushroom-like
2-Furanmethanol	Toast-bread like	Diethyl disulfide	Cooked onions-like
Heptanoic acid	Sour	Acetophenone	Sweet almond
2,3-Pentanedione	Sweet	Ethyl 2-methylbutyrate	Pear-like
2-Phenylacelaldehyde	Flowery	Tetradecanoic acid	Coconut-like
3-Methyl-2-butenal	Cherry-like	Pentanal	Fermented-like
Dimethyl sulfide	Lactone-like, sweet	Isopropyl alcohol	Musty
2,3-Butanedione	Butter, diacetyl vanilla	Nonanal	Rosy
Butyl acrylate	Tropical fruity	2-Methyl-1-propanol	Wine-like

Sources: [2–5].

Table 1.
Some identified volatile compounds in yogurt with their description of odors.

Technique	Dairy products	Analytical principle	Pros	Cons
High-vacuum distillation	Yogurt, cheese, sour cream	Involves the use of organic solvents for extracting minute volumes of concentrated aqueous volatiles	Thermal decomposition of compounds is prevented due to process operating at ambient or sub-ambient temperature	Labor and time-intensive process and requires high sample volumes
Simultaneous (steam) distillation extraction (SDE)	Yogurt, milk fat, skim milk powder	Involves a continuous process utilizing organic solvents with extremely low boiling solvents for concentrating volatile compounds	High yield due to extraction rate	Heat-labile volatiles risk breakdown if extraction is not controlled at low pressure
Dialysis	Yogurt, milk	Separation process is based on the diffusivity potential of volatiles through a membrane resulting in a concentration gradient	Yields high concentration gradients	Labor and time-intensive process
Molecular distillation	Butter, cheese	Similar process to the high-vacuum distillation procedure but only requires transfer of volatiles from a matrix to a chilled and condensed system	Ideal for heat-labile volatile compounds	Volatile requires short distance between the condensation system and the food sample under a high-vacuum environment
Dynamic headspace/purge trap	Yogurt, milk, ice cream, hard cheese	Restricted to the number of bases	Minimal use of sample, rapid process and minimized use of thermal artifacts	Time-sensitive and involves use of expensive equipment
Solvent-assisted vapor evaporation (SAFE)	Butter, milk, whey protein	Requires mixing sample in a selected organic solvent and liquid is centrifuged or evaporated	High yields of volatile compounds devoid of thermal process	Limited to only unique glassware use
Stir-bar sorptive extraction	Human milk, cheese	Volatiles are concentrated without using solvents	Highly immiscible in fluids	Labor-intensive
Solid-phase microextraction (SPME)	Yogurt, milk, ice cream	Employs a fiber system that absorbs volatiles and desorbs into a gas chromatograph injection port	Highly sensitive, and requires small sample volumes	Volatiles could be altered during extended thermal application process
Mass spectrometry	Yogurt, milk, cheese	Detects the mass-to-charge ratio of volatiles	Volatiles are detected based on reference spectra	Extremely expensive
Flame ionization	Yogurt, milk, cheese	Detects volatiles in a stream of gas	Broad spectrum for detecting volatiles	Requires reference standards for comparison

Sources: [3, 4, 6].

Table 2.
Analytical techniques for the determination of volatile compounds in fermented dairy products.

knowledge of the aromatic-metabolic profile of the starter cultures used and the influence of the profile on the sensory characteristics of the fermented products is essential, both for the individual consumer and for the food industry. It should be noted that an important consideration in the selection of starter cultures for the production of yogurt and other dairy products is the ability of the starter culture strains to produce metabolites that shape the sensory qualities of the product. Thus, in the selection of strains included in the composition of starter cultures used for the production of dairy products, the metabolic profile of each strain is examined, particularly since some metabolic products involved in the formation of the aroma have antimicrobial activity.

In this chapter, we present a comprehensive review of the general aromatic components that are present in yogurt products. First, we introduce the role of lactic acid bacteria with regard to yogurt flavor. Next, we present the aromatic compounds and group them based on the chemical structure into carbonyl compounds, organic acids, alcohols, and esters as major compounds. We then go on to discuss advanced instrument techniques for yogurt analysis. It is these techniques that could help us to reach a more advanced level of understanding of the impact of specific yogurt strains for obtaining the desired sensory qualities of yogurt products and other fermented lactic acid products.

2. The role of lactic acid bacteria in yogurt flavor

Yogurt is one of the most popular fermented dairy products worldwide nowadays. Moreover, consumption of yogurt has been increasing globally as a result of its pleasing sensory qualities, including texture, color, and flavor. Being one of the key food preservation methods, fermentation has significantly increased the nutritional value, shelf life, and sensory qualities of foods. This process involves a variety of microorganisms that break down the biochemical components of the food's basic materials (carbohydrates, proteins, and lipids), improving catabolism (digestion), taste, and enhancing the pharmacological and nutritional benefits of the food [8]. Most of the flavor compounds found in yogurt are a result of the activity of microbes in starter cultures, lactic acid bacteria (LAB). Microbes found in this starter culture carry out three key biochemical tasks during fermentation, which include the breaking down of milkfat into free fatty acids (lipolysis), caseins into peptides and free amino acids (proteolysis), and carbohydrates into lactic acid or other metabolites (glycolysis) [7]. Flavor is very important in food; consumers consider flavor to be one of the most significant aspects of food since it affects how well a particular product is liked and its overall acceptability.

2.1 Metabolic pathways of flavor compounds formation in yogurt

During fermentation, lactic acid bacteria processes create flavor precursors that are then transformed into flavor compounds. Enzymes hydrolyze several dietary components, including carbohydrates, proteins, and lipids. Carbohydrate metabolism (glycolysis), amino acid metabolism (proteolysis), and fatty acid metabolism (lipolysis) are the three main metabolic processes of LAB that lead to the formation of volatile compounds [9].

2.1.1 Flavor compounds from LAB carbohydrate metabolism

Lactic acid bacteria use the sugar lactose that is present in milk as their primary source of energy and carbon [10]. In fact, the distinctive acidic flavor of yogurt can be attributed to the conversion of lactose to lactic acid by LAB. The two distinct

carbohydrate fermentation pathways in LAB—homo-fermentation and hetero-fermentation result in various metabolic end products, depending on the LAB species, substrate, and environmental factors. Homofermentative LAB, which include *Pediococcus*, *Lactococcus*, *Streptococcus*, etc., use the Embden-Meyerhof-Parnas (EMP) pathway to produce lactic acid as the main by-product. However, heterofermentative LAB such as *Leuconostoc*, *Oenococcus*, *Lactobacillus*, etc., use the phosphoketolase pathway (PKP), which also produces other end products, such as ethanol, carbon dioxide, acetic acid [9]. Homofermentative metabolism could also switch to a mixed-acid metabolism with a variety of molecules under specific situations such as carbon limitation, carbon excess of slowly metabolized sugars, aerobic conditions. The metabolic by-products of this metabolism would include multiple flavor compounds such as acetaldehyde, ethanol, and diacetyl. Acetaldehyde, for example, dominates the flavor of yogurt in its normal form and helps in its distinctive flavor. Pyruvate is a crucial metabolic precursor that is usually catalyzed by aldehyde dehydrogenase or α-carboxylase to produce acetaldehyde. The characteristic flavor of yogurt is produced in fermented dairy products by a variety of C4 molecules such as diacetyl, acetoin, and 2, 3-butanediol [7]. These molecules may be produced by the citrate or glycolysis metabolism of certain LAB (**Figures 1**–**3**). Diacetyl is the predominant significant flavor compound among these C4 chemicals, and both *S. thermophilus* and *L. bulgaricus* are capable of producing it. Acetoin, which is diacetyl's reduced form, is important for decreasing the sharpness of diacetyl and also adds to the pleasant, creamy flavor of yogurt.

2.1.2 Flavor compounds from amino acid metabolism by LAB

In order for yogurt to have a pleasant taste and aroma (flavor), proteolysis is a crucial biochemical step. Proteolytic abilities in certain LAB allow them to undergo hydrolysis of proteins, which leads to the production amino acids and peptides [9]. Proteolysis and the breakdown of an amino acid (amino acid degradation) make up the first two phases of this process. The enzyme cell-envelope proteinases (CEPs) help to break down the protein into oligopeptides, causing casein to begin to undergo proteolysis by LAB. The second phase then begins and involves the transport of di-, tri-, and oligopeptides into the cell. Peptidases further hydrolyze casein-derived peptides to amino acids after these casein-derived peptides have been absorbed by LAB cells. In a single bacterial genome, peptidases can be encoded in several copies. Free amino acids generated by the breakdown of proteins (proteolysis) may be transformed into a variety of flavoring substances, including those ammonia, amines, aldehydes, phenols, indole, and alcohols, and these compounds all have imparted the flavor of the yogurt. The primary sources of flavor substances obtained from milk protein are mostly branched-chain amino acids such as Val, Leu, Ile, aromatic amino acids such as Phe, Tyr, Trp, and sulfuric amino acids such as Cys, Met [11]. Transamination of amino acids to their respective α-keto acids is the first stage of amino acid breakdown. The α-keto acids then go through several enzymatic processes, such as reduction to produce flavorless α-hydroxy acids and decarboxylation to produce aldehydes that can subsequently be reduced to an alcohol, or oxidative decarboxylation to produce acyl-CoA, and finally, carboxylic acids [7]. After that, esterases or acyltransferases catalyze the formation of esters or thioesters in processes involving alcohols and carboxylic acids [12]. As a member of a different class of lyases, threonine aldolase may convert threonine straight into acetaldehyde.

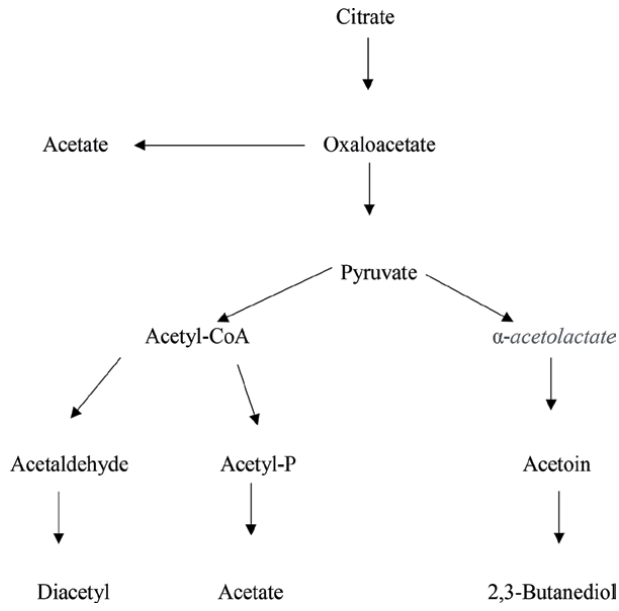

Figure 1.
Pathways of citrate metabolism by lactobacillus [1, 7].

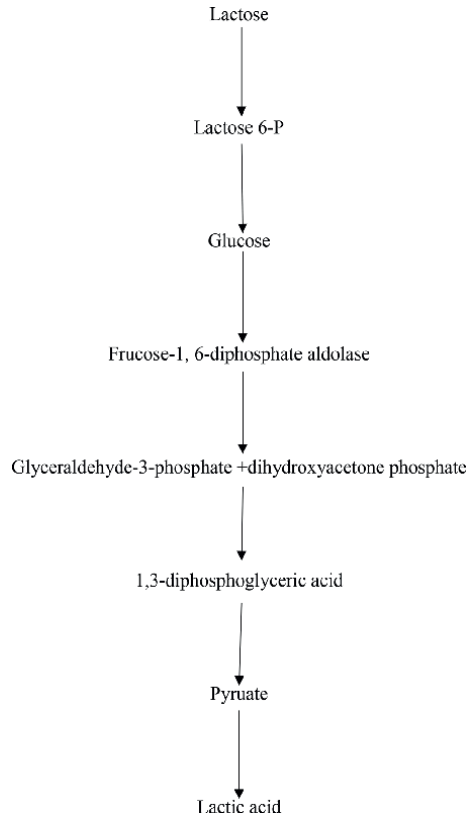

Figure 2.
Pathways of lactic acid production by lactobacillus [1, 7].

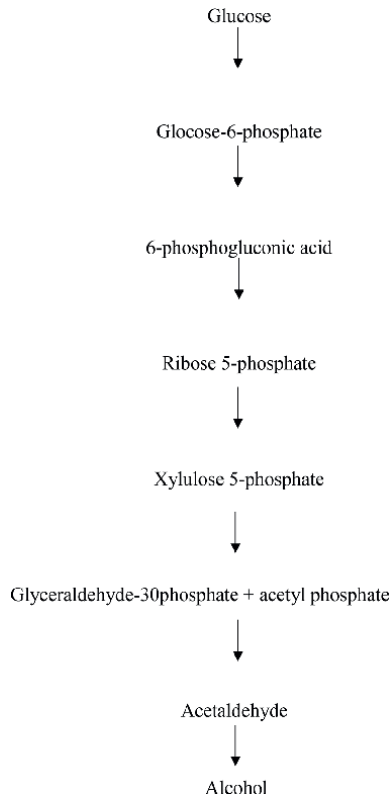

Figure 3.
Pathways of alcohol production by glucose metabolism [1, 6, 7].

2.1.3 Flavor compounds from LAB lipid metabolism

The two major processes that produce flavor components in fermented foods are lipolysis and fatty acid oxidation. Most fermented foods contain free fatty acids as key aroma components produced by the breakdown of lipids (triglycerides, diglycerides, and monoglycerides) [11]. The strains of Lactobacillus exhibit lipases in quite high concentrations, which then produce these free fatty acids [11]. Free fatty acids, particularly saturated and unsaturated fatty acids, serve as catalysts for catabolic processes that result in the oxidation of lipids and the generation of a variety of volatile compounds, including alkanes, methyl ketones, esters, secondary alcohols, and lactones [13]. Unsaturated fatty acids are oxidized by two different pathways, one of which is the formation of hydroxyperoxides via β-oxidation of unsaturated fatty acids in the presence of free radicals. The synthesis of 4-5-hydroxy acids, which are transformed into α-δ-lactones that emit strong fruity aromas, might result from another pathway of unsaturated fatty acid metabolism [14]. In addition, a variety of esterases found in LAB may directly generate flavor ester from glycerides and alcohols through an alcoholysis process. For example, in order to create ethyl butanoate and ethyl hexanoate, LAB can esterify ethanol with butyric and hexanoic acids [11].

2.2 Effect of different lactic acid bacteria on yogurt flavor

The starter cultures, processing conditions, sources of milk, and some other ingredients all have an impact on the flavor of yogurt [15]. However, within

these parameters, the development of the flavor components in yogurt is mostly influenced by the starter cultures used. The culture used for yogurt is primarily composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus. As a result, some nations only allow the use of the word "yogurt" for products prepared using starters that include bacteria from both of these strains [16]. The symbiotic connection between these two bacteria is referred to in mixed cultures as proto-cooperation and makes them mutually advantageous during fermentation even though each grows well in milk on their own [16]. Due to their associative development and mutual stimulation, the number of flavor constituents in the mixed cultures is significantly higher than that in either of the two individual cultures. It was discovered that the largest percentage of flavor compounds, including acetaldehyde, diacetyl, acetoin, acetone, ethanol, and 2-butanone (**Figure 3**), were produced when mixed cultures were utilized during lactic acid fermentation [7]. Additionally, the levels of methylated sulfides and dimethyl trisulfide were extremely low in *Lactobacillus bulgaricus* and *S. thermophilus* monocultures, suggesting that perhaps the mixed culture's higher levels were the result of interspecies interaction. The proto-cooperation of the mixed cultures is also significantly influenced by proteolytic activity. Compared to pure cultures or cultures with proteolytic *S. thermophilus*, this combination will generate higher aroma volatiles and nonvolatile metabolites. Thus, the combination of both microorganisms influences the synthesis of volatile and nonvolatile compounds essential to flavor development. The Lactobacillus strains are the most widely used commercial probiotics in yogurt. In addition to imparting yogurt's flavor and enhancing organoleptic qualities, these strains also provide health benefits. Yogurt flavor development is influenced by a variety of environmental factors, such as the composition of the culture medium, competition for nutrients, and interactions between microorganisms.

2.3 Metabolic engineering application for flavor enhancement

A significant approach for genetically modifying strains in order to increase the production of flavor compounds such as acetaldehyde, diacetyl, and esters is metabolic engineering. The changing of one or more genes or enzymes is a popular method for producing many different flavor molecules [11]. For example, formation of acetaldehyde by yogurt bacteria occurs via a variety of pathways, with threonine aldolase likely being the primary enzyme in this process. Serine hydroxymethyltransferase (SHMT), which is produced by the glyA gene in *S. thermophilus*, also has threonine aldolase activity. According to Chaves et al. [17], overexpression of the glyA gene in *S. thermophilus* increases acetaldehyde synthesis by 80–90%, whereas inhibition of the gene completely abolishes acetaldehyde formation. The main goal of these numerous metabolic engineering solutions for LAB has been to efficiently produce diacetyl for its significance in yogurt flavor. The als or ilvBN genes, ldh gene, and aldB gene that are coded for the enzymes α-acetolactate synthase, lactate dehydrogenase, and α-acetolactate decarboxylase, respectively, are the main enzymes involved in the synthesis of diacetyl.

2.4 Correlation between lactic acid bacteria and flavor compounds in yogurt

In several studies, certain volatile compounds found in yogurt have been linked to LAB species, demonstrating that LAB significantly affect the flavor of many

fermented foods, including yogurt. Yogurt and other dairy products are often fermented from milk from various plants and animal sources. The characteristic LAB species found in these fermented dairy products come from the genera Lactobacillus and are naturally prevalent in a variety of environments; however, they are mostly used for fermentation purposes [18–20]. Lactobacillus was found to be the most prevalent species in most samples of fermented yak milk. Microbial analysis, as well as the flavor profile of the product, revealed that these bacteria were significantly correlated with flavor compounds such as ethanol, benzaldehyde, ethyl acetate, 2, 3-pentanedione, and benzaldehyde [21]. Through correlational analysis using bidirectional orthogonal partial least square, it was determined that bacteria contribute more to flavor production than fungus. The majority of studies that compared the relationship between the LAB community and volatile chemicals found a strong correlation between LAB and the development of flavor in yogurt and other fermented foods. In yogurt, the main species, *Lactobacillus*, is predominant and helps create esters, aldehydes, acids, ketones, and alcohols.

3. Volatile and aromatic compounds in yogurt

Flavor is one of the most important properties of food products and is an important factor determining consumer acceptability. With regard to dairy products, their sensory properties largely depend on the relative balance of flavor compounds derived from fat, protein, or carbohydrates in the milk. For example, the distinctive flavor of yogurt is contributed by lactic acid and a complex mixture of flavor compounds that include the volatiles already present in the milk and specific compounds produced during lactic fermentation [22]. More than 100 different volatiles have been identified in yogurt, including carbohydrates, alcohols, aldehydes, ketones, acids, esters, lactones, sulfur-containing compounds, pyrazines, and furan derivatives [3].

Characterization of the volatile compounds allows for examination of the mechanism of formation of the aromatic profile of the product. Knowledge of the primary flavor compounds and their origin will thus support the production of dairy products of consistent quality that will be more readily accepted by consumers. For example, routine analysis of the primary aroma compounds can be used for quality monitoring during yogurt production. In addition, the profile of volatile compounds in yogurt can be used as a parameter to provide consumers with a better quality and safer food [3].

One major pathway for the production of flavor compounds in yogurt is through lipolysis or oxidation of the fatty acids in milk fat. Unsaturated fatty acids are oxidized in the presence of free radicals to form hydroperoxides, which rapidly decompose to form hexanal or unsaturated aldehydes. Unsaturated fatty acids also lead to the formation of 4- or 5-hydroxyacids, which readily cyclize to γ- or δ-lactones and odd-carbon methyl ketones by decarboxylation of β-keto acids. Another major pathway would be the microbiological transformation of lactose (and produced lactate) and citrate by acid-producing bacteria into acetaldehyde, diacetyl, acetoin, and ethanol. The alcohols in the yogurt can then combine with the free acids to form esters such as ethyl acetate and butyl acetate. In addition, biogenic amines and nitrogen-containing compounds can be transformed from proteins and amino acids, and sulfur compounds can be derived from organosulfur compounds [23].

However, not all volatile components found in foods are important for the foods' organoleptic properties. For example, in most studies, despite the long list of volatile

compounds found in yogurt (**Table 1**), only a few had relatively high concentrations. Only acetaldehyde, ethanol, acetone, diacetyl, and 2-butanone exert a strong influence on the desired aroma and are also present in amounts detectable by common laboratory techniques. The main volatile compounds commonly reported to be responsible for imparting the desired aroma to yogurt are the carbonyl compounds—acetaldehyde, diacetyl, acetone, acetoin, and 2-butanone. Although present in small amounts in yogurt, these compounds are important organoleptic factors.

The primary volatile components involved in the formation of the aroma of typical Bulgarian yogurt are acetaldehyde, acetone, 2-butanone, diacetyl, ethyl acetate, and ethanol. Kaminarides et al. [24] found that acetic acid, acetaldehyde, acetone, diacetyl, 2-butanone, acetoin, and 3-methyl-2-butanone were the primary volatile aroma compounds in yogurt made from sheep's milk. The primary aroma components in Swiss yogurt as determined by GC-sniff technique are acetaldehyde, diacetyl, 2, 3-pentanedione, methional, 2-methyltetrahydrothiophen-3-one, 2-neonal, 3-methylbutyric acid, guaiacol, benzothiazole, and two unidentified compounds [3]. The aromatic compounds in Swiss yogurt were investigated and found that few major compounds that had high-impact yogurt flavor, these compounds are acetaldehyde, dimethylsulfide, Diacetyl, 2, 3-pentanedione, L-limonene, and undecanal. However, other major constituents (fat, protein, and carbohydrates) in yogurt could play a major factor in the release of volatiles compounds. The aromatic components produced by the starter culture can be grouped into separate classes as carbonyl compounds, organic acids, alcohols, and esters, depending on their respective chemical structure.

3.1 Carbonyl compounds

The quality of yogurt is heavily reliant on the relative balance of volatile compounds including carbonyl substances derived from fat, protein, and carbohydrate in the milk base during the fermentation process. Carbonyl compounds are the primary aromatic substances in fermented yogurt where more than 38 of these compounds have been detected [3]. They are composed of aldehydes and ketones. The type and level of compounds derived during fermentation depend on the starter culture, variety of milk, and the conditions of the fermentation process. The metabolism of citric acid and amino acids by lactic acid bacteria—*Lactobacillus acidophilus*—and *Streptococcus thermophiles*, both of which are commonly used in the yogurt industry, produces the flavor compounds characteristic of yogurt products. **Table 3** shows the most common carbonyls compounds in yogurt and typical concentrations in yogurt products [3].

Several carbonyl compounds including diacetyl, acetoin, and butanediol are derived from citrate metabolism while several amino acids are converted into the intermediate metabolite pyruvate and finally acetaldehyde or directly into acetaldehyde.

In citric acid metabolism (**Figure 1**), citrate is converted into acetate and oxaloacetate with the presence of citric acid lyase catalyze. Next, oxaloacetate is decarboxylated and produces pyruvate and carbon dioxide. Subsequently, pyruvate is metabolized by lactic acid bacteria to produce different end products, including diacetyl, acetoin, and butanediol [25].

The crucial role of carbonyl compounds in yogurt can be identified when considering the sensory attributes of yogurt. Although each of these carbonyls is responsible for its characteristic flavor or aroma, the ultimate sensory properties of yogurt are decided by a relative balanced mixture of all flavored substances as well as their dominant properties.

Type	Typical level in yogurt (mg/kg)
Acetaldehyde	23–40
Diacetyl	0.2–3
Acetoin	1.2–28.2
Acetone	0.3–4.0
2-Butanone	0.1–7

Table 3.
List of common carbonyl compounds found in yogurt.

Acetaldehyde is an essential aroma and flavor compound found in fermented yogurt and provides the essential unique green apple or nutty flavor in fermentation by *L. bulgaricus* and Streptococcus thermophiles. However, a proper concentration level of acetaldehyde is required in order to obtain the most desired sensory quality. For example, although acetaldehyde gives a pleasant fruity aroma at diluted concentrations, high levels can result in a pungent irritating odor [3].

Diacetyl, which produces a characteristic buttery flavor in yogurt, is derived by fermentation of the citrate present in milk. It is equally as important as acetaldehyde with regard to the sensory quality of yogurt. The preferred typical yogurt flavor would thus be obtained by a 1:1 mixture of acetaldehyde and diacetyl. However, when the acetaldehyde level in yogurt is low, diacetyl contributes to producing a delicate, full flavor and aroma in the product. At higher concentrations, diacetyl can act as a flavor and quality enhancer as well [3].

Another flavored substance commonly available in yogurt is acetoin, which gives a mild creamy, slightly sweet, butter-like flavor. While acetoin is converted from diacetyl by the diacetyl reductase enzyme [5, 7], its flavor properties are also similar to those of diacetyl. A proper combination of both substances thus results in a typically mild, pleasant, buttery yogurt taste. Moreover, acetoin tends to reduce the harshness of diacetyl.

Acetone and 2-butanone reportedly have similar flavor characteristics with regard to minor but important flavor compounds found in yogurt. Both compounds make a positive contribution to sweet, fruity aroma and flavor qualities. Typical concentrations of acetoin in yogurt range from 1.2 to 28.2 mg/kg [26, 27]. Diacetyl in combination with acetoin is responsible for the soft, pleasant, fatty taste of yogurt that is crucial to yogurt's widespread appeal.

Acetone and 2-butanone are two volatile compounds with a minor contribution to aroma in dairy products [1, 24, 27, 28]. For example, acetone has a sweet fruity aroma and is known to affect the flavor and taste of yogurt. Small amounts of acetone typically originate from milk, but certain amounts are produced by bacteria in yogurt and the concentration of acetone in yogurt ranges from 0.3 to 4.0 mg/kg [27]. The taste characteristic of 2-butanone is similar to that of acetone and the concentrations in yogurt range from 0.1 to 7.0 mg/kg [24, 27]. Gallardo-Escamilla et al. [28] reported that 2-butanone is important for the aroma development of yogurt and contributes to its fruity flavor.

However, many of the carbonyl compounds also play a role in the loss of yogurt taste stability by developing off flavor during storage. For example, reactions from carbonyl compounds can generate off-flavor chemicals. Lipid oxidation in milk results in an undesirable stale, oxidized flavor. Moreover, some malodorous compounds such as 2, 4, 5-trimethyloxazole can be generated from diacetyl and acetaldehyde in

the presence of ammonia [29]. Due to their off-character and low aroma and taste thresholds, these compounds can lead to serious taste and aroma defects.

3.2 Organic acids

The most perceptible chemical compound in yogurt in flavor detection is carbonyl compound followed by organic acids. Degradation of polysaccharides by lactic acid bacteria during fermentation produces monosaccharaides and acids. Organic acids contribute significantly to the sensory properties in fermented yogurt, especially with regard to acidity. For example, changes in acid concentration lead to the development of a characteristic flavor and aroma along with desirable consistency. Lactic acid is the major organic acid found in fermented yogurt, and it has both positive and negative impacts on taste (**Figure 2**). Approximately 20–40% of lactose present in milk base is metabolized into lactic acid, which increases the acid concentration up to 0.9%.

Lactic acid bacteria utilize lactose and then glucose as the carbon source to produce pyruvate through glycolysis. Lactic acid is produced by lactate dehydrogenase. Taste and mouthfeel of the final product can vary with the concentration of lactic acid regardless of the flavor compound contained [30]. Moreover, formation of acid directly involves the texture development. To obtain desirable consistency, it should reach the optimum pH level. Typical pH level in yogurt is 4.4 [3].

Acetic acid, folic acid, and longer-chain organic acid are generated during yogurt fermentation in addition to lactic acid for example, acetic acid amounts range from 0.5 to 18.8 mg/kg in typical products. However, high levels of acetic acid impart an unacceptable vinegar-like taste [31]. Folic acid is mainly derived by *Streptococcus thermophiles* by amino acid utilization. Accumulation of folic acid stimulates the growth of other lactic acid bacteria including *Lactobacillus acidophilus* in the fermentation medium. Longer-chain acids such as octanoic acid develop a characteristic soap-like flavor [3].

Thus, in order to obtain a yogurt with desirable properties, acid production should be controlled. Extended acidification during fermentation or in storage results in the development of off-flavors. Syneresis, the most common issue associated with the sensory quality of yogurt, is a qualitative defect in the yogurt structure that tends to lower consumer acceptability by weakening the appearance, texture, and consistency of the product. Syneresis develops as a result of post acidification, which causes some leakages of whey proteins.

Post acidification depends on the type of strain, microbial ratio in the yogurt starter culture, the storage temperature, and the storage time. Post acidification manipulation can be done changing the microbial ratio, and it increases the shelf life of the yogurt. Volatile acids are also important from a nutritional and therapeutic point of view in addition to their influence on organoleptic properties of the products.

In addition to lactic acid, other acids are produced during the fermentation of yogurt, both by lipolytic processes and by bacterial fermentation. For instance, acetic acid is an important compound produced by lactic acid starter cultures [26]. Acetic acid has been reported at a concentration of 0.5–18.8 mg/kg in yogurt [26]. High levels of acetic acid impart a vinegar-like taste that may not be accepted by consumers [32]. Longer-chain acids (e.g., octanoic acid) may contribute to the characteristic soap-like aroma [33].

3.3 Alcohols

In addition to carbonyl compound and acid, another volatile compound generated during yogurt fermentation is alcohol. However, the contribution of alcohol

compounds in flavor development is comparatively less. A total of eight to nine alcohol compounds associated with fermented yogurt have been detected [34, 35]. The type and concentration of compound primarily depend on the starter culture used (**Figure 3**).

Ethanol is considered to be the principal alcohol derived in lactic acid fermentation. It is produced by breakdown of glucose and catabolism of amino acids. In the ethanol production pathway, glucose breaks down into lactic acid, ethanol, and CO_2 with the presence of ATP. As acetaldehyde degradation occurs during alcohol production, the amount of acetaldehyde in the medium is reduced.

As ethanol has an effective olfactory and trigeminal stimulus, it can act as a flavor enhancer to some extent. In typical yogurt made by cow milk fermentation, ethanol content ranged from 0.2 to 9.9 mg/kg [3] while yogurt made by other milk contained a lower amount. In addition to ethanol production, 1-hexanol and 1-heptanol production was also detected during fermentation. However, production of high levels of alcohol, particularly ethanol, was measured during storage while a reduction in acetaldehyde was reported. The level of ethanol ranged between 8.13 and 10.99% throughout the storage period [34, 35].

3.4 Esters

Esters are another volatile compound found in fermented yogurt and have a similar role to alcohol and acid in flavor development. A total of three to six ester compounds were detected in fermented milk [34, 35] depending on the starter culture. This included ethyl ester, butyl ester, and ethenyl ester. Ethyl ester, which is significant among other esters in flavor development, is derived from the enzymatic or chemical esterification of acids with ethanol. Ethyl ester adds unique fruity and floral aroma and flavor while minimizing the sharpness and bitterness imported by fatty acids and amines [1, 33]. Esters contained in yogurt are in a low amount and are able to withstand extended storage periods.

3.5 Sulfur compounds

Volatile sulfur compounds are significant contributors to the off-flavors of yogurt. As a class, sulfur compounds are typically present in foods at extremely low concentrations and have low sensory detection thresholds. Some of these compounds provide background sensory nuances to the flavor, while others provide unique flavor characterizing identities to the products. Sulfur compounds including methanethiol, dimethyl disulfide, and hydrogen sulfide can be detected in fermented milk products, and their presence contributes to the strong, unacceptable aroma [33–35].

The sensory properties of yogurt depend largely on the relative balance of chemical compounds derived from carbohydrates, protein, or fat in the milk base. The flavor components of yogurt include the volatile compounds naturally present in the milk and specific compounds produced from milk fermentation. It has been reported that more than 100 different volatile compounds have been identified in yogurt, including carbonyl compounds, alcohols, acids, esters, and sulfur-containing compounds.

The major compounds responsible for imparting the desirable flavor in yogurt are acetaldehyde diacetyl, acetoin, 2-butanone, and lactic acid. Moreover, the optimum flavor of yogurt results when proper levels of these compounds are produced. The desirable concentrations of acetaldehyde in yogurt ranged from 23 to 40 mg/kg, while

lesser concentrations resulted in weak flavor and higher acetaldehyde led to an off-flavor. Additionally, as with many other dairy products, yogurt is prone to deterioration, especially under improper storage conditions. The generation of volatile byproducts leads to off-flavors, which make the product unsatisfactory for consumers.

4. Extraction and analysis of volatile aromatic compounds in yogurt

4.1 Solid-phase microextraction (SPME)

The chemical analysis of aroma compounds in yogurt products is typically a complicated process. For example, such analysis requires an extraction stage and, despite the outstanding importance of aroma as an indicator of product quality and conformity, it is still difficult to separate aroma compounds based on common properties such as polarity or volatility. This is particularly true since most volatile organic compounds are present only in small concentrations (μg/kg to mg/kg) in yogurt [36, 37]. As a result, it is often necessary to isolate the volatiles from the complex matrix and concentrate these volatiles for analyses. Unfortunately, the extraction and concentration of volatile aroma components from yogurt products present a major analytical challenge. The most significant problems encountered during this process are:

i. the tendency of the compounds to decompose or transform in the presence of heat and/or oxygen.

ii. potential formation of secondary volatiles by enzymatic reactions.

iii. incomplete recovery of polar/semi-volatile aromatic components.

Classical techniques for preconcentration of volatiles such as steam distillation direct extraction simultaneous steam distillation and extraction with a solvent static headspace [26, 33, 36] and dynamic headspace [30] have been applied to the extraction and concentration of volatile aromatic compounds in yogurt. In recent years, solid-phase microextraction (SPME)-based methods have been used to analyze yogurt flavors [38, 39]. Unlike conventional extraction techniques, SPME is more sensitive to experiment conditions. Any change in experiment parameters that affects the partition coefficient and adsorption rate will also affect the amount adsorbed onto the SPME fiber and the corresponding reproducibility [40].

Solid-phase microextraction (SPME) methods were developed in the 1990s by Arthur and Pawliszyn as a rapid and useful technique for volatile compound analysis. SPME coupled with GC-MS can provide high sensitivity with a small sample volume; consequently, it can be used to analyze the aroma profile of a wide variety of substances. Recently, this technique has been used to study the volatile profiles of fermented camel milk [41], grapes and wine [42], and dried fermented sausage [43].

The amount of analyte extracted on the fiber depends on the polarity and thickness of the stationary phase of the fiber, time and temperature of the extraction, agitation and pH of the sample solution, addition of salt to the sample, and the concentration of the analyte in the sample. These SPME parameters must be optimized for each analyte and matrix. Various fiber coatings are available with thicknesses from 7 to 150 μm. Although fibers coated with thicker films require longer time to reach

extraction equilibrium, they can be more sensitive because they can extract larger amounts of analyte [44].

Solid-phase microextraction onto silica fibers externally coated with a suitable stationary phase is used in combination with GC and is also directly coupled to HPLC for the analysis of low-volatile or thermally labile compounds that are not subject to GC analysis. The SPME device consists of a stand with an integrated extraction fiber inside a needle and an assembly holder. Silica fibers (1 or 2 cm long) coated on the outer surface with a thin film of an extraction phase consisting of a liquid polymer and/or a solid sorbent are commercially available. StableFlex fibers consist of a flexible condensed silicon core and are less fragile. Although SPME has maximum sensitivity to the equilibrium distribution, there is a proportional relationship between the amount of analyte adsorbed by the SPME fiber and its initial concentration in the sample prior to partition equilibrium. As a result, complete equilibrium is not necessary for quantitative analysis by SPME [44].

4.2 Gas chromatography with mass spectrometry (GC-MS)

Chromatographic methods are widely used in the identification of various aromatic metabolites. In lactic acid products, these methods include Fourier transform infrared spectroscopy, electron impact ionization-mass spectrometry (EI-MS), electrospray ionization-mass spectrometry (ESI-MS), and nuclear magnetic resonance (NMR) spectroscopy. Mass spectrometers are generally more sensitive and more selective than any other type of detector. Prior to MS analysis, metabolites must be separated, and the separated compounds must be ionized. Ionization techniques can vary, especially for GC-MS and LC-MS [45]. Each of these techniques has advantages and limitations, and no single analytical technique is yet available for the complete study of the metabolome [46].

GC-MS-based metabolome analyses have been developed and applied for metabolic profiling in plants and microorganisms. The aforementioned studies clearly demonstrated the utility of GC-MS for non-target metabolite profiling in a variety of matrices [47].

GC-MS is a combined system where volatile and thermally stable compounds are first separated by GC after which the eluted compounds are detected traditionally by mass spectrometry. In metabolomics, GC-MS has been described as the gold standard [48, 49].

Instrumental analysis of volatile compounds in yogurt is almost exclusively performed by gas chromatography (GC), although high-performance liquid chromatography (HPLC) has also been used in a limited number of cases. Various detectors, including ionization detectors (FID), thermal conductivity detectors (TCD), electron capture detectors (ECD), photoionization detectors (PID), and mass spectrometry (MS) can be used to detect volatiles [3]. In particular, GC-MS is the most popular technique used in the analysis of aromatic volatile components due to its ability to detect and quantify known compounds, identify unknown compounds, and elucidate the chemical properties of molecules. Although the sensitivity of MS depends on the nature of the analytes and the type of equipment used, the detection limits of the charged species can typically range down to picogram levels or even less. In addition to direct calibration, the quantification of volatile compounds can be performed by matrix addition of the labeled compounds or by addition of the so-called internal standard [34, 35].

The application of GC-MS has boosted research on the aroma of yogurt and other products, especially when coupled with SPME as a pretreatment method. The

primary advantages of SPME are its simplicity, low cost, ease of automation and in situ sampling. SPME coupled with GC-MS has been widely used to assess the aroma chemical profiles of volatile components derived from a wide variety of matrices, including fermented milk [50], fruit and mango juice [51], grapes and wine [42], dry fermented sausage [43], and alcoholic beverages [52, 53].

5. Aromatic components produced by *Lactobacillus delbrueckii* subsp. *bulgaricus*

Recent work has focused on the isolation and characterization of *L. bulgaricus* with a particular interest in the metabolite profile of these strains. For example, eight strains were examined for the metabolic profiles and found at least 47 different aromatic compounds that were recently identified (**Table 4**) [5]. These aromatic compounds were divided into six main groups based on the chemical structure of each, as follows: 1. organic acid group 2. alcohol group, 3. aldehyde group, 4. ketone group, 5. ester group, and 6. aromatic group. As expected, the primary aromatic component, acetaldehyde, was produced by all of the bacterial strains. Importantly, acetaldehyde is recognized as a major flavor component in yogurt and provides the traditional strong, fruity aroma, sometimes described as green-apple-like that is characteristic of yogurt products. All of the tested strains produced pentanoic acid along with octanoic acids and acetone in a wide range of concentrations. We also observed that using different combinations of yogurt cultures led to the formulation of a wide range of unknown aromatic compounds and higher levels of acetaldehyde. Results showed that the interaction between strains generated a favorable yogurt volatile profile

Volatile compounds chemical formula	
Aldehyde compounds:	
Acetaldehyde	C_2H_4O
Furaldehyde	$C_5H_4O_2$
3-Hydroxybutanal	$C_4H_8O_2$
Benzaldehyde	C_7H_6O
Benzacetaldehyde	C_8H_8O
Ethylbenzaldehyde	$C_9H_{10}O$
2-Octenal	$C_8H_{14}O$
Decanal	$C_{10}H_{20}O$
Ketone compounds:	
2-Pentanone	$C_5H_{10}O$
Acetoin	$C_4H_8O_2$
2,3-Butanedione	$C_4H_6O_2$
2-Acetylfuran	$C_6H_6O_2$
2-Nonanone	$C_9H_{18}O$
2-Heptanone	$C_7H_{14}O$
3-Methyl-2-butanone	$C_5H_{10}O$
2-Undecanone	$C_{11}H_{22}O$

Volatile compounds chemical formula	
Acid compounds:	
Formic acid	CH_2O_2
Butyric acid	$C_4H_8O_2$
Acetic acid	$C_2H_4O_2$
Hexanoic acid	$C_6H_{12}O_2$
Pentanoic acid	$C_5H_{10}O_2$
Benzoic acid	$C_7H_6O_2$
Octanoic acid	$C_8H_{16}O_2$
1,2-Benzenedicarboxylic acid	$C_8H_6O_4$
Alcohol compounds:	
2-Furanmethanol	$C_5H_6O_2$
Ethanol, 2-(octyloxy)-	$C_{10}H_{22}O_2$
3-Methyl-2-butanol	$C_5H_{12}O$
2-Undecanol	$C_{11}H_{24}O$
Ester compounds:	
Propanoic acid, ethenyl ester	$C_5H_8O_2$
2(5H)-Furanone, 5-methyl-	$C_5H_6O_2$
Benzoic acid, 2-ethylhexyl ester	$C_{15}H_{22}O_2$
3-Methyl-2-butenoic acid, tridec-2-ynyl ester	$C_{18}H_{30}O_2$
Ethanone, 1-(2,4-dimethylphenyl)-	$C_{10}H_{12}O$
4-Ethylbenzoic acid, methyl ester	$C_{10}H_{12}O_2$
Aromatic hydrocarbons:	
3-Carene	$C_{10}H_{16}$
Undecane	$C_{11}H_{24}$
Tridecane	$C_{13}H_{28}$
3-Heptene, 2,2,4,6,6-pentamethyl-	$C_{12}H_{24}$
2-Methylundecane	$C_{12}H_{26}$
2-Pentene, 2,4,4-trimethyl	C_8H_{16}
Tetradecane	$C_{14}H_{30}$
2,4,6-Trimethyldecane	$C_{13}H_{28}$
Nonadecane	$C_{19}H_{40}$
Pentadecane	$C_{15}H_{32}$
Hexadecane	$C_{16}H_{34}$
Octadecane, 3-ethyl-5-(2-ethylbutyl)	$C_{26}H_{54}$
Octadecane	$C_{18}H_{38}$
Source: [5]	

Table 4.
Aromatic components produced by symbiotic starter cultures of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus.

resulting in the highest numbers of compounds identified as key-aroma compounds that are desirable for an acceptable organoleptic yogurt quality. Moreover, a different starter culture preparation could also lead to the production of different metabolites. Preparation steps would include bulk growth media, media additives, protein sources, and amino acid composition (especially glycine). The use of direct-to-vat culture or a lyophilized starter culture could also impact the aromatic profile of yogurt products. Our work also demonstrated that there are synergistic effects among the aromatic compounds that contributed to the overall yogurt flavor.

6. Conclusion

In this chapter, we reviewed the volatile flavor compounds in yogurt products. In addition, we discussed yogurt starter cultures, major aromatic compounds, and modern instrument analysis of yogurt flavor. Currently, yogurt sales are among the fastest growing in the dairy industry. Consequently, a greater knowledge of enhanced manufacturing procedures and consumer preferences would be highly useful in helping the yogurt industry to improve on its current products and develop additional innovative products. For example, the use of advanced techniques such as synthetic fiber solid-phase extraction and gas chromatography combined with mass spectrometry could help to identify a large number of aromatic compounds in yogurt. This would open up the possibility for more comprehensive exploration of the importance of specific strains in obtaining desired sensory qualities. Moreover, it would support the selection of production strains and the flavor producers. On a more advanced level, such analyses could allow us to develop rapid methodologies for quality control and authenticity of lactic acid products based on the aroma-metabolite profile of the starter cultures in the final product. There remains a lack of sufficient data related to the importance of specific process parameters and strain specificity for aroma formation in lactic acid products. As a result, qualitative and quantitative analysis of volatile aromatic compounds should merely be the first step toward achieving this goal. By determining the relationship between key flavor compounds and the sensory properties of yogurt, we will have a better understanding of how yogurt flavor is affected by the presence of critical flavor compounds. This will enable us to facilitate the production of more uniform yogurt products for enhanced consumer acceptance.

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Chapter 8

Lactic Acid Bacteria: Review on the Potential Delivery System as an Effective Probiotic

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Abstract

Lactic acid bacteria are gram-positive microorganisms that are characterized by the production of lactic acid as a key fermentation product. LAB, specifically *Lactobacillus delbrueckii* subsp. *bulgaricus*, are essential dairy starter cultures for the manufacture of several fermented dairy products such as yogurt. Some LAB are useful microorganisms and are well known to have probiotic effects to provide foods with unique sensory qualities such as aroma and taste. Probiotic strains help to strengthen the human immune system, increasing the body's resistance to diseases. Additionally, probiotics and postbiotics improve gut microbiome balance and prevent health issues. Postbiotics are substances that are produced by microbes' metabolic activities and have a positive impact on diseases, either directly or indirectly. Extensive research has shown that postbiotics possess immunomodulatory and significant clinical effects. Their use has been found to enhance general health and alleviate symptoms of various disorders in healthy individuals. Furthermore, postbiotics exhibit anti-inflammatory, antioxidant, and anticancer properties. Therefore, this chapter presents an overview and the importance of LAB as a probiotic and its importance to human health, metabolic fermentation, and antioxidant potential. The review also discusses different biotechnological methods that improve the survival rate of probiotics during processing and GIT transit like microbial encapsulation.

Keywords: lactic acid bacteria (LAB), probiotics, lactobacillus, postbiotics, bifidobacteria, delivery system, microorganisms

1. Introduction

Lactic acid bacteria (LAB) are significant microorganisms that primarily generate lactic acid as a byproduct of metabolic processes. In the agricultural, pharmaceutical, food, and medical industries, lactic acid bacteria serve a wide range of purposes. One of the most common methods of preserving and prolonging the shelf life of food is the use of LAB, which is used in many food fermentations [1]. Lactobacillus

is a gram-positive, rod-shaped bacteria commonly found in fermented foods that are beneficial for human health [2]. *L. bulgaricus* is among the most significant LAB, as it is recognized as one of the two bacteria needed for yogurt production. *L. bulgaricus* was isolated from Bulgarian yogurt, and today, the yogurt manufacturing industries use it in addition to *Streptococcus thermophilus* for fermentation. This LAB (*L. bulgaricus*) plays a significant role during yogurt production regarding organoleptic, hygienic, and perhaps probiotic characteristics; as a result, it is a safe probiotic with several advantageous qualities [1]. According to the FAO/WHO [3], probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host." These organisms are commonly found in foods such as yogurt, kefir, and sauerkraut, as well as in dietary supplements. Mani-López et al. [4] reported that the food sector, especially the dairy industry, is looking to enhance its knowledge of different probiotic bacteria to produce products that offer health benefits at a reasonable cost. However, the development of starter cultures for fermented dairy products depends on the microbial symbiosis of several lactic acid bacteria with excellent fermentation abilities [5]. Research confirms that commercial yogurt starter cultures' most significant fermentation properties include rapid acidification, specific flavor compounds, weak post-acidification, and health benefits with minimal dietary needs [1].

One of the key challenges in using Lactobacillus as a probiotic is ensuring its safe and effective delivery. Therefore, various delivery systems have been developed for Lactobacillus, and these delivery systems protect the bacteria from extreme conditions such as temperature, processing, storage, and the ability to withstand stomach acids and bile salts to colonize and exert their beneficial effects [6]. The success of a delivery system depends on several factors, including the viability of the bacteria during production and storage, and the ability of the delivery system to protect the bacteria during transit through the digestive system [6]. Advances in technology and research have led to the development of more effective delivery systems for Lactobacillus, which can potentially improve human health in various ways. Therefore, in this review, we discuss lactic acid bacteria focusing on Lactobacillus and their beneficial effects on human health applications as well as their effective delivery system to withstand harsh conditions such as temperature, processing, and storage, to maintain their viability.

2. Lactobacillus

Lactobacilli are fastidious lactic acid bacteria (LAB) of the family Lactobacillaceae that are mostly utilized in the dairy industries due to their fermentative properties. According to Ibrahim and Ouwehand [2], these microbes are non-spore-forming, aerotolerant anaerobes, which are rod-shaped, gram-positive, and part of the phylum Firmicutes, class Bacilli, and the order Lactobacillales. The aerotolerant property of Lactobacillus makes it possible for the organism to thrive in both anaerobic and aerobic environments. Lactobacilli are found to be part of the lactic LAB; therefore, they are usually distinguished based on their ability to create and produce lactic acid as a byproduct during glucose metabolism, which helps contribute to the preservation of the food due to the acid produced after the metabolism. They also contribute to individual sensory qualities such as flavor (aroma and taste) and texture and also enhance the organoleptic properties of foods [7], thus being classified to be the most important bacteria group amongst LAB in applied microbiology [8, 9]. Lactobacillus

organisms play two key roles in the ecosystem, and these two distinct roles include being used as starter cultures in the dairy industries and being used as probiotic cultures. Lactobacillus species are usually used in the food industries (especially dairy industries) with other bacteria species such as streptococcus as a starter culture for fermentation during the manufacture of products such as yogurt, cheese, and sour milk. This is due to the ability of the lactobacillus to convert the sugar lactose into lactic acid to produce dairy products. Lactic acid is the major byproduct, which forms the larger percentage (at least 85%) of metabolic end products in numerous species, including *Lactobacillus acidophilus, Lactobacillus casei, and Lactobacillus plantarum*, indicating that glucose metabolism is primarily homofermentative. Many Lactobacillus strains are also isolated from different sources to be used as probiotic agents; however, it is unlikely that each species/strain possesses all the desired characteristics that will make it a suitable probiotic. Lactobacillus species are also used in the production of pickles and sauerkraut (fermented vegetables), wines and juices (beverages), and other products such as sourdough bread and sausages. The widespread utility of this microbe (Lactobacillus) is generally due to its Generally Recognized as Safe (GRAS) status, which makes a lot of people like its products. Lactobacilli are fastidious LAB and can be found in a variety of nutrient-rich environments such as dairy environments, microbial-rich host habitats such as human and other mammals' mucosal surfaces, and natural ecological niches such as decaying organic matter, soil, and insects' gastrointestinal (GI) system [2]. Also, Lactobacillus species are mostly found in various fermented foods, such as yogurt, as well as in nutritional supplements. Lactobacillu*s* can also be usually found in the crop and ileum flora of poultries, since these are good probiotics for the poultry thus promoting the growth of the bird and suppressing the growth of diseases causing bacteria in the poultry [8].

2.1 Taxonomic classification of lactobacillus

Lactobacillus was first classified by scientists based on its observable type of characteristics such as optimal growth and sugar utilization [10]. According to Ayivi et al. [1], new classifications of Lactobacillus have recently been approved by scientist due to the extent to which the new lactobacilli genera made it difficult to differentiate and categorize between the various lactobacilli species. These novel classifications are made of 25 genera, and this genus is of 262 species (as of March 2020), all of which are phenotypically, ecologically, and genotypically varied [10]. The reclassification comprises host-adapted species *L. delbrueckii* group and *Paralactobacillus* with the other 23 new species being *Amylolactobacillus, Apilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacillus, Loigolactobacilus, Lacticaseibacillus, Latilactobacillus, Holzapfelia, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lactiplantibacillus, Furfurilactobacillus, Bombilactobacillus, Paucilactobacillus, Limosilactobacillus, Fructilactobacillus, Acetilactobacillus, Levilactobacillus, Secundilactobacillus, and Lentilactobacillus*. This new classification represents the microorganism's evolutionary relationships, and environmental, biological, and metabolic properties [10].

2.2 Ecological niches of *Lactobacillus*

Lactobacillus species are among the most "sensitive" and fastidious microorganisms, which are usually located in nutrient-rich settings and other environments. These nutrient-rich habitats are classified as fermented/spoiled foods and animal

feed, and the environment includes plant surfaces, soil, and the bodies of vertebrates, invertebrates, and humans [8]. Lactobacilli are the most common microorganism found in the microbiota of fermented foods. Lactobacillus is prominent in fermented foods such as cheese, yogurt, and milk (dairy products) because lactobacilli species are the primary bacteria employed in dairy fermentation. Lactobacillus has also been identified in fermented foods such as meats, veggies, and sourdough [8]. Due to that, the Lactobacillus species *L. plantarum* is the most common bacterial species that occurs naturally in vegetables like cabbage and lettuce [11]. Lactobacilli can also be found in the natural microbiota of the host animals, occupying numerous niches. They are healthy bacteria and therefore found in the human's gastrointestinal, urinary, and genital systems without causing any harm to the human body or producing disease [2]. They are also present in the mouth cavity and vaginal canal in humans forming part of the human microbiome and imparting great benefits to the human system such as the prevention of the growth of other harmful bacteria [12].

Lactobacillus can also be found in sewage and soils, where it causes fecal pollution. Soil samples have yielded isolates of *L. plantarum, Lactobacillus paracasei* subsp. *paracasei,* and *Lactobacillus brevis* species [13]. Lactobacillus has also been found and isolated in plants where sugar traces can encourage their growth.

2.3 Characterization of *Lactobacillus*

Lactobacillus is a LAB that is known for producing lactic acid as a byproduct of glucose metabolism. Considering the characteristics of microorganisms, Lactobacillus is perhaps the most important LAB being utilized widely in the food industries as starter cultures to perform key responsibilities such as the production of fermented foods and their possible probiotic effects on humans [14]. These LAB (Lactobacillus) cells have rod-shaped with a size in the range of 0.5–1.2 × 10^{-10} μm; however, under certain growth conditions, the cells assume a coccoid-like shape [15]. Lactobacilli are auxotrophic and therefore grow best in nutrient-rich media, with the best growth temperature being 30–40°C; however, they can survive in a range of 5–53°C. The best (optimum) growth pH range for these LAB is 5.5–5.8 but can also grow at a pH < 5 [15]. The survival and viability of probiotic bacteria in adequate amounts in a product are the most significant requirements when considering the characteristics of probiotic strains in food. These Lactobacillus probiotic strains are not deficient in these characteristics and therefore make them a very good strain for the food industries. The characteristics of Lactobacillus that make them suitable probiotic microbe for use in the food industry include their resistance to bile and acid, adherence to human epithelial cells, colonization of the human intestine, production of antimicrobial substances, favorable growth characteristics, and favorable effects on human health. All these "rich" characteristics make it ideal for the effective utilization of the Lactobacillus strain in industries [16].

3. Health benefits of lactobacillus

Lactobacilli are useful probiotics that have a wide range of benefits ranging from health, industries (both pharmaceuticals and food), and fermentation of food substances to induce good sensorial properties such as flavor, texture, and increase the shelf life of the food (preservation). These LABs also form part of the human microbiota and induce a good effect on the human body system preventing the

proliferation of other harmful and pathogenic microorganisms. Lactobacillus, for example, can aid in the digestion of food, the absorption of nutrients, and the fight against disease-causing microbes. For that reason, lactobacillus is said to be the most frequent bacteria used to treat diarrhea, including viral diarrhea and diarrhea caused by medications. Lactobacillus is also used to treat general digestive issues, newborn colic, and a variety of other stomach and intestinal ailments. Discussed below are some health benefits of Lactobacillus.

3.1 Protection of vaginal ecosystem

Microorganisms such as bacterial species have been shown to invade and create complex communities, or microbiota, in various human body parts (**Figure 1**). These microorganisms being present in the human microbiome appear to have a crucial role in the human body such as development, physiology, immunity, and nutrition [18]. The vulvar cleft in humans (females) connects the uterine cavity, endocervix, and vagina to the outside. These canals aid blood flow during menstruation, but they are frequently inhabited by a diverse mix of indigenous microbial flora. Lactobacillus species have long been recognized to exist in the vaginal canal of humans and serve a significant and perform essential role in maintaining and improving health by limiting the proliferation of harmful organisms by creating various defensive mechanisms [19]. Lactobacillus can provide these important health benefits to the individual by producing lactic acid, bacteriocins, and hydrogen peroxide, all of which limit the growth of bacteria, being harmful or not and fungal pathogens that cause urogenital tract infections [20]. This argument of Lactobacillus being present in a female's vagina canal to improve health agrees with the fact that Döderlein isolated Döderlein's

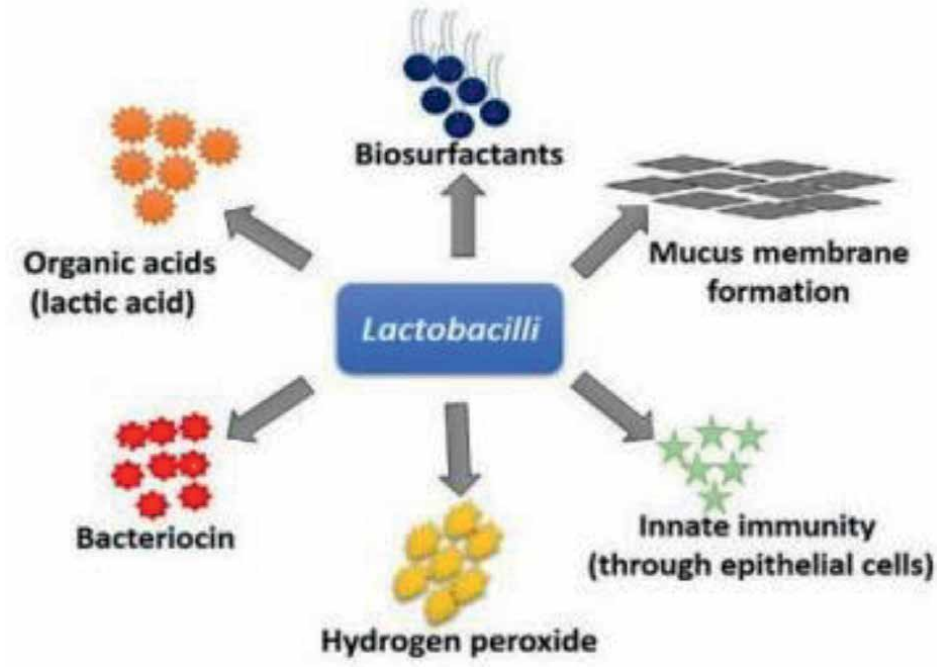

Figure 1.
Action mechanism of vaginal lactobacillus species [17].

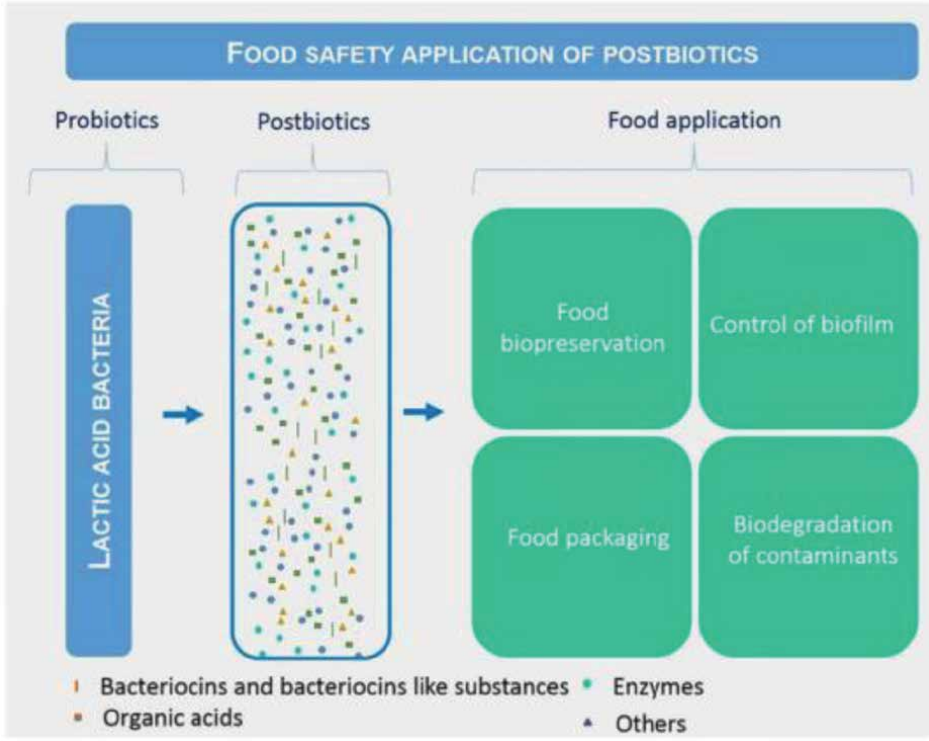

Figure 2.
The potential applications of postbiotics in food commodities [22].

bacillus from a healthy pregnant lady's vaginal samples and called it Lactobacillus [21]. It is generally known that the Lactobacillus being present in large quantity in the female genital organ's canal is "healthy" or "normal," and this is attributed to the vagina's excellent defense mechanism against other harmful bacteria, whereas a low or absent amount is deemed "abnormal" [17]. Lactobacillus species produce lactic acid as a product of glycogen digestion in follicular cells of the vaginal epithelium, resulting in an acidic environment (pH 4.5) [21]. A healthy vagina's acidic environment is an essential defense mechanism against the multiplication and growth of many potential diseases and infections [17]. Inhibitory substances produced by Lactobacillus to protect the vagina are shown in **Figure 2**.

The vaginal Lactobacillus functions by releasing substances that include lactic acid, bacteriocins, hydrogen peroxide, and biosurfactants to prevent the proliferation of harmful microorganisms and regulate the immune cells [17].

4. Carbohydrate metabolism of lactobacillus

The first and most essential step in food fermentation is the breakdown (catabolism) of carbohydrates by LAB. LAB as a group has a tremendous ability to break down carbohydrates and related substances. Lactic acid (>50% sugar carbon) is the primary product. It should, however, be noted that LAB adapts to different

environments and adjusts their metabolism accordingly. This could result in dramatically different final product patterns [23].

4.1 fermentation of glucose

Fermentation of glucose with LAB occurs in two pathways which include either the pentose phosphate pathway or the Embden–Meyerhof–Parnas (glycolysis) pathway. These two major pathways are used to phosphorylate and metabolize glucose sugar [24]. Except for *Leuconostocs*, the obligately heterofermentative Lactobacilli, *Gonococci,* and *Weissellas,* the Embden–Meyerhof–Parnas pathway is found in all LAB. In this process, 1 mole of glucose produces 2 moles of lactic acid and results in a net gain of two ATPs [23]. According to de Oliveira [25], glycolysis can result in heterotactic fermentation under certain conditions, and some homofermentative LAB utilizes the pentose phosphate pathway to metabolize substrates. The phosphoketolase split of xylulose-5-phosphate to glycerol aldehyde-3-phosphate (GAP) and acetyl-phosphate is regarded as an essential and important step in the pentose phosphate pathway. After that, GAP is converted to lactate, and acetyl-phosphate is also metabolized to acetate and ethanol [24]. In sourdough fermentations, Lactobacilli are largely heterofermentative and use the pentose phosphate pathway to break down glucose. Oxygen and fructose may both be used as electron acceptors in microaerophilic circumstances, resulting in the creation of other metabolites such as acetate and mannitol [23].

4.2 Fermentation of fructose

Fructose, a hexose sugar abundant in many plants, is one of the most important monosaccharides for bacterial growth in most plant-associated habitats, and it is usually fermented by the two Lactobacilli metabolic pathways [26]. *Lactobacillus sanfranciscensis* and *Lactobacillus pontis* can thus use fructose as a carbon source; nonetheless, when maltose is present, they utilize it most as an electron acceptor to create mannitol [27], especially when oxygen is limited [27]. The acetate kinase process generates additional ATP during the conversion of fructose to mannitol, resulting in a shorter lag phase with a greater growth rate and biomass production. The predominant product, acetic acid, has a molar ratio of 4:1 (fructose: maltose) [27]. Fructose is converted to mannitol by *L. sanfranciscensis,* but a little amount of lactic acid and ethanol are produced by *L. pontis* [28].

4.3 Fermentation of lactose

Lactose is mostly regarded as the major carbohydrate in dairy products, and it is the only carbohydrate that is usually used by Lactobacilli for the rapid growth, development, and production of acid [7]. The utilization of lactose by Lactobacillus is usually determined by the system of transport and enzymes involved in the hydrolyzation of the lactose sugar. The Lactobacillus species transports the lactose sugar into the cell through the permease and after the transportation sugar is then hydrolyzed (broken down) into glucose and galactose by an enzyme known as β-galactosidase. As the result of lactose fermentation, the simple glucose and galactose are next metabolized using the Embden–Meyerhof pathway to release lactic acid. This lactose fermentation process can also use a pathway known as 6-phosphogluconate, and in this process lactic acid, CO_2, and ethanol are produced as the end product [29].

4.4 Fermentation of pentose

Pentoses like arabinose, ribose, and xylose, among other related carbohydrates, like gluconate, may all be broken down by several LABs. These compounds are processed through the phosphoketolase pathway after being taken in by cells through permeases [23, 24]. Pentose phosphate is then metabolized by epimerases or isomerases who phosphorylate the pentoses to create ribulose 5-phosphate or xylulose 5-phosphate [23].

4.5 Fermentation of disaccharides

Disaccharides are substances composed of two simple sugars (monosaccharides). LAB breaks down (metabolize) and splits disaccharides into simple sugars (two monosaccharides) for easy absorption into the bloodstream. Lactose, cellobiose, maltose, melibiose, sucrose, and other disaccharides are among those digested by LAB [24]. These sugars are transferred as free sugars or phosphorylated sugars passing through the cell membrane and then splitting into two monosaccharides or a monosaccharide and a monosaccharide phosphate [30]. Even though disaccharide fermentation appears to be more complicated than monosaccharide fermentation, some LABs prefer disaccharides as growth substrates; lactose fermentation by dairy LAB and maltose fermentation by sourdough LAB are two examples [24].

5. Lactobacillus as probiotics

5.1 History of probiotics

The term probiotic was taken from the Latin word (pro) and the Greek word (bios), both of which means "for life." The probiotics theory was introduced in the early twentieth century, according to Zoumpopoulou et al. [31]. A German chemist known as Werner Kollath came up with the term "active chemicals which are important and required for the healthy growth and development of life" in 1953 and that was when probiotics were first introduced. Probiotics were also defined by Lilly and Stillwell in 1965 as "substances released by an organism that boost the growth of another organism." In 1992, Fuller, on the other hand, also came up with a definition that "probiotics are live microbial feed additive with great benefits to the host by enhancing gut microbial balance" [32]. All these concepts are linked to the belief that probiotics are beneficial to human health. Despite all the above definitions for probiotics, FAO/WHO in [3] described probiotics as "live microorganisms that, when administered in suitable proportions, impart a health benefit on the host," and this FAO/WHO definition for the term "probiotics" was the most generally accepted. However, the relationship of being beneficial to health was not overlooked in the FAO/WHO definition, implying that probiotics are extremely beneficial to human health.

The first food makers used bacteria and yeasts to ferment this milk into fermented dairy products, although they were unaware of the presence of these microorganisms [33]. Scientists such as Hippocrates and others considered fermented milk to be more than just a food product but also to have useful medicinal properties. This observation supported the fact that sour milk was, however, later prescribed for curing stomach and intestinal disorders [34]. Louis Pasteur found the microorganisms

responsible for the fermentation of this milk and other fermented products in the early 1900s, whereas Eli Metchnikoff (Russian scientist) was also the first to discover the positive influence of these microbes on human health [35]. As a result, he proposed his longevity without aging theory and linked it to the Bulgarian bacillus discovered by Stamen Grigorov (Bulgarian physician), and later suggested that lactobacilli might help mitigate the putrefactive effects of gastrointestinal metabolism that contributed to illness and aging [35]. He also stated that toxins produced by bacterial putrefaction in the large intestine and released into the circulation are the cause of aging.

"According to Metchnikoff, the intestinal bacteria' need for food allows humans to develop techniques to change the flora in our bodies and replace harmful microorganisms with healthy ones." This then explains in detail the "probiotic concept." Lactobacilli are considered probiotics by Metchnikoff (as opposed to antibiotics, which are damaging to the host's life), and probiotics may improve health and delay aging. Metchnikoff is based on these scientific theories in the development and expansion of the French dairy industry. The Pasteur Institute's Henry Tissier also isolated bacteria (now known as *Bifidobacterium bifidum*) from the feces of healthy breastfed newborns and advised recommending it to babies with diarrhea [23].

5.2 Probiotic consumption

Probiotic consumption is critical since they provide numerous advantages to the body, particularly the gastrointestinal tract (GIT). The most essential issue, however, is how it is introduced into the biological system. In the late 1960s, some viable probiotic strains such as Lactobacillus (some species) and *B. bifidum* "group" were introduced as cultures into dairy products in Germany due to their ability to adhere to and adapt to intestinal walls, as well as their useful property of producing mildly acidified products such as yogurts [36]. Though these fermented milky products were popular in Germany and were known as mild yogurts or "bio-yogurts," an alternative product known as acidophilus milk was also popular in the United States at the time (s). The incorporation of these probiotic strains into these fermented milk products and other commodities results in a variety of ways of administration and consumption into the human system, including meals, mostly fermented, and pharmaceutical items, such as capsules or microencapsulated form. According to Salminen et al. [37], if probiotic bacteria are a specified part of a food, Functional Food Science in Europe (FUFOSE) defines it as "live constituents of a food that exert beneficial effects on health."

Probiotic foods are always in demand in the general market because people are beginning to recognize their value; these foods currently account for between 60 and 70 percent of the overall functional food industry. This causes the dairy food sector to expand its market niche since a continual increase in dairy-type probiotic foods is noticed; nevertheless, nondairy items including vegetables, fermented meats, and fruit juices also contribute to the same amount of probiotic food products. Currently, there is no evidence that a high number of probiotics are harmful to human health; in some situations, they may even be quite useful and provide significant advantages to human health [38].

5.3 Origin and sources of probiotics

Probiotic bacteria have been discovered and isolated in a wide range of environments, including foods (fermented foods), animals, plants, and humans. These

probiotics are found in these various sources for a variety of reasons, including their fermentative and preservation impact (mainly in foods), contribution to good health in the human ecosystem (in humans), and so on. According to Ayivi et al. [1], the Lactobacillus and the Bifidobacterium species are the most frequently used probiotics. These two LAB strains have been identified to dominate the human intestinal wall due to their high adhesion capacity and are also generally considered safe for use (GRAS). According to Quigley [39], these safe Lactobacillus strains are well suited for gastrointestinal supplementation because Bifidobacterium is a prominent constituent as far as the large intestine is concerned, whereas Lactobacillus also being a major inhabitant of the small intestine. Streptococcus thermophilus, nonpathogenic strains of *Escherichia coli, Enterococcus, Bacillus,* and yeasts such as *S. boulardii* are also bacteria strains that are as good as these lactobacilli and *Bifidobacterium* [1]. Together with other probiotics, some strains particularly *E. coli* provide health benefits such as efficiently treating constipation and other related gastrointestinal diseases [40].

One of the most common sources of these probiotics is yogurt, which contains diverse bacterial strains such as *L. bulgaricus* and *S. thermophilus*. Yogurts are frequently fortified with strains that have favorable health effects on the human system when consumed. *S. thermophilus* has probiotic qualities, according to Pieniz et al. [41], and thus, its fortification in yogurt products is highly important to induce health advantages to the body. The strains utilized, on the other hand, are chosen based on their ability to survive in the product throughout its shelf life as well as during their transit to the stomach and the rest of the GI tract, allowing them to reach the distal tract, where they largely exercise their function.

5.4 Probiotics and human health

Probiotics are usually linked to several health benefits, with most studies concentrating on the gastrointestinal (GI) tract but also showing promise in other regions of the body such as the respiratory system, vaginal tract, and subcutaneous tissue [42]. These advantageous characteristics vary depending on the strain, and while different attributes may be linked to a particular strain, numerous activities are frequently carried out through microbe interaction, either with one another or with the host [43]. They produce chemicals (antimicrobial substances) such as bacteriocins, which are naturally occurring antimicrobial substances that are typical of a proteinaceous nature with a lipid or carbohydrate moiety, or organic acids (specifically lactic and acetic acids, hydrogen peroxide, and so on) that lower the pH of the system during their colonization as well as other activities such as growth and metabolism [44]. These natural antimicrobials help to suppress infections. They can improve epithelial and tissue integrity and functionality during their stay in the gut, primarily by producing low levels of nitric oxide (NO), increasing mucus production, improving gut epithelial cell proliferation, inhibiting carcinogenic substance production or elimination through detoxification, and producing nutrients, particularly short fatty acids and vitamins [45]. Many different Lactobacilli and Bifidobacterium strains are presently available for use by humans in the inhibition and treatment of gastrointestinal (GI) infections [46]. Probiotics can promote intestinal health by regulating microbiota, stimulating and building the immune system, synthesizing and improving nutrient bioavailability, reducing symptoms of lactose intolerance, and lowering the risk of a variety of diseases [1]. During hydrolysis, several of these probiotics produce enzymes that have been demonstrated to improve protein and fat absorption. LAB, for instance, has been proven to boost vitamin B complex concentration in fermented

foods [47]. Microorganisms acting in the digestive system or during the manufacture of cultured foods have been shown to increase the digestibility or absolute amounts of several dietary components, according to [47]. Probiotics offer a wide range of health and developmental benefits, some of which are discussed below. During fermentation, probiotics such as Lactobacillus can create enzymes such as lactase, which aid in the breakdown of lactose in dairy products. Lactose is a disaccharide that can induce intestinal distress in people with low levels of the intestinal enzyme lactase, resulting in bloating, gas, and abdominal pain [47]. Lactose intolerance is a genetic condition that affects around 75% of the world's population [48]. This condition limits the use of dairy products in a certain group of people. Lactobacillus, however, produces the enzyme known as lactase during fermentation, which hydrolyzes lactose into glucose and galactose for absorption. Kim and Gilliland discovered in 1983 that feeding lactose-intolerant individuals with fermented milk resulted in a significantly lower level of hydrogen in the breath when compared to individuals being fed with unfermented milk, and this low level of hydrogen indicates that lactose was metabolized before entering the large intestine.

Several probiotics have also been discovered to be added to the feed of animals to enhance the weight of these domestic animals. This weight gain was attributable to illness management and increased nutritional digestibility in the animals. Robinson and Thompson [49] conducted a study on humans and discovered that infants gained 21.9 oz. on average during their first month of life when fed a regular formula and 26.5 oz. when fed a special formula fortified with *L. acidophilus.* This research confirms that probiotics are useful in stimulating growth and development in young animals and individuals, according to evidence from different studies. By enhancing gut microbiota, restoring antioxidant systems, reducing insulin resistance and inflammation, and improving gut microbiota, probiotics also play a crucial role in the prevention of metabolic diseases including obesity, diabetes, and cardiovascular disease [50]. By competing with and sticking to the mucosal surface of the intestine and stimulating immunological responses, probiotics reduce intestinal infection and inhibit the development of Candida and *Helicobacter pylori* [51]. Some recommended microorganisms used as probiotics in both humans and animals and their health benefits are grouped in **Tables 1** and **2**, respectively.

5.5 Probiotics in cancer prevention

The gastrointestinal tract (GIT) of humans serves as a "storage" for the rich and diverse collection of microorganisms (gut microbiota), predominantly bacteria. Through homeostasis and illness throughout human life, this group of microorganisms generally has a significant impact on the host [63]. The human system has around 10 times more prokaryotic cells than eukaryotic ones because of the presence of gut bacteria. Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus, and Enterococcus, as well as some *Bacillus* and *Saccharomyces* strains, are among the numerous microorganisms found in the GIT [64]. However, many of the products from probiotic products currently found in the market these days contain more LAB from the genera Lactobacillus and Bifidobacterium [65]. These beneficial probiotic microorganisms impart and play a variety of roles in the human system, including cancer prevention and treatment. Cancer is defined as a disease that emerges because of uncontrollable cell growth and spreads to other sections of the body. This killer sickness has been one of the human battle diseases that, if not treated properly, can usually lead to death. However, there has been a scientific basis and proof that

Lactobacillus	*Bifidobacterium*	*Streptococcus*	**Other bacteria**	**Yeast and molds**
acidophilus	*bifidum*	*lactis*	*Leuconostoc*	*Saccharomyces cerevisiae*
plantarum	*infantis*	*cremoris*	*Pediococcus*	*C. pintolopesii*
casei	*longum*	*alivarius subsp. thermophilus*	*Propionibacterium*	*A. niger*
rhamnose	*thermophilum*	*intermedius*	*Bacillus*	*A. Oryza*
delbrueck subsp. Bulgaricus	*adolescents*		*Enterococcus*	
GG	*animalis*		*E. faecium*	
reuter	*angulatum*			
fermentum	*pseudocantenulatum*			
brevis				
lactis				
cellobiosus				
johnsonii				
paracasei				

Table 1.
Microorganisms used as probiotics for humans and animals [50].

probiotic bacteria, such as the above-mentioned strains, usually prevents or minimizes the formation of some group of cancers in the human body, and this is because members of the gut microflora can create carcinogens such as nitrosamines [1]. Numerous studies have also shown the possibility of probiotics for the prevention and treatment of cancer. Most of these studies focused on the modulation of the microbiota, the immune system, the reduction of bacterial translocation, the improvement of gut barrier function, the anti-inflammatory and antipathogenic activity, and the effects of probiotics on tumor growth and metastasis [65]. Verhoeven et al. [66] discovered that regular intake of probiotics (drink) for about 6 months increased the clearance of HPV and cervical cancer precursors in 54 women. Another group of researchers discovered that probiotics or synbiotics significantly reduced the activity of intestinal procarcinogen enzymes linked to colonic carcinogenesis in experimental animal models [67]. All these findings from different research sources propose and portray probiotics to be a good source of cancer inhibition and reduction in the human system, thus making these useful microbes for consumption especially strains from the Lactobacillus and the Bifidobacterium genera.

Additionally, LAB may influence the maturation of immune cells and their products not only in the gut but also in systemic immunological organs like the lymph node and spleen, resulting in tumor inhibition [65]. These data suggest that probiotics could be useful dietary supplements against neoplastic susceptibility due to their wide influence on the host's local and systemic immune mechanisms [68].

Another encouraging research was published in 1980 by Goldin and Gorbach, who were among the first to correlate Lactobacillus-rich diets to a decreased risk of colon cancer (by 37 percent compared to controls). Many *in vitro* studies have also indicated

Probiotic strain	Health benefit	Reference
Lactobacillus rhamnosus GG	Prevention or alleviation of infantile diarrhea Prevention of early atopic disease in children at high risk	Salminen et al., [37], Szajewskah and Mrukowicz [52]
Bifidobacterium lactis HN019, L. rhamnosus HN001	Enhancement of natural immune function in healthy people	Arunachalam et al., [53]
E. coli Nissle 1917, S. boulardii, Lactobacilli, *Bifidobacterium*	Maintenance of remission of ulcerative colitis Treatment of Crohn's disease	Hart et al., [54], Marteau et al., [55]
Lactobacilli	Improvement of lactose digestion	Goldin [47]
Lactic Acid Bacteria	Reduces risk of bladder cancer	Ohashi et al. [56]
L. rhamnosus GG, Bifidobacterium species 420, L. acidophilus 145	Reduction of the occurrence of pathogenic bacteria in the nose	Gluck and Gebbers, [57]
L. casei strain Shirota, B. longum BB536	Reduces the severity of constipation and improvement of bowel movement and stool consistency. Increase defecation frequency and stool softness	Chen et al., [58]
Lactobacillus, Bifidobacterium	Treatment of *H. pylori* infection	Kuo et al., [59]
Enterococcus faecium, S. thermophilus	Decrease in LDL-cholesterol	Sanders and Marco, [60]
L. acidophilus NCFM	Minimizes risk of diabetes and obesity	Andreasen et al., [61] Sanchez et al., [62]

Table 2.
Probiotic bacteria and their health benefits.

probiotics' therapeutic properties in regulating the growth and death of cancer cells such as gastric, colonic, and myeloid leukemia cells [69]. Despite this, many researchers have discovered that the *L. rhamnosus GG* strain has a significant antiproliferative effect and/or induces apoptosis in mus musculus colon carcinoma (HGC-27) and human colonic cancer cells (Caco-2, DLD-1, and HT-29) [70] as well as lowering the level of IL-8 [71].

The ability of probiotics to prevent the growth of colon cancer (colorectal) may be attributed to several mechanisms, some of which include modification of the intestinal microflora, inactivation of cancer-causing agents, competition with putrefactive and pathogenic microbiota, improvement of the host's immune system, antiproliferative effects such as regulation of apoptosis and cell differentiation, fermentation of undigested food, and inhibition of tyrosine [72].

6. Lactobacillus as a starter culture for the dairy industry

Lactobacillus and other related species have been utilized as starter cultures for fermentation operations in a wide range of industries. Starter cultures are therefore regarded as an important component of basically fermented foods produced commercially, and nonetheless, these starter cultures are composed of useful microbes

such as *Lactobacillus* which are directly introduced into the various food components, which help to produce the desired and predict the occurrence and characteristics in the finished product, which is the food [73].

Several fermented foods can indeed be produced without the need for a starter culture; however, adding a concentrated number of microorganisms such as *Lactobacillus* as a starter culture makes a difference and ensures that the food has desired characteristics such as the extension of shelf life, increased nutritional value benefits, altered sensory aspects, and an increase in economic value. According to Durso and Hutkins [73], several local methods for creating starter cultures for LAB involve backslopping or inoculating (introducing into) a fresh batch with a little quantity of the completed, particularly preserved product.

Other ways for starter culture production include harnessing microorganisms naturally found on the product and using specific containers that allow the starter culture microbes to survive within cracks and holes. These traditional fermentation procedures, however, allow for the creation of a variety and distinct fermented foods and drinks, and they are still utilized in small- to medium-scale manufacturing facilities, as well as in developing nations. These techniques of creating starter cultures, on the other hand, are prone to delay or failure fermentations, contamination, and variable product quality [73]. Following the discovery that pure cultures could be used to mature milk in the late 1880s by a team of scientists led by Storch of Denmark, Weigman of Germany, and Conn of the United States, the significance of flavor-producing bacteria (i.e., citrate-fermenting diacetyl-producers) was quickly established. Therefore, Christian Hansen established a starter culture business in 1978 based on this knowledge, and it has since grown to become a significant starter culture provider to industries such as brewing, dairy, baking, wine, and meat industries. In the past, producer-created starter culture strains were made by growing pure strains in heat-sterilized milk. These liquid cultures are still useful and well-liked today, despite having a short lifespan because of the loss of cell viability activities due to fermentation. Another technique of culture preparation, known as the crude dry culture preparation method, was devised; nevertheless, this method required multiple milk transfers to activate and respond to the culture. Freeze-dried cultures that were first produced in the 1960s are now noted to be one of the dairy cultures being used widely in the food industries. These cultures now dominate the starter culture industry because of the major advancements in freezing and freeze-drying methods. Given that starter culture bacteria's primary function is to ferment sugars and create acids, the capacity of LAB to metabolize carbohydrates is very essential.

7. Postbiotic

"Postbiotics" is known to be a new term in the biotic field, and therefore, they are still not common to the public. Therefore, in contrast to pre- and probiotics, it has, however, become more challenging to get a consensus on the true definition of postbiotics in the available literature. Nevertheless, Tsilingiri and Rescigno [74] defined postbiotics as any chemical released/created by the metabolic activity of a bacterium that directly or indirectly benefits the host. Postbiotics were also described by Blazheva et al. [6] as "a preparation of inanimate microbes and their constituents which is beneficial to the health of the host." Postbiotics are therefore functional bioactive substances that are created in a matrix during fermentation and are employed to support the health of individuals [6]. Several of the suggested health benefits

of probiotic, prebiotic, and synbiotic additions depend on the possible production of short-chain fatty acids and components such as microbial fractions, functional proteins, secreted polysaccharides, extracellular polysaccharides, cell lysates, teichoic acid, peptidoglycan-derived muropeptides, and pili-type structures [75–77]. These understandings contributed to a reappreciation of food fermentation and gave rise to the theory of postbiotics. Postbiotics can also be described as functional fermentation chemicals, such as the ones mentioned above, that can be combined with dietary elements to enhance health [64]. Paraprobiotics and fermented infant formulas (FIFs) are two examples of postbiotics that are frequently mentioned. These days, the term "paraprobiotics," also known as "ghost probiotics," "non-viable probiotics," or "inactivated probiotics," is frequently used to refer to nonviable or inactivated microbial cells that, when provided in adequate proportions, benefit the host [64], whereas FIFs are baby or follow-on formulae that have been fermented with lactic acid-producing or other bacteria and often do not contain living bacteria [64]. The potential for postbiotics to boost the effectiveness of active microorganisms or transform them into useful components is possible. Additionally, postbiotics get around the technical difficulties of maximizing colonization and preserving the viability and stability of the microorganisms at high doses in the product. Postbiotics can also be employed in circumstances where it is more difficult to regulate and maintain the conditions for manufacture and storage, such as in underdeveloped nations [64]. Additionally, it has been suggested that using postbiotics in critically ill patients, young children, and premature newborns may be a desirable substitute for other "-biotics" [78]. Food, microbiology, and customized medicine may become even more intertwined according to the postbiotics idea [64].

In general, all substances created because of microbial fermentation might be "postbiotics." Additionally, the concept of these postbiotics is often established on the concept that these microbiotas release a variety of metabolites during/after fermentation, and these metabolites impact positive effects on the health of humans. In addition to treating various types of diarrheas, postbiotic consumption in healthy people has been shown to improve general health and alleviate the symptoms of a variety of illnesses, including atopic dermatitis in adults and colic in newborns. Numerous studies have shown that postbiotics can have clinically significant effects as well as immunomodulatory effects. In addition, postbiotics also have antioxidant, anti-inflammatory, and anticancer capabilities [79]. Postbiotics have some significant functions, which include boosting active microorganisms to work more effectively or transforming them into useful components. This might perhaps speed up the transport of active substances to the intended location in the gut and extend the shelf life of these chemicals [80].

Postbiotics may also impart several benefits in food safety, and one of the advantages of adding probiotics to food is that they interact with pathogenic bacteria, which may inhibit pathogen growth by competing with them for resources such as nutrients or by secreting antimicrobial compounds [81]. Probiotic bacteria generate antimicrobial compounds known as postbiotics, which have the potential to play a significant role in food safety by preventing the development of pathogens in food and enhancing consumer health. Numerous bioactive metabolites, including organic acids, short-chain fatty acids, carbohydrates, antimicrobial peptides, enzymes, vitamins, cofactors, immune-signaling compounds, etc., are present in postbiotics made from LAB [82, 83]. In almost every study pertaining to food safety, the authors created a CFS solution that contained biological compounds produced by target bacteria and employed as postbiotics. Several LAB strains can be thought of as probiotics, and

their postbiotic products frequently provide consumers with similar or complementary health benefits [84]. To enhance the technical qualities and lengthen the shelf life of foods, LAB has been widely employed, whether as the primary starter or as the secondary starter [22]. Postbiotics have several qualities that distinguish them from probiotics and make them important components. For instance, postbiotics have advantages over parent bacterial live cells in that they have a longer shelf life, safer structures, cannot spread antibiotic resistance, do not produce biogenic amines (BA), are simple to use and store, are stable in a wide range of pH and temperature, and have broad-spectrum antimicrobial activity [22, 85]. The difficulty in using live starter cultures directly in food products is ensuring that they will grow and survive in a variety of food matrices and settings. In this regard, the direct addition of a postbiotic mixture or individual postbiotic prevents unfavorable interactions between live primary and adjunct starters for antimicrobial purposes [86]; however, there are some challenges in the use of individual postbiotic of starters and protective cultures. Despite the high expense of bacteriocin separation and purification, antimicrobial metabolites have a restricted spectrum, making it possible for infections that have been treated with some of them, such as bacteriocins, to acquire resistance. In addition to a postbiotics mixture's excellent heat stability, food may fully benefit from its broad-spectrum antibacterial activity and the synergistic interactions between organic acids and other metabolites. Individual postbiotics perform a variety of well-known and newly discovered food safety roles, such as food biopreservation and packaging, control, and elimination of the biofilm of foodborne pathogens, biodegradation of dangerous chemical contaminants (such as mycotoxins, pesticides, and BAs), and much more. According to Moradi et al. [22], the kind of target microbe or pollutant, the concentration and method of administration, and the properties of the food matrix all affect how effective postbiotics are in food systems.

7.1 Antioxidants potential of probiotics and postbiotics

7.1.1 Probiotics

Antioxidants are chemicals that significantly slow down or stop an oxidizable substrate from oxidizing when present in a low amount as compared to the other substrate [87]. According to Blazheva et al. [6], oxidative stress is a pathological situation that occurs when reactive oxygen species (ROS) overtake the body's antioxidant defenses, causing tissue damage, accelerated cell death, and oxidative modification of biological macromolecules (such as lipids, proteins, and DNA). Oxidative stress is just a body-wide imbalance between free radicals and antioxidants. In this circumstance, reactive nitrogen species (RNS), which cause oxidative stress, and ROS are mostly captured by antioxidants [6]. Antioxidants act as scavengers of ROS and RNS, protecting living organisms from the damaging effects of oxidative stress; as a result, compounds having antioxidant activity are of special importance. Probiotic bacteria thus have special properties that can give antioxidant effects to humans, according to recent studies, and this helps to avoid disorders linked to oxidative stress. Additionally, probiotic bacteria influence the intestinal barrier's permeability and inhibit the overabundance of dangerous bacteria in the gut microbiota [6].

Bifidobacteria are one category of microorganisms that are often regarded as probiotics since they live naturally in the human GIT and have been linked to a healthy colon microbiota [6]. Because Bifidobacteria are strict anaerobes, the oxygen being present in the GIT acts as a stressor, causing it to create antioxidant molecules

to scavenge these free oxygen radicals. Nonetheless, the amount of these antioxidants produced has not been reported in the literature [6]. Bifidobacteria can produce a variety of chemicals that can impede free radical oxidation processes and decrease oxidized molecules. These chemicals and other metabolites are known as the "post-biotics," and they help these probiotics in their specific actions. Some Bifidobacteria produce conjugated linoleic acid metabolites that exhibit the capacity to shield cells from damaging oxidative activity. Different species of Bifidobacteria have genomes that contain a gene for linoleic acid isomerase [88]. As byproducts of the fermentation of plant materials, Bifidobacteria are also capable of producing polyphenols, lignans, and flavonoids, all of which have an antioxidant impact and contribute to the probiotic function of the microbe [6]. A total of 25 Bifidobacterium strains were evaluated by Braune and Blaut [89] for their ability to produce lignan and flavonoid aglycones from flaxseed and soybean extracts. Most of these *Bifidobacterium* strains increased the levels of apigenin, daidzein, genistein, naringenin, and secoisolariciresinol. Additionally, the Bifidobacterium *pseudocantenulatum* and *Bifidobacterium* breve strains produced quercetin and quercetagetin, enhanced the quantity of kaempferol, and exhibited significant levels of herbaceous synthesis. The *Bifidobacterium* strains converted a wide variety of flavonoids' glycosides into their aglycones, boosting their antioxidant activity and bioavailability. According to Mayo and Van Sinderen [90], studies on the biosynthesis of vitamins by Bifidobacteria have revealed that *B. bifidum, B. breve, Bifidobacterium adolescentis, B. longum subsp. infantis,* and *B. longum subsp. longum* can all produce the vitamins nicotinate, thiamine (B_1), pyridoxine (B_6), and folate (B_{12}). Vitamin B_6 is a cofactor of glutathione and plays a significant part in the antioxidant process. Folic acid (B_9) also boosts the lipoproteins' resistance to oxidation [90].

The gut microbiome includes another useful microbe called *Lactobacillus* as well, and they both possess strong antioxidant properties. It has been demonstrated that some strains of lactobacilli, although being facultative anaerobes or microaerophilic, may use oxygen as a substrate in processes mediated by flavin oxidases and in specific circumstances can create a minimal respiratory chain. The production of several antioxidant proteins is the main factor affecting lactobacilli's antioxidant abilities. Very infrequently, lactobacilli produce the enzyme superoxide dismutase (SOD), which neutralizes superoxide anion [6]. Included in the antioxidant abilities of lactobacilli are the same proteins that control the chelation of iron and copper ions. Numerous antioxidant substances, including riboflavin, vitamin B_{12}, and carotenoids, are produced by different Lactobacillus strains. In addition, Lactobacilli shows antioxidant traits for the microorganisms they are symbionts for. The genes and proteins that protect lactobacilli from free radicals and their antioxidant qualities are well understood; however, little is known on the parts of lactobacilli cells and their metabolites that shield other microorganisms from free radicals. According to studies, certain strains of lactobacilli's culture fluid exhibit these characteristics in the form of exopolysaccharides, linoleic acid metabolites, histamine, vitamin K_2, and soluble proteins [6]. Because probiotic bacteria from the Lactobacillaceae and Bifidobacterium families have historically been used by people to ferment food, they are widely regarded as safe (GRAS). Additionally, probiotic bacteria in the human gut have significant antioxidant activity and encourage the synthesis of antioxidant enzymes to aid in the removal of reactive oxygen species and so lessen oxidative damage. To protect cells from oxidative stress-related damage, probiotic bacteria that build up in the GIT can boost the activity of antioxidant enzymes and reduce systemic circulatory oxidative stress. In addition to being utilized to treat early stages of disorders such as ulcerative

colitis, irritable bowel syndrome, and allergic diseases, strains with antioxidant qualities can help the body's antioxidant status return [6].

7.1.2 Postbiotics

Research on the antioxidant properties of postbiotics has just become known. In an evaluation of the several postbiotic types of medications, Zólkiewicz et al. [79] investigated bacterial lysates, exopolysaccharides, enzymes, cell wall fragments, short-chain fatty acids, cell wall fragments, cell-free supernatants, and metabolites produced by the gut microbiota. According to Coelho et al. [13], *Liquorilactobacillus satsumensis, Leuconostoc mesenteroide,* and *S. cerevisiae* all have antioxidant activity that inhibited 2, 2-diphenyl-1-picrylhydrazyl (DPPH) by 20 to 28%.

Exopolysaccharide from *Lactococcus lactis subsp. lactis* has been studied *in vivo* as a postbiotics with reports of increased antioxidant enzyme levels (e.g., catalase, superoxide dismutase, and glutathione peroxidase activities) and decreased levels of lipid peroxidation in serum and mice livers [91]. Additionally, findings from *in vivo* studies support the same approach, showing that postbiotics have the capacity as an antioxidant and other health advantages. Postbiotics of lactic acid bacteria isolated from traditionally fermented sausages be potential agents of innovative pharmaceutical therapy for several illnesses associated with oxidative stress and are less dangerous alternatives to living microorganisms [6]. Studies have shown that the antioxidants of postbiotics vary and are influenced by factors that include the chelating ability of the metal ions, the antioxidant enzyme system, and the antioxidant metabolites present in them. As a result, postbiotics can be used as a treatment and feed supplements to minimize inflammation caused by disorders related to oxidative stress [6]. The above *in vivo* and *in vitro* studies provide crucial information regarding the ability of postbiotics to protect against oxidative stress, which is thought to be either a major or secondary cause of many cardiovascular illnesses, as well as to protect against damage caused by free radicals [92]. Postbiotics have also been studied for their potential antibacterial, antiviral, antioxidant, anti-obesity, anti-diabetes, antihypertensive, anti-proliferation, anti-mutation, and anticancer characteristics, and all these benefits have been shown *in vitro* and *in vivo* [93]. The most predominant benefits of postbiotics as natural antioxidants are generally their clinical (safety), technological (sustainability), and economical (low production costs) benefits [6].

8. Improvement of probiotic stability through biotechnological method

Probiotics must always maintain their viability and functionality during the process of production, storage, and ingestion to have their positive benefits. The manufacturing conditions (temperature, oxidation, shear stress, etc.), storage conditions (moisture/low water activity, packing, oxidation, temperature, etc.), and GIT conditions (low pH, bile salts, digestive enzymes) are the main principal factors that often impact these strains' capacity to survive. Encapsulation has been examined by several researchers as one of the direct methods for avoiding or lessening the impact of these factors. These probiotic bacteria are therefore delivered to their target places using this technology by establishing a microenvironment in which they can survive. Encapsulation is the process of shielding probiotics in particles to protect them from

the environment by incorporating their microbial cells and/or their biologically active ingredients. To effectively perform its tasks, the capsules used for the encapsulation covering must be thin, semipermeable, and mechanically stable. By selecting a substance for the capsule that can create a "friendly" environment in the stomach and shield the probiotic strains from the stomach's acidic conditions and the bile salts secreted from the pancreas, however, it can also be made to release the probiotic cells in a specific location on the body [6]. Depending on the required features, which include polymers, several methods and materials were devised for the encapsulation of the probiotics. Considering the qualities, the encapsulation technique and the intended use of the finished product can help you choose the best shell or carrier material for this encapsulation. It must be suitable for industrial purposes, widely accessible, and simple to deal with. Polysaccharides of the plant including those from cellulose, pectin, gum arabic, agar-agar, alginate, carrageenan, inulin, and maltodextrin and those from animals (chitin, chitosan, hyaluronic acid, etc.) are the most often utilized encapsulating materials [6]. Probiotics are additionally encapsulated using resistant starch, oligosaccharides, as well as other fibers from fruits, vegetables, cereals, bran, and husk. Animal (casein, whey protein, and albumin) and plant (soy protein, pea protein, etc.) protein encapsulation ingredients are usually included in this category. However, their primary disadvantage is that digestive enzymes can degrade them, although this problem can be solved by coating them with another polymer [6].

8.1 Freeze drying

Freeze drying/lyophilization is a technique in which water is removed from a product after it has been frozen and put under a vacuum, allowing the ice to convert directly from solid to vapor without going through a liquid phase. Probiotic stability is stabilized by freeze-drying (lyophilization), which is typically done with cryoprotectants present and with the water being sublimated under a vacuum [6]. The purpose of the cryoprotectants is to increase microbial survivability and stabilize the product during storage. Probiotics' viability and shelf life are also enhanced by lyophilization, although this does not affect how long they survive in the digestive system. As a result, it is widely used as a second step in microencapsulation processing to increase the shelf life of probiotic bacteria by drying them out after they have been encapsulated in emulsions or gels [6]. Sugars like glucose, mannose, lactose, trehalose are among the most popular low-molecular-weight cryoprotectants. Most of the molecules with large molecular weight are polysaccharides and proteins, such as soy protein, milk, gelatin, and maltodextrin [94]. Wang et al. [95] coated sodium alginate microcapsules containing *L. plantarum* CCTCC M 2014170 cells with an inner layer of inulin and an exterior layer of skim milk before freeze-drying the mixtures. The findings demonstrated that the probiotic's resilience to the GIT's simulated environment was enhanced by encapsulation. In the stomach, *L. plantarum's* viability remained unchanged after 2 hours, but in the 1% bile salt solution, there was a 1.21 log drop in cfu/mL. The microencapsulation of probiotics *L. acidophilus* and *L. casei* by freeze drying was also studied by Bora et al. [96] utilizing whey protein isolate, fructo-oligosaccharides, with a combination at ratio (1: 1). In contrast to lose cells, which had a low survival rate after 90 minutes of incubation at pH 2, the probiotic that had been encapsulated had a high encapsulation yield (98%) and was not significantly impacted by the simulated stomach juice [96].

8.2 Spray drying

Another technique used in food processing is spray drying, which is employed for a variety of purposes including enhancing probiotic stability. Due to its suitability for large-scale industrial applications, affordability, and ease of scaling up, it is frequently utilized in the production of food products and bioactive chemicals [97]. When probiotics are dried using a spray technique, the liquid feed used for the encapsulating wall and the probiotics are both atomized into a hot gas drying chamber where the wet droplets are heated to a high temperature [6]. Emergence of dried solid particles is caused by the rapid creation of crusts. The primary disadvantage to this approach is the application of osmotic stress and high temperatures, which might result in reduced viability and loss of activity. After being sprayed-dried in feed solutions with varied amounts of gum arabic, the viability of *L. acidophilus NCDC 016* cells was examined in a study [98]. The input air temperature increase caused a drop in encapsulation yield, which was addressed by raising the gum arabic content. It was once believed that the proteins in gum arabic were what caused the microbial cell wall to produce a protective layer. *S. cerevisiae var. boulardii* was also encapsulated by Arslan et al. [99] spray drying temperatures. Gelatine and gum arabic were the most viable wall materials for *S. boulardii* microencapsulation based on viability tests following spray drying and subsequent tests under simulated gastrointestinal tract conditions.

8.3 Extrusion

Probiotic cells are also encapsulated through extrusion that involves the suspension of the bacteria in droplets of liquid which later gel or have membranes developed on their surfaces. Using coaxial air or liquid flow, submerged nozzles, vibration technologies for the jet break-up, and other methods, one can obtain the droplets pouring from a nozzle [6]. The droplets then enter a solution that will harden them. Particles of different sizes, morphologies, and mechanical strengths are created by varying the processes and materials used to produce gel beads. This technique of encapsulation is simple, affordable, and highly kind to microbial cells. However, its slowness restricts its use on an industrial scale. The targeted release potential of the gel beads was proven by the encapsulated *B. adolescentis* cells' log reduction values, which ranged from 2.0 to 2.6. In addition, the gel beads disintegrated when exposed to circumstances simulating intestinal juice. The probiotic *L. acidophilus KBL409* was encapsulated in alginate and alginate-chitosan, and then, all samples were incubated for 1 or 2 hours at 37°C with simulated stomach fluids (pH 1.5) before being exposed to simulated intestinal fluids (pH 6.5) for the next 2 hours. The largest percentage of survivors were found in the alginate/chitosan capsules [100]. To protect the microbial cells and extend shelf life, Apiwattanasiri et al. [101] suggest using silk sericin as a wall material and coating layer for probiotic encapsulation.

8.4 Emulsion-based system

Two immiscible liquids combine to produce an emulsion when an emulsifier (stabilizing agent) is present. The technique of creating an emulsion is simple and comprises the rapid mixing of the two phases (continuous and dispersed phases)

while adding one phase over the other, which, if appropriate, also contains an emulsifier. Although it is easily scaled up, this process' primary limitation is that it generates particles of various sizes. Zhang et al. [102] developed secondary emulsions to contain *Ligilactobacillus salivarius.* The main emulsion was created by the emulsification of melted anhydrous milk fat with whey protein isolate or sodium caseinate in a neutral aqueous phase. This emulsion increased the encapsulation efficiency by up to 90% and improved the thermal and storage stability of *L. salivarius.* The probiotic survived the simulated gastric and intestinal digestions at a higher rate due to more cross-linking with calcium ions [102]. When *Lacticaseibacillus paracasei* was encapsulated in a milk-based water/oil emulsion, El Kadri et al. [103] found that the probiotic was more viable than free cells throughout 28 days of storage at 4°C, and this is due to the fact during storage the emulsion remained stable, and the encapsulated *L. paracasei* had a survival rate that was much greater than the free cells. To encapsulate the probiotic *L. casei* on an alginate matrix, flaxseed mucilage was employed. The encapsulation effectiveness was more than 95%, and stability and survival rates of the probiotic in simulated gastrointestinal conditions were evaluated [104]. As a result, *L. casei's* resistance to the negative effects of the simulated digestive system was strengthened by the introduction of flaxseed mucilage [104].

9. Conclusion

LAB, a class of widely distributed probiotic bacteria, plays a key role in the fermenting of different food products. LABs, specifically probiotics, have also been proven to impart a therapeutic effect on the remedy of several foodborne-related diseases. Thus, the role of these beneficial probiotics in fostering healthy microbiota and boosting resistance to illnesses and infections is crucial. Therefore, the importance of including probiotics in human diets cannot be overemphasized, given the numerous derived therapeutic health benefits. The survival rate of probiotics throughout processing, storage, and GIT transit has been improved by numerous authors using a variety of microbial encapsulation techniques. The gathered information demonstrates that these methods considerably boost the stability of various species and strains of probiotics. The effectiveness of such encapsulated microorganisms in the body must yet be evaluated through additional study. Even though the precise processes are still being completely understood, postbiotics may help to promote host health. For postbiotics to be shown to have a positive impact on health, well-designed randomized placebo-controlled intervention trials are required in addition to preclinical and *in vitro* research that focuses on the mechanisms of action. Additionally, a new era of "-biotic" research is beginning because of improvements in measuring the makeup and function of the gut microbiome. As a result, the variety of substances with potential health advantages that may be used in specialized nutrition has already increased and will continue to do so. Given that postbiotics face fewer difficulties in terms of storage and shelf life than viable probiotics, they can be a superb and secure way to improve health. Probiotics' potency may be effectively increased by postbiotics and bioactive substances, transforming them into useful components or medicinal agents. However, the nomenclature, regulatory considerations, and safety in use are the remaining postbiotics application-related issues that need further investigation by researchers to create global standards and regulations, which will then open new opportunities to produce healthier, safer, and more sustainable products [22].

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List of abbreviations

LAB	lactic acid bacteria
ROS	reactive oxygen species
RNS	reactive nitrogen species
GIT	gastrointestinal tract
FAO	Food and Agricultural Organization
WHO	World Health Organization
GRAS	generally recognized as safe
ATP	adenosine triphosphate
GAP	glycerol aldehyde-3-phosphate
GI	gastrointestinal
HPV	human papillomavirus
FIFs	fermented infant formulas

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The dairy industry faces challenges that impact the quality, safety, nutrition, and consumer acceptability of dairy products. The most important dairy product is milk, which is one of the most nutritious of all foods and is widely consumed worldwide. As such, it is important to optimize processes and innovation to convert milk into value-added products to ensure consumer well-being and food security for the growing global population. This book provides a comprehensive overview of the science and technology of dairy processing. It includes eight chapters that discuss recent issues, technology updates, and unique topics in the field. Topics include starter cultures in fermentation, camel milk, milk-borne infectious disease, lactic acid bacteria, and much more.

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