Methods and Protocols in Food Science

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Editors

Probiotic Foods and Beverages

Technologies and Protocols

Humana Press

Methods and Protocols in Food Science

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Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

Volumes and chapters will be organized by field and presented in such way that the readers will be able to reproduce the experiments in a step-by-step style. Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol will be clarified.

Probiotic Foods and Beverages

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Preface to the Series

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated and used by food and related scientists, as well as theoretical basis are provided for each protocol. Aspects related to improvements in the protocols, adaptations and further developments in the protocols may also be approached.

Methods and Protocols in Food Science series aims to bring the most recent developments in research protocols in the field as well as very well established methods. As such the series targets undergraduate, graduate and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by a comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the do's and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The Methods and Protocols in Food Science series will fill an important gap, addressing a common complain of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

Campinas, Brazil ***Anderson S. Sant'Ana***

Preface

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have defined probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The global probiotics market was valued at USD 58.17 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 7.5% from 2021 to 2030. The health promotion provided by these microorganisms has been the main driving force of this market niche. Also, an emerging functional food discipline in this field is using postbiotics and paraprobiotics in food and beverages. Paraprobiotics and postbiotics can express health benefits in addition to the inherent viability of probiotics, proving that not all mechanisms, nor clinical effects, are directly related to viable bacteria and broadening the current concept of what probiotics are. Furthermore, paraprobiotics and postbiotics have valuable potential for developing biotechnological products with functional ingredients and are more stable, allowing for easier use on an industrial scale.

Protocols in Technology of Probiotic Foods and Beverages is a book that addresses the latest relevant state-of-the-art protocols to manufacture functional probiotic foods and beverages. In addition, this book combines, as comprehensibly as possible, well-established protocols and procedures used by many laboratories in academia and industry.

Regarding dairy products, Chap. 1 provides information about the material, main processing procedure, and packaging steps for processing fermented milks. At the same time, Chap. 2 discusses probiotic strains used to manufacture different cheese types and the survival of those probiotics, regarding actions taken to increase their viability. The limitations from research to industrial limitations and the main factors to consider for appropriate probiotic strain selection for industrial application are pointed out. Chapter 3 is a practical guidance for probiotic ice cream manufacture, presenting the steps and amount of probiotic addition into ice cream production. Finally, Chap. 4 is a practical guidance for probiotic butter manufacture, discussing ways of adding probiotics.

Regarding non-dairy products, Chap. 5 deals with plant-based beverages, demonstrating the process of obtaining soy, oat, and rice extracts and the fermentation process to obtain probiotic beverages. At the same time, Chap. 6 describes the process of obtaining probiotic plant-based cheeses, such as pea cheese, tofu, soy-based cream cheese, and chickpea petit Suisse cheese. Chapter 7 describes a method incorporating probiotic bacteria encapsulated in an alginate matrix using an emulsification process as a pretreatment into fruit juices. Furthermore, techniques for morphological analysis by scanning electron microscopy, as well as the characterization of the juice and the evaluation of cell viability against simulated gastric conditions, are provided. Chapter 8 describes the process of obtaining probiotic-fermented vegetables, such as pickles, sauerkraut, and natto. Chapter 9 describes two preparation methods of Kombucha using a symbiotic culture of bacteria and yeast or a synthetic microbial community as a starter. Moreover, the determination of bioactive compounds, including organic acids, sugars, and catechins, has been introduced. Chapter 10 provides a guideline on preparing a probiotic beer that can be used for researching new probiotic microorganisms and highlights essential points to be considered when developing probiotic beers. Chapter 11 describes a protocol for probiotic Friolano-type sausage. Furthermore, the possible sources of defects in producing probiotic salami and the best alternatives to overcome them are presented. Chapter 12 proposes the design of two independent protocols for the delivery of probiotics through bakery products: (I) a probiotic bread by adding microorganisms directly to the dough and (II) an edible probiotic film based on sodium caseinate and chia mucilage for application in bread surface. Furthermore, for both protocols, the function of each reagent/ingredient and the chemical reactions involved are described in detail, indicating the possible issues, sources, and the best alternatives to overcome them. Finally, Chap. 13 has up-to-date and detailed information on the production of different probiotics and synbiotic chocolate.

Regarding proposals for increasing probiotic survival in food products, Chap. 14 describes in detail the main methods of encapsulation of probiotics, including emulsion, extrusion, and spray-drying techniques.

However, in recent years, researchers have observed that viability may not be necessary for some health effects, and products with inactivated microorganisms have been developed. In this way, Chaps. 15 and 16 provide detailed protocols for obtaining potential paraprobiotics and postbiotics for use in food and beverages.

Finally, following new health effects associated with probiotic cultures, Chap. 17 describes protocols for elaborating on a food product with psychobiotic potential in detail. In addition, the most used behavioral tests for preclinical trials that can be applied to confirm the psychobiotic effect are also discussed.

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Chapter 1

Probiotic Fermented Milk

Shibo Ma, J. K. Vidanarachchi, and Chaminda Senaka Ranadheera

Abstract

Probiotic fermented milk is a product made by appropriate microbial growth using milk as the substrate which contains mainly live microorganisms. Fermented milk has been consumed for thousands of years worldwide, and the incorporation of probiotics has pushed it in a novel direction. The substrate selection includes cows, buffalo, goats, sheep, yak, horses, camel, and others' milk. The various substrate has their uniqueness, and typical traditional products, including kefir, koumiss, etc., are made from them. Further, the range of probiotics is vast, and commonly used genera contain *Lactobacillus* and *Bifidobacterium*. The primarily incorporated method is to inoculate it into the starter culture to co-ferment substrate with traditional fermentation culture. Other methods include fermenting substrate directly or adding it back into the product. The typical products include ambient-temperature fermented milk or probiotic fermented milk beverage. The basic processing method of probiotic fermented milk is similar to traditional fermented milk, where the incorporation of probiotics into the fermented milk product is unique due to the special incubation requirement of each probiotic. Commonly seen additives include sweetener, thickener (thickening technology), and prebiotics which were introduced in this chapter, which could give a comprehensive vision of the current fermented milk production and the indication of applying these additives to the fermented milk considering the existence of probiotics. Some novel and popular fermented milk products and their manufacturing methods were briefly introduced in this chapter, such as ambient-temperature fermented milk, roasted flavor fermented milk, and probiotic fermented milk beverage. General products' quality issues and legal compliance were also mentioned. Still, the most critical way to determine the manufacturing procedure and parameter is by running a pilot test based on the designation of the product, which could give a clear indication of the material, method, and post-manufacturing issues.

Key words Probiotic fermented milk, Manufacture process, Probiotics, Special milk, Sweetener, Prebiotics, Thickening technology

1 Introduction

Probiotic fermented milk is a product derived from traditional fermented milk. Fermented milk is a milk product made via appropriate microbial growth and/or enzymatic conversions of milk [1]. Here, the probiotic fermented milk should go further, where it requires the existence of probiotics in the fermented milk. It was recognized that probiotic fermented milk should contain live

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microorganisms [2]. However, the recent research regarding parabiotics and postbiotics broadened the scope of the products [3], where the importance of the viability of probiotics had been assimilated. In this chapter, *probiotic fermented milk*, referred in a broad sense, is a cluster of fermented milk products containing probiotic strains, live or not. More detailed introduction about parabiotics and postbiotics will be given in Chaps. 15 and 16. Fermented milk has been consumed for thousands of years. It was originated from various places, such as Mongolia, Egypt, Caucasian areas, etc., where multiple products were developed to fulfill the local requirements. For a clear written record, Greek and Roman are the first to mention this type of product (yogurt) in their history, around 100 BC [4]. For probiotics, its health effect had been realized and applied for dozens of centuries, combined with fermented milk consumption [5]. However, its mystery hadn't been revealed until modern times for their existence and taxonomy. In 1857, Pasteur discovered lactic acid bacteria (LAB) for their role in the fermentation of yogurt. In 1908, Elie Metchnikov proposed the idea of probiotics' health effect in his book *The prolongation of life: optimistic studies* (where the word "probiotic" haven't been proposed yet) [5, 6]. In 1953, German scientist Werner Kollath proposed the term "probiotic," which has been further used [7]. For currently admitted and used probiotic definition and effect, it was determined and published by FAO/WHO in 2001 and slightly modified in 2014 by Hill et al. [8] who confirmed that the *probiotic* should be "live microorganisms which could confer a health benefit on the host, when being administrated in appropriate amount." This definition differed the probiotic fermented milk from other traditional fermented milk (relatively different, traditionally used microorganisms for fermentation were sometimes regarded as probiotic in some situations), where the probiotic in the products should be capable of conferring benefit to humans after consumption. Firstly, the probiotic should tolerate gastric, bile, and intestinal fluid, and could colonize and proliferate in the gastrointestinal tract (GI tract). The safety and viability of probiotics are critical to the selection criteria, where the evaluation procedure has been clarified recently. China has published a new Group Standard names *Probiotic Food* by China National Food Industry Association (CNFIA) to define the requirement of probiotics used in food and the evaluation procedure to evaluate their safety and viability (T/CNFIA 131–2021) (*see* **Notes 1** and **2**) [9]. The standard also requires the precise strain number and source, and the completion of whole genome sequencing and random clinical trial to support its efficacy based on scientific articles. Other scholars also believe the probiotics used in the fermented milk (food) should exist in the GI tract originally, and genetically modified (GM) strain/species should not be used [10]. Meanwhile, there are a lot of strains or species that were tested and claimed to possess probiotic potential. Still, the authorities did not have explicit consensuses to determine which strain/species or groups can be regarded as probiotics. For example, China and Canada had a list showing the possibility of adding these species into foods as probiotics (Table 1).

Table 1
The list of microbial strains available to be used in foods in China and other countries [89–92]

Genera	Species
Bifidobacterium	*Bifidobacterium adolescentis**,^,# *Bifidobacterium animalis* subsp. *animalis* *,^,# *Bifidobacterium animalis* subsp. *lactis**,^,# *Bifidobacterium bifidum**,^,# *Bifidobacterium breve**,^,# *Bifidobacterium longum**,^,# *Bifidobacterium longum* subsp. *Longum**,^,#! *Bifidobacterium longum* subsp. *Infantis* *Bifidobacterium longum* subsp. *Suis**,^,#!
Bacillus (*Assessed* case-by-case in AU)	*Bacillus subtilis*^! *Bacillus cereus*^!
Companilactobacillus	*Companilactobacillus farciminis* #!
Debaryomyce%	*Debaryomyces hansenii*#!
Enterococcus (*Assessed* case-by-case in AU)	*Enterococcus faecium*^! *Enterococcus faecalis*^!
Fructilactobacillus	*Fructilactobacillus sanfranciscensis* #!
Lacticaseibacillus	*Lacticaseibacillus casei*# *Lacticaseibacillus paracasei* # *Lacticaseibacillus rhamnosus*#
Lactiplantibacillus	*Lactiplantibacillus paraplantarum* #! *Lactiplantibacillus plantarum*#
Lactobacillus	*Lactobacillus acidophilus**,^,# *Lactobacillus amylolyticus**,^,#! *Lactobacillus crispatus**,^,# *Lactobacillus delbrueckii*#! *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*)# *Lactobacillus delbrueckii* subsp. *Delbrueckii*#! *Lactobacillus delbrueckii* subsp. *Lactis*# *Lactobacillus gallinarum*#! *Lactobacillus gasseri*# *Lactobacillus helveticus*# *Lactobacillus johnsonii*# *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*#
Streptococcus	*Streptococcus salivarius* subsp. *thermophilus*
Lactococcus	*Lactococcus Lactis* subsp. *lactis* *Lactococcus cremoris* *Lactococcus Lactis* subsp. *Lactis* biovar diacetylactis
Latilactobacillus	*Latilactobacillus curvatus* # *Latilactobacillus sakei*

(continued)

Table 1
(continued)

Genera	Species
Lentilactobacillus	*Lentilactobacillus buchneri* #! *Lentilactobacillus hilgardii*#! *Lentilactobacillus kefiri* #!
Propionibacterium	*Propionibacterium freudenreichii* subsp. *Shermanii*^,# *Propionibacterium freudenreichii*^,#!
Acidipropionibacterium	*Acidipropionibacterium acidipropionici* #
Leuconostoc	*Leuconostoc mesenteroides* subsp. *Mesenteroides*# *Leuconostoc citreum*#! *Leuconostoc lactis*#! *Leuconostoc pseudomesenteroides*#!
Levilactobacillus	*Levilactobacillus brevis*#!
Ligilactobacillus	*Ligilactobacillus salivarius*#
Limosilactobacillus	*Limosilactobacillus fermentum* ^,# *Limosilactobacillus mucosae* #! *Limosilactobacillus panis*#! *Limosilactobacillus pontis*#! *Limosilactobacillus reuteri* *,#
Loigolactobacillus	*Loigolactobacillus coryniformis* #!
Mammaliicoccus	*Mammaliicoccus vitulinus*
Oenococcus	*Oenococcus oeni*#!
Kluyveromyces%	*Kluyveromyces lactis*#! *Kluyveromyces marxianus*#
Pediococcus	*Pediococcus acidilactici*# *Pediococcus pentosaceus*#
Staphylococcus	*Staphylococcus xylosus* *Staphylococcus carnosus*
Saccharomyces%	*Saccharomyces bayanus*#! *Saccharomyces boulardii**,#! *Saccharomyces cerevisiae*#! *Saccharomyces pastorianus*#!
Schizosaccharomyces%	*Schizosaccharomyces pombe*#!
Weizmanni	*Weizmannia coagulans*
Xanthophyllomyces%	*Xanthophyllomyces dendrorhous*#!

*: genera or species available in foods as probiotic (or showing health effect) in USA, symbols marked at species column, separated with other symbols using comma (,)

^: genera or species available in foods as probiotic (or showing health effect) in Australia (includes those that were not authorized by China, which was marked as ^!), symbols marked at species column, separated with other symbols using comma (,).

#: genera or species available in foods as probiotic (or showing health effect) in Canada (includes those that were not authorized by China, which was marked as #!), symbols marked at species column, separated with other symbols using comma (,).

%: yeast, marked at genera column.

Table 2
The approximate composition of various typical probiotic fermented milk products [47, 93–96]

	Yogurt	Kefir	Koumiss	Ymer (Denmark product)	Skyr (Iceland product)
Protein, %	5	3	2.2	5–6	12.7
Fat, %	7.5	0.2	1.9	3.5	0.2
Acidity, %	0.8	1			
Total solids, %	18.5	10.6–14.9		14.5	17.5
Carbohydrate, %		6	2.8	3.5	3.9
Alcohol, %		1	2.2		0.3–0.5
Ash, %		0.7			0.8
Others		1.97 g/L CO_2			

Many fermented milk could contain probiotics, such as yogurt, kefir, koumiss (kumys, kumis, kumiss, coomys), sour cream, and fermented buttermilk. Besides these traditional probiotic fermented milk products, some novel fermented dairy beverages containing probiotics have been developed recently, and the most famous one is Yakult®. The main difference among them is the product status (fluidity) and intrinsic microbial environment (multi vs. single strain) (*see* **Note 3**). They have different substrates, processing procedures, and storage conditions, where the most important is their proximate composition (Table 2). By the time of quality detection, the parameter measured had been regulated by the authorities from various countries. Table 3 summarizes the regulation parameters and numbers of the parameters which the products should achieve.

As mentioned above, the strict definition of probiotic fermented milk should contain live microorganisms in their product matrices. However, recent product development has combined the inactivation of live cells into the processing procedure to extend the shelf life or more stable quality, such as ambient-temperature yogurt (pasteurized fermented milk) and other products. They apply various live-cell inactivation methods to limit or eliminate the activity of viable microorganisms in the products to prolong the shelf life of the products for a farther distribution range or more manageable storage conditions. The inactivation methods include radiation, heating, high pressure, etc. (*see* **Note 4**). There are also coupled designs for these sterilized products about packaging material and style. General packaging uses plastic cups/containers (set) or bottles (stirred) to package fermented milk. For premium products, the glass jar is acceptable to package the product as well.

Table 3
The compilation of fermented milk standards from various countries [27, 38, 97–100]

	Codex Alimentarius	China	USA	Canada	Australia
Fat, %	$\leq 10^{acd}$, 15^{b}	3.1^{a}, 2.5^{e}	$\geq 3.25^{ab}$	——	——
Non-fat-solid, %	——	8.1^{a}	$\geq 8.25^{ab}$	$\geq 9.5^{b}$, 7.6^{e}, 6.5^{f}	——
Protein, %	Min 2.7^{abcd}	2.9^{a}, 2.3^{e}	——	$\geq 2.8^{b}$, 2.2^{f}	$\geq 3^{a}$ (cow's milk)
Acidity, %	Min 0.3^{a}, 0.6^{bc}, 0.7^{d}	——	$\geq 0.5^{a}$, 0.7^{b} (or $\leq$ pH 4.6)	$\geq 0.7^{b}$	$\leq$pH 4.5^{a}
Acidity, °T	——	70	——	——	——
Microbial load, cfu/g(mL)	Min $10^{6,\ ab}$ (total), $10^{4,\ cd}$ (yeast)	$\geq 10^{6,\ ae}$	$\geq 10^{7,a}$	$\geq 10^{7,b}$	$\geq 10^{6,\ a}$
Ethanol, %vol./w	Min 0.5^{d}	——	——	——	——
Document number	CXS 243–2003	GB19302–2010	FDA-21 CFR 131.112, 131.200	National Dairy Code, Part III	Australia New Zealand Food Standards 2.5.3, F2015L00413

[a]Fermented milk/Cultured milk.
[b]Yogurt, alternate culture yogurt, acidophilus milk (Yogurt: fermented milk using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* as culture; alternate culture yogurt: using *Streptococcus thermophilus* and any *Lactobacillus* species; acidophilus milk: using *Lactobacillus acidophilus* as culture).
[c]Kefir.
[d]Kumys.
[e]Flavored fermented milk (with sugar or fruit component addition).
[f]Yogurt drink (drinkable fermented milk).
——: Not mentioned or required by such standards.

However, novel tetra packaging was developed to comply with the requirement of ambient-temperature fermented milk to assist its prolonged storage time. The shelf life of regular fermented milk (with or without probiotics) is around 21–28 days. For plastic packaged products, some of them can be shortened to 14 days (it is worth noting that the shelf life does not have a severe relationship with the preservation ability of LAB or the health effect of probiotics). The optimal storage condition of such products is around 4 °C, requiring fully cold-chain logistics. For ambient-temperature fermented milk, the shelf life can be extended to 6 months at ambient temperature (around 25 °C).

Moreover, there are vast amounts of products commercially available in the market. Still, they can be characterized according to several criteria, such as matrix status (set/stirred), product additive (natural, sweetened (flavored), nutritionally enhanced), post-fermentation processing (condensed, frozen, carbonized, spray-dried), fat content (full-fat, partially skimmed, skimmed, and Greek yogurt) [11]. Nevertheless, their material, main processing procedure, and packaging step are very similar, with a slight difference in additive, post-fermentation, and packaging steps. These will be described in detail in **Part III**.

2 Material

Materials used for probiotic fermented milk production can be divided into several groups: raw milk and milk substrate, starter culture and probiotic strains, sweetener and additive. They have different effects on the probiotic fermented milk, which should be focused on during processing.

2.1 Raw Milk and Milk Substrate

The substrate and primary material of probiotic fermented milk should be various milk originating from multiple breeds or species of mammals. Commonly seen dairy animal species include cows, goats, sheep, buffalo, donkeys, and camels, where cows are the most used for raw milk production. Bovine milk is the most consumed milk by humans. Various cattle breeds have been domesticated by humans for milk production (some of the breeds are for both milk and beef). These temperate breeds include Ayrshire, Guernsey, Brown Swiss, Shorthorn, Jersey, and Holstein Friesian. Among them, Holstein Friesian is the only most important breed for milk production. Holstein Friesian originated from the Netherlands and had been exported widely to the world due to its adaptability. It has excellent milk production capability, where its average milk yield is 25–35 kg/day [11]. This yield is far from other dairy breeds. Holstein Friesian has a lower milk fat content than other temperate breeds except for Shorthorn [11]. The typical appearance of Holstein Friesian is black and white color. Besides, other species have their characteristics, such as Jersey has a high milk fat (4.95%) content and dry matter (14.54%) content with low yield (19–25 kg/day), and shorthorn has a high protein-fat ratio but low yield as well (17–25 kg/day) [11]. Therefore, the selection of raw milk sources would significantly affect the final product's quality.

Notably, the quality of raw milk produced by different animals can be affected by various factors. Of which the most important and controllable are milking season, feeding (water and fodder), and equipment. The raw milk composition could be varied significantly following the milking season (lactation season) change, but the lactose in the milk could be stable. Protein and milk fat have a solid response to season change, where the lowest content occurred in summer (3.21% for protein, 4.1% for fat) and the highest content occurred in winter (3.38% for protein, 4.57% for fat), respectively [12]. It had been reported that the raw milk yield and composition were negatively related to environmental temperature [13–17]. This phenomenon is reasonable and explainable due to the Holstein Friesian originating from a cool area, which has a stress reaction to heat. Heat stress is one of the most significant issues in cows, especially Holstein Friesian husbandry. Lactation season could also affect raw milk yield and composition, whereas Holstein Friesian's lactation season could be over 200 days. Raw milk yield and composition have fluctuated over a long period [17]. The raw milk yield increases and reaches a peak during the early lactation period but goes lower following the lactation period [17]. The fat content has a real controversial tendency compared with yield [17]. It went lower at the beginning of the lactation period and turned to increase, accompanied by lactation progress [17]. Milk protein also has higher content at the beginning of the lactation period [17].

Water and forage feed could be crucial factors that impact the raw milk quality, where the contaminant and odor components, such as heavy metals, animal drugs, and toxins, could be transmitted to the milk through cow's milk secretion [18]. The type and quality of forage could also affect the milk fat content and composition, where the involvement of phytochemical composition in the forage attracts attention [19]. The feeding method could influence the quality of raw milk as well. Grazing cows have lower raw milk yield than feedlot cows, but the fat content in grazing cows' milk is higher than in feedlot cows' milk. The difference between the protein content is negligible [18, 20]. It is worth noting that the fatty acid composition in the milk produced from grazing or feedlot cow is also different. In summary, it is wise to determine the source of raw milk regarding the abovementioned factors before adopting it in fermented milk production for better product quality.

Apart from species, breed, and lactation season, and feeding material quality and method, milking sanitation and equipment are also critical to raw milk quality, especially microbial load. Essential sanitation of the cows' udder (or other dairy animals) is necessary as the microbe in the raw milk strongly correlates with teat skin sanitation. Research proved that the microbial composition is significantly different between raw milk and teat skin due to the both-way contamination. However, 92.1% of the bacteria in the raw milk come from the teats' skin (genetically connected) [21]. An efficient way to sanitize the udder is teat dipping (pre and post), in which the teat was sanitized via iodine solution. The same research also revealed that the microbial composition of teat skin is significantly similar to raw milk, which means the both-way contaminations were halted, and the microbial was only transferred from raw milk to teat [21]. This result proved that iodine sanitation is an efficient way to intercept teat-raw milk contamination. Sanitation of milk equipment is also a pivotal step in ensuring the quality of raw milk. Research indicates that appropriate sanitized equipment could reduce raw milk's thermophilic spore load [22]. Other factors that have relationships with low spore load include farming environment, husbandry scale, regular udder massage, and others [22]. These factors also confirmed that appropriate farming methods, feeding (fodder and silage), housing conditions, and even the cow's mood influence the raw milk quality, which needs attention.

Milking is an essential step for raw milk collection, where the equipment evolution has served this step well. Machine milking has far higher efficiency than manual milking, which has improved the raw milk yield significantly [11]. Recently, automatic milking equipment (robotic milking) was developed to avoid excess stress on cows and save human labor. This equipment ensures the animal welfare of cows and eases their nervousness, anxiety or other negative moods to prevent low-quality raw milk. Usually, the cows were tagged and managed via ear tag, where the information of each cow can be collected when they enter the milking robot for milk tracing. The cost of milking also decreased compared with traditional milking. This automated milking machine has attracted the attention of farmers from developed countries, such as the USA, Australia, The Netherlands, and New Zealand, to apply this system for better raw milk production.

After milking, the raw milk should be tested before production. Some standards or codes require the quality of raw milk. The most crucial parameters are microbial load and somatic cell count (SCC). For microbial load, the USA requires that the raw milk for direct consumption should not contain more than 15,000 total viable bacteria/mL and < 10 coliform bacteria/mL [23]; China has a 2×10^6/mL total viable microbial count limitation of raw milk, whereas the EU limited the total viable microbial count to 1×10^6/mL [24]. For somatic cells, it is not required by China, but the USA and EU had limited the count below 6×10^6 and 4×10^6 cells/mL, respectively [23, 25]. Somatic cell count (SCC) is vital for raw milk quality. It indicates the health status of cows or other dairy animals.

SCC was influenced by parities, calving season, and lactation period, and the yield will drop dramatically when the SCC goes higher [26]. Research proved that the composition of raw milk reached the lowest amount when the SCC exceeded 5×10^6/mL; hence the researcher recommended that the SCC in raw milk should not be above 5×10^6 cell/mL [26].

Besides the microbial count and SCC, many other parameters should be satisfied, including fat, protein, and non-fat milk solids in many countries. For industry raw milk collection, many essential tests need to be performed to ensure the quality of raw milk and perspective products. These include sensory tests, ethanol tests, clot-on-boil tests, titratable acidity, density test, microbial (dye reduction methods)/somatic cell/antibiotic test, composition determination, and adulterant tests [11]. Among them, the ethanol test is a rapid method to determine whether the raw milk is fresh or not, based on the acidity of raw milk [11]. This is a very fast and easy method to be applied in the industry due to the simple phenomenon, equipment, and indicative capability. For fresh raw milk, there will be no phenomenon when ethanol (68%, 70%, 72%) is added to the raw milk, where the coagulation of casein (protein) will occur when the raw milk deteriorates [11, 18]. Notably, a microbial/somatic cell/antibiotic test is necessary, especially for fermented milk production. Besides the microbial count, excess antibiotic in the raw milk is crucial for fermented milk production due to their inhibitory effect on the starter culture cultivation and growth, especially probiotic, which requires a strict environment. The source of antibiotics is vast, but it may come from cattle disease treatment, fodder additive residue, and milking contamination [18]. Addition of antibiotics purposely is rarely seen, but it affects the quality significantly, which needs strict regulation. Developed countries require that antibiotics should not be tested in raw milk [18]. However, a trace amount of antibiotics is still allowed in developing countries [27], indicating that raw milk should be appropriately tested and treated when applied to produce fermented milk in these countries.

After collection, pre-treatment should be performed to ensure the quality of raw milk for further production. Usually, pre-treatment includes filtration, purification, cooling, pre-pasteurization (optional), and deaeration (optional) [11]. Filtration and purification could efficiently remove physical contaminants and excessive microbial and somatic cells to reduce observable contaminants by the naked eye. However, rapid cooling is essential for the stable quality of raw milk during storage before processing. Usually, freshly collected milk has cow's body temperature, which should be cooled around 4–6 °C as soon as possible. The growth of microbes could be attenuated or inhibited at this temperature. If its temperature could be cooled down to 2–3 °C, the growth of the microorganism could be near completely halted, and it can be stored for about 7 days [11]. Pre-pasteurization should be performed if the raw milk is not used immediately to avoid quality deterioration.

2.2 Starter Culture and Probiotic Strains

Starter culture is essential for probiotic fermented milk production. It usually contained lactic acid and polysaccharides producers, such as *Lactobacillus* (L) and *Streptococcus* spp. (S). The ratio of L/S is around 1:1 or 1:2, where the overwhelming of *Lactobacillus* will result in excess lactic acid content and unacceptable flavor [11]. Detailed starter culture production will not be mentioned here. Still, the type of starter culture and production of starter culture are described in Table 4 and Fig. 1, respectively. It is worth noting that adding probiotics as a starter culture is the main method to incorporate probiotics into fermented milk. Hence, the cultivation of probiotics needs further attention. The synergistic or antagonistic bio-relationship between conventional starter culture (*Lactobacillus & Streptococcus* (L&S)) and probiotic could affect the success of fermentation [28]. For example, the difference between the growth rate of L&S and probiotic leads to desired microorganism cultivation failure, or the metabolites of each species could promote or inhibit the growth of other species (hydrogen peroxide, oxygen content, carbonized, etc.) [28–34].

Table 4
Various types of starter cultures used in fermented milk production [11, 40, 47]

Classification criteria	Type of starter culture	Notes
Preparation of Starter cultures	LAB pure culture	Primary strains included in the culture (step 1)
	Mother starter culture	Proliferation of primary strains (step 2)
	Bulk starter culture	Proliferation of mother culture, used for manufacture directly (step 3)
Strain composition of Starter cultures	Mixed strains starter culture	Contains more than one strains for synergistic fermentation
	Single strain starter culture	Contains only one strain, mixed when applying
	Supplemented strains starter culture	Contains one or more strains for special purpose, includes exopolysaccharides production, aroma component production, and probiotics
Status of Starter culture	Liquid starter culture	Easy to operate and cheap, but the viability can be weakened
	Powder starter culture	Better viability and stability than liquid form
	Frozen starter culture	Highly concentrated, highest viability, direct usage

LAB lactic acid bacteria
Steps 1, 2, 3: The steps required for starter culture application during production procedure. These steps were performed according to factories in situ application.

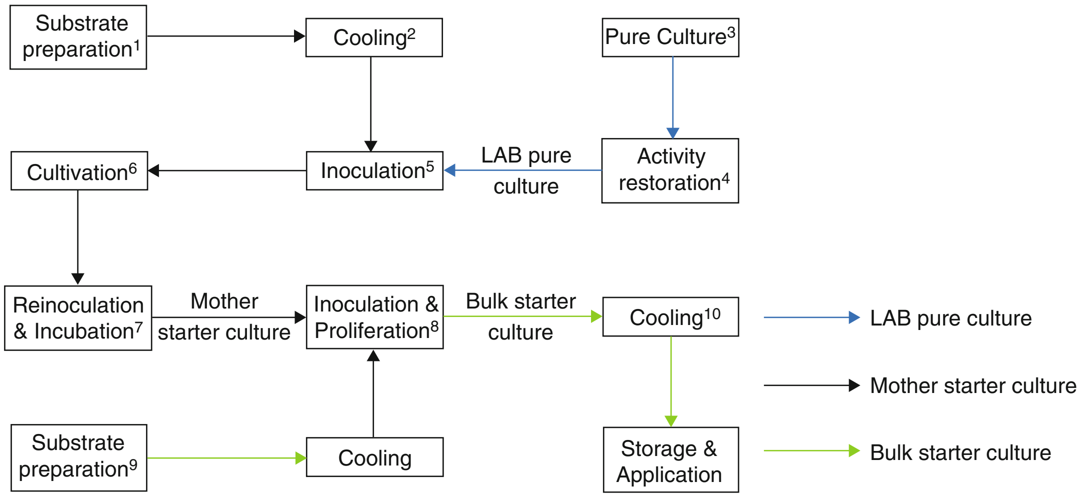

Fig. 1 The flow chart of starter culture preparation [11, 40]. (1) Reconstituted skimmed milk (10–12% solid), heated 90–95 °C for 30–40 min or 121 °C for 15 min. (2) Mesophilic culture: 20–30 °C; theromophilic culture: 42–45 °C. (3) 0–4 °C storage, subculture every 1–2 weeks; random purification is needed. (4) Restoration for 2–3 times. (5) 1–2% addition amount. (6) Temperature determination according to strain characteristics; Time: 3–20 h. (7) Same condition or 2–3 times. (8) At 42 °C, stop when acidity >0.8%. (9) Same substrate treatment condition, but using product raw material as substrate, 1–2% of total raw material. (10) Use within 6 h: 10–20 °C; use after 6 h: 4–5 °C

Due to the growth rate, the time and form of probiotic addition are crucial. As for the preservation of the viability of probiotics, many ways are used to protect probiotics and assist them in reaching the GI tract without severe weakening due to lactic acid in fermented milk or harsh condition in the GI tract. Encapsulation is a commonly used method to protect probiotics. Probiotics can be encapsulated (usually microencapsulated) in different wall materials or matrices to maintain viability (*see* Chap. 14 for more details). Different wall material has various properties, such as protection, lyse, texture alteration, etc. There is a study that showed that the addition of microencapsulated probiotics could affect the texture of yogurt (smoothness), which needs attention (alginate-starch as wall material, which can affect the texture) [35]. Other materials used for microencapsulation include whey protein (an useful by-product of cheese production), gellan gum (polysaccharides), etc. The microencapsulation method includes drop-out, emulsification, extrusion, coacervation, and others. Compared with extrusion, emulsification has a higher encapsulation rate [36]. Microencapsulated probiotics can shorten the fermentation time of fermented milk as well [36], but this phenomenon needs further clarification to differentiate between bacteria synergistic effect or microencapsulation promotion. Besides, the strong buffer capacity of the substrate (neutralized pH) or the firm texture of fermented milk (gel) (prevents acid contact with probiotics) can protect probiotics efficiently as well [28].

2.3 Sweetener and Additives

Many additives can be used in probiotic fermented milk, where the sweetener is the most important one. Sweeteners could provide a sweet taste to the consumer to assimilate or cover the harsh taste of lactic acid in the fermented milk. A commonly used sweetener is sugar (sucrose), which is accepted by most consumers. Recently, artificial sweeteners, such as sucralose and aspartame, were used to provide a more intense sweet taste and reduce cost. However, the health requirement of customers had forced the producer to replace artificial sweeteners with natural sweeteners, hence stevia, erythritol, and mogroside have come into sight of the producers. These selections have broadened the horizon of sweeteners from a health perspective and increased the acceptability and functionality of fermented milk. Besides, there are other additives, such as fruit components (jam, crushed or pulp), thickener/stabilizer/emulsifier, essence, pigment/colorant, etc. [11], that can be added into the fermented milk in accordance with local regulations.

It is worth noting that some unique carbohydrates, such as dietary fiber, resistance starch, oligosaccharides, and inulin, were added to the probiotic fermented milk to acquire its health benefit and probiotic promoting capability (synbiotic ability). These substances are called as prebiotics. Prebiotics is a type of food component that could not be digested by the endogenous host enzymes yet could exert benefit on the host by modulating gut microflora [37]. In this case, the type, purity, chain length, percentage of prebiotic, target probiotic/microflora, product formula and characteristic, and storage conditions need to be considered when applying prebiotic in probiotic fermented milk [37]. Prebiotics can significantly affect the probiotic viability and the physiochemical (texture and rheology), organoleptic and functional properties of the products [37]. However, the effect (positive, negative, or neutral) is still under debate, which needs more attention when utilizing them in the products [37]. More detailed availably of thickener (thickening technology) and prebiotic selection will be discussed in **Notes 5** and **6**.

3 Method

The production method of probiotic fermented milk is similar to yogurt production, which involves pre-treatment (standardization, pre-heating, homogenization, heating, cooling), inoculation, fermentation, additive addition, and packaging. The flow chart of the processing procedure is shown in Fig. 2. Here, it is notable that the order of fermentation, packaging, and additive use is different between set-fermented milk and stirred-fermented milk. Detailed order is shown in Fig. 2 as well. In the following paragraph, each step will be discussed separately, and the combination of such steps should be performed as per product and in situ requirements.

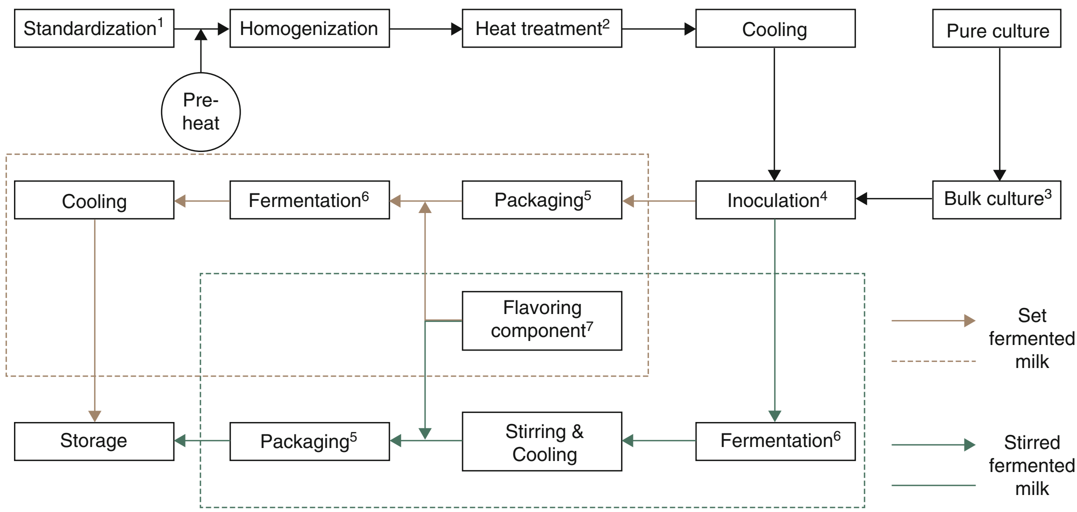

Fig. 2 The flow chart of fermented milk processing [11, 40, 47]. (1) Milk solid, includes protein, cream, thickener, sweeteners were added here; filtration may be applicable for unsolved substances, critical control point 2 (CCP 2) for both set and stirred fermented milk. (2) Significantly important for product quality control, CCP3 for both set and stirred fermented milk. (3) The hygienic condition of starter culture is important, CCP4 for both set and stirred fermented milk. (4) The control of hygienic condition and relative parameter is critical for this step, CCP5 for both set and stirred fermented milk. (5) The hygienic condition of environment and packaging container is critical, CCP6 for set fermented milk, CCP 7 for stirred fermented milk. (6) The fermentation temperature and time are critical for the success of products processing, CCP 7 for set-fermented milk, CCP 6 for stirred fermented milk. (7) Includes fruit component (pulp or jam), essence substances, etc

3.1 Pre-Treatment

Pre-treatment includes raw milk standardization, homogenization, heat treatment, and inoculation steps. Firstly, the raw milk pumped from the storage tank should be standardized to fulfill the requirement of local regulations where the factory resides, or the product will sell. In general, any product should satisfy the requirement of FAO/WHO regulation [38] for global distribution and retail selling. Fat and protein content should be less than 10% and more than 2.7%, respectively. Hence, any raw milk that does not meet this requirement should be standardized to achieve this limitation. Usually, the fermented milk fat content is between 0.5–3.0% [11], depending on whether it is skim or not, where the addition of cream is necessary to adjust this content to not only fulfill the regulation but also to guarantee the sensation of such product. Besides, the non-fat-solid of milk will be fortified, if necessary, whereas the skimmed milk powder should be used here. These components (cream, skimmed milk powder) can be provided within the factory from other product lines to utilize the by-product and make the best value of it. The sugar and stabilizer should be added here to favor the growth and fermentation of