

METHOD FOR PREPARING FERMENTED MILK WITH LIVE BACTERIA AT NORMAL TEMPERATURE

[0001] This application claims the priority of Chinese Patent Application No. 202210224369.5,
5 filed with the China National Intellectual Property Administration on March 7, 2022, and titled
with “METHOD FOR PREPARING FERMENTED MILK WITH LIVE BACTERIA AT
NORMAL TEMPERATURE”, the disclosure of which is hereby incorporated by reference in
entirety.

10 FIELD

[0002] The present disclosure relates to the technical field of dairy products, and specifically
relates to a method for preparing an ambient-temperature fermented milk with viable bacteria.

BACKGROUND

15 **[0003]** Fermented milk is a popular dairy product known for its rich nutritional value and
unique flavor. Low-temperature fermented milk refers to fermented milk that has a short shelf life
and must be kept under refrigerated conditions throughout transport sales. Ambient-temperature
fermented milk is produced by pasteurizing the fermented milk after fermentation, and it has no
viable bacteria, allowing it to be stored and transported at ambient temperature. Although
20 ambient-temperature fermented milk can be stored at room temperature for a longer period of
time, the absence of viable bacteria in the product that the benefits associated with fermented
milk are lost.

[0004] The prior art has disclosed a variety of fermented milk beverages containing viable
bacteria. For example, Chinese patent CN101273736A discloses a method of preparing a
25 fermented milk beverage that maintains a high count of viable bacteria at ambient temperature by
adding *Lactobacillus rhamnosus* ATCC 53103 to the fermented milk beverage. However, on the
one hand, the method is limited to preparing a fermented milk beverage with viable bacteria, not
a fermented milk with viable bacteria, and on the other hand, the viable bacteria count and pH

value of the fermented milk beverage prepared by the method decreases rapidly during storage at ambient temperature.

SUMMARY

- 5 **[0005]** In view of this, an object of the present disclosure is to provide a method for preparing a fermented milk with viable bacteria at ambient temperature. The method provide by the present disclosure enables the production of an ambient-temperature fermented milk with viable bacteria, which exhibits no significant changes in viable bacteria count, pH, or acidity over a storage period of 6 months at ambient temperature.
- 10 **[0006]** The present disclosure provides a method for preparing an ambient-temperature fermented milk with viable bacteria, comprising:
- [0007]** fermenting a fermentation base material with a lactose-negative bacterium other than *Lactobacillus rhamnosus* as a fermentation bacterium to obtain a fermented product, pasteurizing the fermented product, and adding *Lactobacillus rhamnosus* to obtain an ambient-temperature
- 15 fermented milk with viable bacteria;
- [0008]** wherein the fermentation base material comprises a proliferation factor other than lactose.
- [0009]** The present disclosure provides a method for preparing an ambient-temperature fermented milk with viable bacteria, comprising:
- 20 **[0010]** inoculating a mixture of *Lactobacillus rhamnosus* and a lactose-negative bacterium other than *Lactobacillus rhamnosus* into a fermentation base material, and fermenting the mixture to obtain an ambient-temperature fermented milk with viable bacteria;
- [0011]** wherein the fermentation base material comprises a proliferation factor other than lactose.
- 25 **[0012]** Chinese patent CN101273736A discloses a method of preparing a fermented milk that maintains a high count of viable bacteria at ambient temperature. This method uses *Lactobacillus rhamnosus* ATCC 53103 as the fermentation bacterium and adds a monosaccharide or disaccharide, other than lactose, that can be utilized by the *Lactobacillus rhamnosus* as a

proliferation factor for fermentation. Since *Lactobacillus rhamnosus* ATCC 53103 does not ferment lactose and does not produce lactic acid, the amount of proliferation factor can be controlled to manage the pH value at the end point of fermentation, thereby effectively controlling the post-acidification of the fermented milk. This allows the fermented milk to be stored at room temperature for extended periods while maintaining a high count of viable bacteria. However, *Lactobacillus rhamnosus* ATCC 53103 has a slow fermentation rate, typically requiring over 12 hours of fermentation to reach the end point of fermentation. It is challenging to maintain a high count of viable bacteria, and the viable bacteria count decreases by two orders of magnitude after storage at ambient temperature for about 28 days. Furthermore, fermented milk prepared using *Lactobacillus rhamnosus* ATCC 53103 often has a less desirable taste and flavor.

[0013] Based on this, the inventors of the present application have creatively discovered that using a lactose-negative bacterium other than *Lactobacillus rhamnosus* as the fermentation bacterium and *Lactobacillus rhamnosus* as the viable bacterium results in an ambient-temperature fermented milk with viable bacteria that not only has a shorter fermentation time and better taste and flavor but also exhibits no significant changes in pH, acidity, and viable bacteria count over a storage period of 6 months at ambient temperature.

[0014] In the present disclosure, a lactose-negative bacterium is defined as a strain that does not ferment lactose but ferments other monosaccharides or disaccharides. Examples include *Lactobacillus rhamnosus*, YoFlex® Acidifix™ 1.0 from Chr. Hansen, and nu-trish® LGG® from Chr. Hansen. The present disclosure uses a lactose-negative bacterium, other than *Lactobacillus rhamnosus*, as the fermentation bacterium to shorten the fermentation time, improve the taste and flavor of the resulting fermented milk, and help maintain stable parameters such as pH value, viable bacteria count, and acidity during storage at room temperature. In an embodiment, the lactose-negative bacterium used as the fermentation bacterium is YoFlex® Acidifix™ 1.0 from Chr. Hansen.

[0015] *Lactobacillus rhamnosus*, used to maintain the viable bacteria count in the fermented milk, is also a lactose-negative bacterium, meaning it does not ferment lactose. In an embodiment, the *Lactobacillus rhamnosus* is nu-trish® LGG® from Chr. Hansen.

[0016] In an embodiment of the present disclosure, *Lactobacillus rhamnosus* can be added to

the fermented milk after fermentation to maintain the viable bacteria count. Alternatively, it can be added to the fermentation base material in combination with the fermentation bacterium for fermentation. Due to the slower fermentation rate of *Lactobacillus rhamnosus* compared to other lactose-negative bacteria such as YoFlex® Acidifix™ 1.0 with fast fermentation rate, it primarily acts as a viable bacterium rather than as a fermentation bacterium.

[0017] In an embodiment, in the step of inoculating a mixture of *Lactobacillus rhamnosus* and a lactose-negative bacterium except for *Lactobacillus rhamnosus* into a fermentation base material, the fermentation rate of the lactose-negative bacterium other than *Lactobacillus rhamnosus* is faster than that of *Lactobacillus rhamnosus*.

[0018] The fermentation base material comprises a proliferation factor, and the proliferation factor is used to control the initiation and termination of fermentation by the lactose-negative bacterium other than *Lactobacillus rhamnosus*. By adjusting the amount of the proliferation factor in the fermentation base material, the fermentation of the lactose-negative bacterium other than *Lactobacillus rhamnosus* can be completed. In an embodiment, the proliferation factor is selected from the group consisting of glucose, fructose, galactose, arabinose, ribose, mannose, rhamnose, fucose, tagatose, sucrose, maltose, cellobiose, trehalose, melezitose, gentiobiose, acetyl glucosamine, white sugar, and a combination thereof. In an embodiment, the proliferation factor is white sugar or glucose. In an embodiment, the proliferation factor is present in an amount of 0.6 wt% to 1.2 wt%. In an embodiment, the proliferation factor is present in an amount of 0.7 wt% to 1.0 wt%.

[0019] The fermentation base material further comprises a milk raw material, and optionally, a flavoring agent, a stabilizer, a proteinaceous ingredient, and water. The milk raw material serves as the primary fermentation base material, rich in proteins, minerals, growth factors, and other nutrients. It provides a carbon source, nitrogen source and trace elements for microbial fermentation and imparts a smooth taste and texture to the yogurt, while supplying various types of essential nutrients for the human body. In an embodiment, the milk raw material comprises at least one of animal-based milk raw material and plant-based milk raw material. Animal-based milk raw material includes, but is not limited to, fresh cow's milk, fresh goat's milk, fresh camel's milk, and reconstituted milk prepared from corresponding milk powders. Plant-based milk raw material includes, but is not limited to, soy milk and oat milk.

[0020] Proteinaceous ingredients serve as nutritional food supplements that can enhance the protein content of the fermentation base material, thereby increasing the nutritional value and quality of the fermented milk. In an embodiment, the proteinaceous ingredient includes, but is not limited to, whey protein, cow milk protein, casein, soy protein isolate, soy protein concentrate, pea protein, peanut protein, walnut protein, and coconut milk.

[0021] The purpose of flavoring agents is to provide flavor and increase the soluble solids content of the fermented milk, contributing to its desirable palatability. Flavoring agents include, but are not limited to, sweeteners and essence other than the proliferation factor. In an embodiment, the flavoring agent is selected from sugar alcohols used as sweeteners, including but not limited to isomaltulose, maltitol, erythritol, xylitol, galactitol, mannitol, and sorbitol.

[0022] Stabilizers help ensure that the protein molecules in the fermented milk are evenly distributed throughout the fermented milk system, effectively improving protein coagulation and achieving a thicker consistency and product stability. In an embodiment, stabilizers include, but are not limited to, pectin, agar, gellan gum, starch, carrageenan, gelatin, xanthan gum, diacetyl tartaric acid ester of mono- and diglycerides, mono- and diglycerides of fatty acids, acetylated distarch phosphate, hydroxypropyl distarch phosphate, and citrus fiber. In an embodiment, the blending of multiple stabilizers can enhance the curd formation of the fermented milk, such as a blend of agar, pectin, gellan gum, and diacetyl tartaric acid ester of mono- and diglycerides.

[0023] In the present disclosure, the milk raw material is firstly standardized and mixed with a proteinaceous ingredient, a proliferation factor, a stabilizer, and a flavoring agent. This mixture undergoes homogenization, sterilization, and cooling to obtain a fermentation base material. Specifically, in the present disclosure, the process involves: firstly, the proteinaceous ingredient, a portion of the proliferation factor, the stabilizer, and the flavoring agent are added to a portion of the standardized milk raw material and mixed; secondly, another portion of the standardized raw milk and the remaining proliferation factor are added for continue mixing; finally, the remaining milk raw material is added to mix well. The mixed material solution is degassed under vacuum, homogenized, sterilized and cooled to obtain the fermentation base material. In an embodiment, the degassing under vacuum is performed at a temperature of 50-65°C, with a vacuum degree of -40 to -70 kpa. In an embodiment, the homogenization is a two-stage homogenization, wherein the second stage is adjusted to achieve 30 bar as indicated by a pressure gauge, then the first stage

is adjusted to achieve 160 ± 5 bar as indicated by a pressure gauge, and the homogenization is performed at a temperature of $50-60^{\circ}\text{C}$. In an embodiment, the sterilization is performed at $132\pm 1^{\circ}\text{C}/4$ s or $121\pm 1^{\circ}\text{C}/60$ s. In an embodiment, the cooling is performed to a temperature of $39-43^{\circ}\text{C}$.

5 **[0024]** In an embodiment, the fermentation base material comprises:

[0025] 80%-99% of the milk raw material,

[0026] 0.6 wt%-1.2 wt% of the proliferation factor,

[0027] 0-2% of the proteinaceous ingredient,

[0028] 0-12% of the flavoring agent,

10 **[0029]** 0-3% of the stabilizer, and

[0030] water to balance.

[0031] In an embodiment, the fermentation base material comprises:

[0032] 80%-99% of the milk raw material,

[0033] 0.8 wt%-1.0 wt% of the proliferation factor,

15 **[0034]** 0-2% of the proteinaceous ingredient,

[0035] 0-12% of the flavoring agent,

[0036] 0-3% of the stabilizer, and

[0037] water to balance.

[0038] After obtaining the fermentation base material, it is inoculated with fermentation
20 bacteria. As described above, the fermentation bacterium is a lactose-negative bacterium other
than *Lactobacillus rhamnosus*, preferably YoFlex® Acidifix™ 1.0. In an embodiment, the
lactose-negative bacterium is present in an amount of 80-150 U/t. In an embodiment, the
lactose-negative bacterium is present in an amount of 100-120 U/t.

[0039] In an embodiment, fermentation is carried out at $38-42^{\circ}\text{C}$ until the pH is reduced to
25 below 4.5. After the titratable acidity is adjusted to 72°T , demulsification is performed, followed
by pasteurization. *Lactobacillus rhamnosus* is then added under sterile conditions, and the

product is filled into containers to obtain an ambient-temperature fermented milk with a high viable bacteria count.

[0040] In an embodiment, the pasteurization is carried out at a temperature of 75°C to 80°C for 20 to 30 seconds. In an embodiment, the pasteurization is carried out at a temperature of 77°C for 25 seconds.

[0041] In an embodiment, the *Lactobacillus rhamnosus* is nu-trish® LGG® from Chr. Hansen. In an embodiment, the *Lactobacillus rhamnosus* is present in an amount of 1.0×10^6 CFU/g to 1.0×10^8 CFU/g. In an embodiment, the *Lactobacillus rhamnosus* is present in an amount of 5.0×10^6 CFU/g to 1.0×10^8 CFU/g.

[0042] In an embodiment where fermentation bacteria and *Lactobacillus rhamnosus* are added simultaneously, *Lactobacillus rhamnosus* and a lactose-negative bacterium other than *Lactobacillus rhamnosus* as the fermentation bacterium are added simultaneously to the fermentation base material for fermentation. The fermentation is carried out until the pH is reduced to below 4.5. After the titratable acidity is adjusted to 72°T, demulsification and filling are performed, followed by pasteurization. *Lactobacillus rhamnosus* is then added under sterile conditions to obtain an ambient-temperature fermented milk with a high viable bacteria count.

[0043] The inventors of the present application have creatively discovered that using a lactose-negative bacterium other than *Lactobacillus rhamnosus* as the fermentation bacterium and *Lactobacillus rhamnosus* as the viable bacterium to prepare an ambient-temperature fermented milk with viable bacteria, whether *Lactobacillus rhamnosus* is added after fermentation or simultaneously with fermentation bacteria, can shorten fermentation time to complete within 7-8 hours. Furthermore, it can yield a fermented milk with good taste and flavor. Most importantly, by adding a proliferation factor to the fermentation base material and controlling the fermentation endpoint, the resulting fermented milk exhibits no significant changes in pH, acidity, and viable bacteria count over a storage period of 6 months at ambient temperature.

[0044] In the method provided in the present disclosure, using a lactose-negative bacterium other than *Lactobacillus rhamnosus* as the fermentation bacterium ferments the milk raw materials without consuming lactose or producing carbon sources available to *Lactobacillus*

rhamnosus. This controls the acid production during *Lactobacillus rhamnosus* fermentation, achieving post-acid stability at ambient temperature with a long shelf life, effectively controlling post-acid, and resulting in fermented milk with a stable taste.

5 DETAILED DESCRIPTION

[0045] The method for preparing a fermented milk with viable bacteria at ambient temperature provided by the present disclosure will be described in detail below in conjunction with examples.

[0046] Example 1

10 **[0047]** The fermented milk was prepared according to the formula shown in Table 1, which shows the formula for the fermented milk prepared in Example 1 and Comparative Example 1 of the present disclosure.

Table 1: Formula for Preparing Fermented Milk in Example 1 and Comparative Example 1 of the Present Disclosure

Fermented Milk	Example 1 (%)	Comparative Example 1 (%)
Raw Cow Milk	933.5	933.5
Erythritol	40	40
Agar	0.7	0.7
Pectin	0.7	0.7
Diacetyl Tartaric Acid Ester of Mono- and Diglycerides	0.3	0.3
Gellan Gum	0.3	0.3
White Sugar	7.5	/
Acetylated Distarch Phosphate	8	8
Glucose	/	7.5
Whey Protein Powder	9	9
YoFlex® Acidifix™ 1.0	100U/t	/
nu-trish® LGG®	0.2	0.2

[0048] Wherein, the amount of nu-trish® LGG® added was 0.02% of the fermentation base material, and the LGG viable bacteria count in the fermentation base material after addition was 5.0×10^6 CFU/g.

[0049] The preparation method was as follows.

5 **[0050]** Preparation of Standardized Milk: Raw cow milk was subjected to separation at 60°C, mechanical impurities removal, and then fat standardization. The cow milk following fat standardization was homogenized at 60°C and 100±5 bar, and then pre-sterilized at 75°C for 15 s to obtain standardized milk.

10 **[0051]** Preparation of Fermentation Base Material: 50% of the formulated amount of the standardized milk was heated to 55°C, and whey protein powder was first added to the standardized milk. The mixture was processed using equipment with mixing, dispersing, shearing and emulsifying functions to form a homogeneous and dispersed mixture at 55°C for 10 min. Then erythritol, agar, pectin, diacetyl tartaric acid ester of mono- and diglycerides, gellan gum, white sugar, and glucose were added to the mixture at 55°C for 20 min. After cooling, the mixture
15 was transferred to another ingredient tank, and the remaining ingredients were mixed with the milk in the tank. This mixture was mixed well, followed by degassed at 55°C and a vacuum of 10 kpa, homogenized at 220bar, sterilized at 115°C for 120 seconds, and pumped into the fermentation tank ready for fermentation.

[0052] Fermentation: The fermentation temperature was maintained at 40°C. When 40% of the
20 mixture remained in the ingredient tank, stirring was initiated at full rotation. YoFlex® Acidifix™ 1.0 purchased from Chr. Hansen was added through a laminar flow hood. Timing began after completion of feeding the base material. Stirring was conducted for 10 min. The mixing effect was judged by the absence of large clumps of bacterial powder in the fermentation tank. The mixture was left for fermentation, and after 7 h of fermentation, pH measurements were started.
25 When the pH of the yogurt was reduced below 4.5, the titratable acidity was adjusted to 72°T, and stirring was resumed for demulsification, with a stirring speed at 25 rpm for 2 min.

[0053] Pasteurization of Yogurt: The yogurt was fed at a temperature of 30°C, pasteurized at 77°C for 25 seconds, and discharged at 40°C. Then the yogurt was stored in a sterile tank.

[0054] *Lactobacillus rhamnosus* nu-trish® LGG® (purchased from Chr. Hansen) was sterilely

added.

[0055] Filling: The filling temperature was 20-25°C.

[0056] The method for Comparative Example 1 differed in that nu-trish® LGG® was used as the fermentation bacteria, and fermentation was carried out for 18 h before pH measurement. The yogurt was filled without pasteurization.

[0057] The results showed that, in Example 1, after 7-8 h of fermentation, the pH of the fermented milk reached 4.4-4.5, with an acidity of 70-75°T; whereas in Comparative Example 1, 18-22 h of fermentation was required for the fermented milk to reach a pH of 4.4-4.5 and an acidity of 70-75 °T.

[0058] Sensory evaluation was conducted on the yogurts prepared in Example 1 and Comparative Example 1. The results are shown in Table 2, which shows the sensory evaluation results of the yogurts prepared in Example 1 and Comparative Example 1 of the present disclosure. The sensory evaluation method was as follows:

[0059] Evaluation Process: An evaluation team of greater than or equal to 50 persons scored several aspects including curd formation, fluidity, smoothness, and oral retention on a 10-point scale, with 1 being strongly disliked and 10 being strongly liked. The final results were the average scores of the 50 persons.

[0060] Curd Formation: In the process of pH reducing and the gradual approach to the isoelectric point of the proteins, the proteins in the cow milk underwent changes in spatial configuration (mainly involving tertiary and quaternary structures). A spatial network structure was formed through hydrogen bonding, resulting in mutual cross-linking of the proteins. The proteins aggregated with each other, and this was observed as curd formation in the macro performance.

[0061] Fluidity: The flow characteristics of the yogurt.

[0062] Smoothness: Over prolonged fermentation, repeated curdling and agitation can cause whey separation and clumping, leading to an unsmooth taste when tasting.

[0063] Oral Retention: The duration the yogurt stays in the mouth..

Table 2: Sensory Evaluation Results of Yogurts Prepared in Example 1 and Comparative

Example 1 of the Present Disclosure

		Example 1	Comparative Example 1
Fermentation time		7-8 h	18-22 h
Sensory Evaluation	Curd Formation	9	6
	Fluidity	7	9
	Smoothness	8	6
	Oral Retention	9	7

[0064] As can be seen from Table 2, the fermented milk obtained by fermentation in this example of the present disclosure was rated higher in terms of curd formation, smoothness, and oral retention than the yogurt prepared in Comparative Example 1.

[0065] The yogurt prepared in Example 1 was stored at ambient temperature, 30°C, 37°C and 45°C for 6 months. The pH values of the yogurt were measured at the initial stage (W0, actual measurement time was three days after sample preparation), one week (W1), two weeks (W2), three weeks (W3), four weeks (W4), five weeks (W5), six weeks (W6), seven weeks (W7), eight weeks (W8), eleven weeks (W11), sixteen weeks (W16), seventeen weeks (W17), twenty weeks (W20), twenty-two weeks (W22), and twenty-eight weeks (W28) of the storage period. The results are shown in Table 3, which shows the pH values of the yogurt provided in Example 1 of the present disclosure.

Table 3: pH Values of Yogurt Provided in Example 1 of the Present Disclosure

Example 1	Ambient Temperature	30°C	37°C	45°C
W0	4.49	4.49	4.49	4.49
W1	4.43	4.45	4.46	4.42
W2	4.42	4.47	4.45	4.33
W3	4.35	4.47	4.45	4.3
W4	4.37	4.49	4.43	4.4
W5	4.38	4.49	4.42	4.45
W6	4.28	4.42	4.34	4.32
W7	4.3	4.48	4.35	4.44
W8	4.32	4.55	4.35	4.43
W11	4.28	4.5	4.4	4.45
W16	4.4	4.5	4.35	4.35
W17	4.35	4.4	4.3	4.4
W20	4.35	4.35	4.25	4.45
W22	4.3	4.35	4.2	4.35
W28	4.2	4.25	4.25	4.3

[0066] As can be seen from Table 3, the pH values of the ambient-temperature yogurt prepared by the present disclosure exhibited minimal decrease over 6 months at ambient temperature, 30°C, 37°C, and 45°C.

5 **[0067]** The yogurt obtained in Comparative Example 1 was stored at 4°C and ambient temperature for 6 months. The pH values of the yogurt were measured at the initial stage (D3), five days (D5), nine days (D9), sixteen days (D16), twenty-two days (D22), two months (M2), three months (M3), four months (M4), five months (M5), and six months (M6) of the storage period. The results are shown in Table 4, which shows the pH values of the yogurt provided in
10 Comparative Example 1 of the present disclosure.

Table 4: pH Values of Yogurt Provided in Comparative Example 1 of the Present Disclosure

	D3	D5	D9	D16	D22	D30	M2	M3	M4	M5	M6
4°C	4.55	4.56	4.57	4.55	4.58	4.6	4.4	4.4	4.3	4.3	4.3
Ambient Temperature	4.55	4.4	4.35	4.3	4.3	4.23	4.2	4.1	4.13	3.87	3.8

[0068] As can be seen from Table 4, the pH values of the yogurt prepared in Comparative Example 1 showed a fast decrease at ambient temperature.

15 **[0069]** The yogurts obtained in Example 1 and Comparative Example 1 were each stored at ambient temperature for 6 months. The viable bacteria count and acidity of the yogurts were measured at the initial stage (M0, actual measurement time was three days after sample preparation), one month (M1), two months (M2), three months (M3), four months (M4), five months (M5), and six months (M6) of the storage period. The results are shown in Tables 5 and 6.
20 Table 5 shows the viable bacteria count of the yogurts provided in Example 1 and Comparative Example 1 of the present disclosure, and Table 6 shows the acidity results.

Table 5: Viable Bacteria Count of Yogurt Provided in Example 1 and Comparative Example 1 of the Present Disclosure

CFU/g	D3	M1	M2	M3	M4	M5	M6
Example 1	5.6E+08	4.3E+08	1.3E+08	8.7E+07	5.5E+07	4.3E+07	4.5E+07
Comparative Example 1	1.3E+09	1.3E+08	9.5E+07	6.2E+07	1.3E+07	6.5E+06	6.3E+06

Table 6: Acidity of Yogurt Provided in Example 1 and Comparative Example 1 of the Present Disclosure

°T	M0	M1	M2	M3	M4	M5	M6
Comparative Example 1	71	76	80	83	90	93	94
Example 1	71	80	89	90	93	96	93

[0070] As can be seen from Tables 5 and 6, the yogurt prepared by the method in the Example of the present disclosure maintained a good viable bacteria count and acidity well at ambient temperature, showing no significant changes.

[0071] Example 2

[0072] The fermented milk was prepared according to the formula shown in Table 7, which shows the formula for the fermented milk prepared in Example 2 of the present disclosure.

Table 7: Formula for Preparing Fermented Milk in Example 2 of the Present Disclosure

Ingredient	Content
Fresh Cow Milk	90.6%
White Sugar	0.75%
Whey protein	1.75%
Agar	0.07%
Gellan Gum	0.03%
Isomaltulose	3.50%
Erythritol	1%
Modified Starch	1.30%
Hydroxypropyl Distarch Phosphate	1.00%
YoFlex® Acidifix™ 1.0	100 U/t
nu-trish® LGG®	0.02%

[0073] Wherein, the amount of nu-trish® LGG® added was 0.02% of the fermentation base material, and the LGG viable bacteria count in the fermentation base material after addition were 5.0×10^6 CFU/g.

[0074] The preparation method was as follows.

[0075] Preparation of Fermentation Base Material: 50% of the formulated amount of fresh cow milk was heated to 55°C, and whey protein powder was first added to the fresh cow milk. The

mixture was processed using equipment with mixing, dispersing, shearing and emulsifying functions to form a homogeneous and dispersed mixture at 55°C for 10 min. Then white sugar, isomaltulose, erythritol, and modified starch were added to the mixture at 55°C for 20 min. After cooling, the mixture was transferred to another ingredient tank, and the remaining ingredients were mixed with the milk in the tank. This mixture was mixed well, followed by degassed at 55°C and a vacuum of 10 kpa, homogenized at 220bar, sterilized at 115°C for 120 seconds, and pumped into the fermentation tank ready for fermentation.

[0076] Fermentation: The fermentation temperature was maintained at 40°C. When 40% of the mixture remained in the ingredient tank, stirring was initiated at full rotation. YoFlex® Acidifix™ 1.0 and nu-trish® LGG® purchased from Chr. Hansen was added through a laminar flow hood. Timing began after completion of feeding the base material. Stirring was conducted for 10 min. The mixing effect was judged by the absence of large clumps of bacterial powder in the fermentation tank. The mixture was left for fermentation, and after 7 h of fermentation, pH measurements were started. When the pH of the yogurt was reduced below 4.5, the titratable acidity was adjusted to 72°T, and stirring was resumed for demulsification, with a stirring speed at 25 rpm for 2 min.

[0077] Filling: The filling temperature was 20-25°C.

[0078] Comparative Example 2

[0079] The method differed from Example 2 in that F-DVS G089 (containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) from Chr. Hansen, with an addition amount of 150U/t. After fermentation, the resulting yogurt was pasteurized at 77°C for 25 seconds, and then nu-trish® LGG® from Chr. Hansen was sterilely added. The yogurt was then filled at 20-25°C.

[0080] The yogurt prepared in Example 2 was stored at 4°C, 25°C, 37°C and 45°C for 1-8 months. The viable bacteria count, pH values, and titratable acidity (TA) of the yogurt were measured at the initial stage (D3), one week (W1), two weeks (W2), three weeks (W3), four weeks (W4), five weeks (W5), six weeks (W6), eight weeks (M2), ten weeks (W10), twelve weeks (W12), four months (M4), five months (M5), six months (M6), and thirty-two weeks (W32) of the storage period. The results are shown in Tables 8, 9, 10 and 11. Table 8 shows the results for the yogurt provided in Example 2 of the present disclosure at 4°C. Table 9 shows the

results for the yogurt provided in Example 2 of the present disclosure at 25°C. Table 10 shows the results for the yogurt provided in Example 2 of the present disclosure at 37°C. Table 11 shows the results for the yogurt provided in Example 2 of the present disclosure at 45°C.

Table 8: Results for Yogurt provided in Example 2 of the Present Disclosure at 4°C

Time	D3	M3 (W12)	M4	M5	M6
Viable Bacteria Count (CFU/g)	3.60E+08	7.45E+06	2.00E+06	4.00E+06	1.20E+06
pH	4.47	4.21	4.23	4.16	4.18
TA (°T)	73.26	89	88.92	90.6	89.7

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Table 9: Results for Yogurt provided in Example 2 of the Present Disclosure at 25°C

Time	M1 (W4)	M2 (w8)	M3 (w12)	M4	M5	M6
Viable Bacteria Count (CFU/g)	8.70E+07	9.40E+07	4.00E+07	8.30E+07	6.10E+07	8.70E+06
pH	4.24	4.29	4.3	4.31	4.23	4.25
TA (°T)	88.98	92.54	96	96.06	97.4	99.02

Table 10: Results for Yogurt provided in Example 2 of the Present Disclosure at 37°C

Time	Viable Bacteria Count (CFU/g)	pH	TA
D3	3.60E+08	4.25	87.92
W1	1.49E+08	4.24	88.5
W2	1.50E+08	4.34	92.76
W3	1.53E+08	4.34	93.5
W4	1.39E+08	4.27	96.86
W5	1.54E+08	4.26	96.9
W6	1.24E+08	4.23	100.2
W8	3.70E+07	4.24	104.06
W10	6.40E+07	4.22	107.5
W12	3.70E+07	4.19	110.5
W32	1.70E+05	4.2	137

10 Table 11: Results for Yogurt provided in Example 2 of the Present Disclosure at 45°C

Time	D3	W1	W2	W3	W4
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Viable Bacteria Count (CFU/g)	3.60E+08	5.80E+07	5.00E+07	4.20E+07	1.25E+07
pH	4.28	4.25	4.3	4.33	4.31
TA (°T)	85.78	89.46	91.48	88.8	93.28

[0081] The yogurt obtained in Comparative Example 2 was stored at 25°C for 6 months. The viable bacteria count, pH values, and titratable acidity of the yogurt were measured at four weeks (W4), five weeks (W5), eight weeks (M2), twelve weeks (W12), four months (M4), five months (M5), and six months (M6) of the storage period. The results are shown in Tables 12 and 13. Table 12 shows the results for the yogurt provided in Comparative Example 2 of the present disclosure at 25°C. Table 13 shows the results for the yogurt provided in Comparative Example 2 of the present disclosure at 37°C.

Table 12: Results for Yogurt provided in Comparative Example 2 of the Present Disclosure at 25°C

25°C	D3	W8	W12	M4	M5	M6
Viable Bacteria Count (CFU/g)	4.50E+08	1.5E+07	8.2E+06	6.5E+04	<1000	<1000
pH	4.48	4.01	3.8	3.6	3.4	3.2
TA (°T)	72	90	120	123	138	138

Table 13: Results for Yogurt provided in Comparative Example 2 of the Present Disclosure at 37°C

25°C	D3	W8	W12	M4	M5	M6
Viable Bacteria Count (CFU/g)	4.50E+08	2.6E+07	<1000	<1000	<1000	<1000
pH	4.48	3.9	3.6	3.4	3.2	3.2
TA (°T)	72	120	132	137	139	135

[0082] As can be seen from Tables 8 to 13, during the 6 months of storage at ambient temperature, the yogurt fermented by the method provided in the present disclosure exhibited small changes in the viable bacteria count, pH and acidity.

[0083] Sensory evaluation was conducted on the yogurts prepared in Example 2 and Comparative Example 2. The results are shown in Table 14, which shows the sensory evaluation

results of the yogurts prepared in Example 2 and Comparative Example 2 of the present disclosure. The sensory evaluation method was as follows:

[0084] Evaluation Process: An evaluation team of greater than or equal to 50 persons scored sourness-sweetness balance and texture on a 10-point scale, with 1 being strongly disliked and 10 being strongly liked. The final results were the average scores of the 50 persons.

[0085] Sourness-Sweetness Balance: Assessing whether the yogurt's sourness and sweetness were well-balanced.

[0086] Texture: Evaluating the visual and textural experience of the yogurt, including preference level.

[0087] Evaluation: Descriptive terms with the highest frequency.

Table 14: Sensory Evaluation Results of Yogurts Prepared in Example 2 and Comparative Example 2 of the Present Disclosure

		Example 1	Comparative Example 1
Sensory Evaluation	Sourness-Sweetness Balance	8	1
	Texture	7	2
Evaluation		Acceptable sourness, minor separation of whey, uniform texture	Sharp acidity unacceptable, severe clumping, separation of whey

[0088] Example 3

[0089] The fermented milk was prepared according to the formula shown in Table 15, which shows the formula for the fermented milk prepared in Example 3 of the present disclosure.

Table 15: Formula for Preparing Fermented Milk in Example 3 of the Present Disclosure

Fresh Cow Milk	85.47%
White Sugar	1.20%
Whey protein	1.10%
Agar	0.60%
Gellan Gum	0.03%

Pectin	0.60%
Isomaltulose	7.70%
Erythritol	1%
Modified Starch	1.30%
Hydroxypropyl Distarch Phosphate	1.00%
YoFlex® Acidifix™ 1.0	100 U/t
nu-trish® LGG®	0.02%

[0090] Wherein, the amount of nu-trish® LGG® added was 0.02% of the fermentation base material, and the LGG viable bacteria count in the fermentation base material after addition was 5.0×10^6 CFU/g.

5 **[0091]** The preparation process was as follows:

[0092] 1. Reverse osmosis (RO) membrane filtration equipment was used to separate the pre-sterilized milk into RO retentate and RO permeate.

[0093] Recommended Protein Content of RO Retentate (%): $5.0 \leq \text{Protein Content} \leq 5.2$

[0094] Recommended Fat Content of RO Retentate (%): $5.7 \leq \text{Fat Content} \leq 6.4$

10 **[0095]** 2. The membrane-filtered RO retentate, comprising 50-55% of the formulated amount of the fermented milk, was heated to 60-65°C. Whey protein powder and stabilizers (excluding pectin and agar powder) were added while stirring. The mixture was maintained and mixed well at 60-65°C for 15-20 min, and the volume was standardized.

15 **[0096]** 3. The base material was homogenized at 30/160 bar and sterilized at 132°C for 4 seconds. It was then transferred to a fermentation tank and fermented at a constant temperature of 40°C using YoFlex® Acidifix™ 1.0 as the fermentation bacteria until the pH dropped below 4.5 and the acidity was above 95°T. The mixture was then stored.

20 **[0097]** 4. Preparation of B2 Gel Liquid: The RO permeate, accounting for 91-92% of the B2 gel liquid ingredients, was heated to 70-75°C. Pectin was added to form the B2 gel liquid. The mixture was processed for 15-20 min using equipment with mixing, dispersing, shearing and

emulsifying functions to be uniformly dispersed and free of visible particles. It was then sterilized at 115-121°C for 40-45 seconds, cooled to 40-45°C, and mixed with the fermented milk at a ratio of 5.5:1.

[0098] 5. Preparation of B3 Gel Liquid: The RO permeate, accounting for 83-84% of the B3 gel liquid ingredients, was heated to 70-75°C. Agar was added and mixed to form the B3 gel liquid. The mixture was sterilized at 115-121°C for 40-45 seconds, then stored sterile at 35-40°C. It was subsequently mixed with the prepared fermented milk and B2 gel liquid at a ratio of 9:1.

[0099] 6. The nu-trish® LGG® was sterilely added.

[00100] 7. Filling: The mixture was filled into sterile cups at 35-40°C to produce sterile cup-stirred yogurt.

[00101] The yogurt obtained in Example 3 was stored at 25°C for 6 months. The viable bacteria count and pH values of the yogurt were measured immediately after preparation (D0), on the third day (D3), and at two months (M2), four months (M4), and six months (M6). The results are shown in Tables 16 and 17. Table 16 shows the viable bacteria count results for the yogurt provided in Example 3 of the present disclosure at 25°C. Table 17 shows the pH value results for the yogurt provided in Example 3 of the present disclosure at 25°C.

Table 16: Viable Bacteria count of Yogurt Provided by Example 3 of the Present Disclosure at 25°C

	D0	D3	M2	M4	M6
Example 3	8.2E+06	3.2E+07	2.8E+07	4.2E+07	1.3E+07
Commercial Yogurt	2.0E+07	4.6E+05	<10	<10	<10

Table 17: pH Value of Yogurt Provided by Example 3 of the Present Disclosure at 25°C

	D0	D3	M2	M4	M6
Example 3	4.47	4.45	4.32	4.27	4.21
Commercial Yogurt	4.35	3.82	3.65	/	/

[00102] Note: Commercial yogurt refers to store-bought, low-temperature cup yogurt.

[00103] After comparison, Example 3 was compared with the commercial cup yogurt. In terms of viable bacteria count, at ambient temperature (25°C), the viable bacteria count in the yogurt prepared in Example 3 was effectively maintained, whereas the viable bacteria count in the commercial yogurt decreased sharply and became undetectable after two months. The pH of the yogurt prepared in Example 3 decreased by only 0.2, which was considered an acceptable reduction.

[00104] The above are only preferred embodiments of the present disclosure, but the scope of the present disclosure is not limited thereto. Within the technical scope disclosed in the present disclosure, any person skilled familiar with the technical field can make equivalent replacements or changes according to the technical solution and inventive concept of the present disclosure, which shall all fall within the scope of the present disclosure.

CLAIMS

1. A method for preparing an ambient-temperature fermented milk with viable bacteria, comprising:

5 fermenting a fermentation base material with a lactose-negative bacterium other than *Lactobacillus rhamnosus* as a fermentation bacterium to obtain a fermented product,

pasteurizing the fermented product, and

adding *Lactobacillus rhamnosus* to obtain an ambient-temperature fermented milk with viable bacteria;

10 wherein the fermentation base material comprises a proliferation factor other than lactose.

2. A method for preparing an ambient-temperature fermented milk with viable bacteria, comprising:

inoculating a mixture of *Lactobacillus rhamnosus* and a lactose-negative bacterium other than *Lactobacillus rhamnosus* into a fermentation base material, and

15 fermenting the mixture to obtain an ambient-temperature fermented milk with viable bacteria;

wherein the fermentation base material comprises a proliferation factor other than lactose.

3. The method according to claim 1 or 2, wherein the proliferation factor is selected from the group consisting of glucose, fructose, galactose, arabinose, ribose, mannose, rhamnose, fucose, tagatose, sucrose, maltose, cellobiose, trehalose, melezitose, gentiobiose, acetyl glucosamine, 20 white sugar, and a combination thereof, preferably white sugar.

4. The method according to claim 3, wherein the proliferation factor is present in an amount of 0.6 wt% to 1.2 wt%.

5. The method according to claim 1 or 2, wherein the lactose-negative bacterium is YoFlex® 25 Acidifix™ 1.0, and

the *Lactobacillus rhamnosus* is nu-trish® LGG®.

6. The method according to claim 5, wherein the lactose-negative bacterium is present in an

amount of 80 U/t to 150 U/t, and

the *Lactobacillus rhamnosus* is present in an amount of 1.0×10^6 CFU/g to 1.0×10^8 CFU/g.

7. The method according to claim 1 or 2, wherein the fermentation base material further comprises a milk raw material, and optionally, a flavoring agent, a stabilizer, a proteinaceous ingredient, and water.

8. The method according to claim 7, wherein the fermentation base material comprises:

80% to 99% of the milk raw material,

0 to 2% of the proteinaceous ingredient,

0 to 12% of the flavoring agent,

0 to 3% of the stabilizer, and

water to balance.

9. The method according to claim 8, wherein:

the milk raw material is selected from the group consisting of an animal-based milk raw material, a plant-based milk raw material, and a combination thereof;

the proteinaceous ingredient is selected from the group consisting of whey protein, cow milk protein, casein, soy protein isolate, soy protein concentrate, pea protein, peanut protein, walnut protein, coconut milk, and a combination thereof;

the flavoring agent is selected from the group consisting of isomaltulose, maltitol, erythritol, xylitol, galactitol, mannitol, sorbitol, essence, and a combination thereof; and

the stabilizer is selected from the group consisting of pectin, agar, gellan gum, starch, carrageenan, gelatin, diacetyl tartaric acid ester of mono- and diglycerides, mono- and diglycerides of fatty acids, acetylated distarch phosphate, hydroxypropyl distarch phosphate, citrus fiber, xanthan gum, and a combination thereof.

10. An ambient-temperature fermented milk with viable bacteria prepared by the method according to any one of claims 1 to 9.