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Research Article

Detection of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in Inactivated Fermented Milk Using Fluorescence Quantitative Loop-Mediated Isothermal Amplification

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Currently, no effective method exists to detect and monitor fermentation probiotics and evaluate the quality of inactivated fermented milk. Therefore, in this study, a fluorescence quantitative loop-mediated isothermal amplification (FQ-LAMP) method was developed to detect *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The specificity of LAMP primers for *L. bulgaricus* and *S. thermophilus* was verified using S-type amplification curves and a single peak at approximately 88.568°C and 83.704°C of the melting curves, respectively. The lowest quantification limits of FQ-LAMP for the two strains in inactivated fermented milk were 8.1×10^3 CFU/g (170 fg/μL) and 6.8×10^3 CFU/g (170 fg/μL), respectively. FQ-LAMP was used to analyse 40 inactivated fermented milk samples from six randomly selected brands. The logarithmic concentration of *S. thermophilus* in all products was between 7.482 and 8.936. The logarithmic concentration of *L. bulgaricus* ranged from 4.590 to 8.277, with no detectable *L. bulgaricus* in three samples. FQ-LAMP has the potential as a rapid, specific, and accurate method for detecting and monitoring *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk during their shelf life.

1. Introduction

Fermented milk is made from raw cow (goat) milk or milk powder that has a reduced pH, achieved through processing procedures such as sterilisation and fermentation [1, 2]. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus,* two fermentation bacteria, are beneficial for the intestinal tract [3, 4]. They can maintain the intestinal microecological balance [5], inhibit the growth and reproduction of harmful intestinal bacteria [6, 7], regulate intestinal immune function [8], and improve intestinal barrier function [9], among other health functions. As most of the lactose in fermented milk is degraded by lactic acid bacteria, their administration is more suitable for people with lactose intolerance, especially Asian populations [10, 11]. Fermented milk can be classified as sterilised (inactivated) and nonsterilised (activated), depending on whether it is sterilised at the end of fermentation [12]. However, the shelf life of activated bacteria-fermented milk is short (generally 21 days), and the acid structures tend to be

easily altered, resulting in the deterioration of the milk. Therefore, a cold chain system is required throughout the storage, transportation, and sales process, which is an inconvenience for manufacturers and dealers [13].

Inactivated fermented milk and its products have several advantages over activated milk, such as a long shelf life (generally 6 months), easy storage and transportation, and no risk of infection for susceptible people [14]. Inactivated probiotics have many beneficial effects on the human body; for example, heat-inactivated *Lactobacillus brevis* can enhance the nervous system and memory [15] and alleviate specific dermatitis symptoms [16]. Moreover, long-term use of products containing inactivated lactic acid bacteria can improve the intestinal environment and intestinal function of the consumers, aiding the treatment of gastrointestinal diseases [17–19]. Given these advantages, several inactivated fermented milk products have been introduced in the market, and the quantity of lactic acid bacteria is the core parameter for quantifying probiotic function [20]. Currently, the method for detecting lactic acid bacteria in inactivated fermented milk is primarily based on the traditional culture method after fermentation and thermal inactivation. However, applying this method is tedious, and it cannot specifically detect mixed fermentation bacteria or achieve real-time monitoring of the number of inactivated lactic acid bacteria during storage [20]. Therefore, establishing a rapid and quantitative method to detect commonly used fermentation bacteria in inactivated fermented milk is important to evaluate the quality of inactivated fermented milk.

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification method developed in 2000 [21]. This method uses four specific primers to identify six specific target gene regions that can be amplified under isothermal conditions. Gene amplification and product detection can be completed in one step with high amplification efficiency (10^9–10^{10}-fold) in 15–60 min. Furthermore, a fluorescent dye (SYBR Green I) can be optimised and added to the LAMP reaction system to produce fluorescence quantitative LAMP (FQ-LAMP) [22]. SYBR Green I binds only to double-stranded DNA grooves, resulting in a fluorescence that is 800–1,000 times stronger than the original. The fluorescence intensity represents the number of double-stranded DNA molecules. During nucleic acid synthesis, SYBR Green I can be used to automatically add double-stranded DNA, and the cycle threshold (Ct) value is obtained by detecting the fluorescence intensity. According to the standard curve, the initial concentration of the bacterial template solution can be determined for quantification. This method has the advantages of simple operation, strong specificity, high sensitivity, good repeatability, low pollution, fast running time, and automatic quantitative analysis and has thus become an important method for probiotic detection [23].

However, effective methods to detect and monitor fermentation probiotics in inactivated fermented milk have not yet been developed. Thus, in this study, we developed an FQ-LAMP method for detecting and monitoring two commonly used fermentation bacteria, *L. bulgaricus* and *S. thermophilus*. In addition, this study analysed the quantities of *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk purchased from Shijiazhuang Supermarket, Hebei Province, China, to provide a basis for ensuring the quality of inactivated milk.

2. Materials and Methods

2.1. Strains and Culture Conditions. Four strains of *L. bulgaricus* and *S. thermophilus* and thirteen common strains usually present in raw milk were used in this study for FQ-LAMP-specific detection (Table 1). All strains were preserved in the R&D Laboratory of Jun Le Bao, Shijiazhuang, China. *Listeria monocytogenes* (ATCC19111), *Cronobacter sakazakii* (ATCC29544), and *Pseudomonas fluorescens* (CICC23246) were cultured in a brain heart infusion broth medium (BHI, Beijing Land Bridge Technology Co. Ltd., Beijing, China) at 37°C for 24 h. *L. bulgaricus* (CICC6097, CICC6047, CGMCC14425, and CGMCC14427), *S. thermophilus* (CICC6063, CICC6222, CICC20174, and CGMCC11672), *Lactobacillus acidophilus* (CICC6081), *Lactobacillus rhamnosus* (CICC6001), *Lactobacillus plantarum* (CGMCC1.1856), *Lactobacillus plantarum* (CICC6009), *Lactobacillus casei* (CICC6117), *Lacticaseibacillus paracasei* (CGMCC4691), *Bifidobacterium animalis* (CICC6250), *Bifidobacterium breve* (CICC6185), *Bifidobacterium adolescentis* (CICC6180), and *Bifidobacterium bifidum* (CICC6173) were cultured separately in a Man, Rogosa, and Sharpe (MRS, Beijing Land Bridge Technology Co., Ltd., Beijing, China) liquid medium at 37°C for 24 h. The four *Bifidobacterium* strains were cultured in an anaerobic environment (Anaerobic gas bag, BioMerieux Company, Lyon, French). The medium and culture conditions for plate counting of *L. bulgaricus* and *S. thermophilus* included incubation in an MRS agar medium at 37°C for 48 h.

2.2. Sample Pretreatment and DNA Extraction. To extract the DNA of *L. bulgaricus* and *S. thermophilus* from inactivated or activated fermented milk, the fermented milk sample was pretreated [24]. Then, the DNA of the pretreated samples was extracted using a bacterial DNA extraction kit(Tiangen Biotech Co., Ltd., Beijing, China). Briefly, 0.2 g inactivated fermented milk was added to a 2-mL centrifuge tube with 1,500 μL deionised water, 200 μL 18% sodium citrate, and 100 μL 1 mol/L sodium hydroxide. Thereafter, the mixture was centrifuged at 4°C for 5 min at 13,523 × g, then the supernatant was discarded, and the precipitate was retained. Next, the genomic DNA of the precipitate was extracted using a bacterial DNA extraction kit.

2.3. Design of Specific FQ-LAMP Primers for L. bulgaricus and S. thermophilus. Sequence data for the *L. bulgaricus recA* gene [25] sequence (LC685718.1) and *S. thermophilus thioredoxin reductase* (NADPH) gene sequence (GenBank Acc. No: AGFN01000211.1) were obtained from the National Center for Biotechnology Information microbial genome

Table 1: Strains used in the study.

Order number	Strain name	Strain number	Results of FQ-LAMP detection	
			L. bulgaricus gene	*S. thermophilus* gene
1	*L. bulgaricus*	CICC6097	+	−
2	*L. bulgaricus*	CICC6047	+	−
3	*L. bulgaricus*	CGMCC14425	+	−
4	*L. bulgaricus*	CGMCC14427	+	−
5	*S. thermophilus*	CICC6063	−	+
6	*S. thermophilus*	CICC6222	−	+
7	*S. thermophilus*	CICC20174	−	+
8	*S. thermophilus*	CGMCC11672	−	+
9	*L. acidophilus*	CICC6081	−	−
10	*L. rhamnosus*	CICC6001	−	−
11	*L. plantarum*	CGMCC1.1856	−	−
12	*L. plantarum*	CICC6009	−	−
13	*L. casei*	CICC6117	−	−
14	*L. paracasei*	CGMCC4691	−	−
15	*B. animalis*	CICC6250	−	−
16	*B. adolescentis*	CICC6180	−	−
17	*B. breve*	CICC6185	−	−
18	*B. bifidum*	CICC6173	−	−
19	*P. fluorescens*	CICC23246	−	−
20	*L. monocytogenes*	ATCC19111	−	−
21	*C. sakazakii*	ATCC29544	−	−

Note. CICC strains were purchased from the China Center of Industrial Culture Collection, Beijing, China. CGMCC strains were purchased from the China General Microbiological Culture Collection Center, Beijing, China. ATCC strains were purchased from the American Type Culture Collection, Rockefeller, Maryland, USA.

database (https://www.ncbi.nlm.gov), and the gene sequences were aligned, followed by primer design. Conserved sequences were determined and used to design primers. Primers were designed using PrimerExplorer V5 (https://primerexplorer.jp/lampv5e/index.html). Information on the specific primers used is listed in Table 2. The two sets of primers included the forward and backward inner primers (FIP/BIP), the forward and backward loop primers (FL/BL), and the forward and backward outer primers (F3/B3).

2.4. Reaction System and Reaction Conditions of FQ-LAMP. The volume and concentration of the optimised FQ-LAMP reaction system for *L. bulgaricus* and *S. thermophilus* are listed in Table 3. A 25-μL reaction system, as described in Table 3, was placed in a polymerase chain reaction (PCR) tube, gently vibrated for mixing, and instantaneously centrifuged (25°C). Next, mineral oil (20 μL) was added to the reaction system to cover the reaction surface and prevent cross-contamination between samples, which can reduce the accuracy of the results.

Amplification reactions were performed using Applied Biosystems QuantStudio 3 (Applied Biosystems, Waltham, MA, USA) at 63°C for 40 min. Melting-curve analysis was performed at the end of FQ-LAMP assays by heating the reaction mixtures to 95°C for 15 s, cooling to 60°C for 60 s, and then increasing the temperature to 95°C for 15 s.

2.5. Specificity of FQ-LAMP. FQ-LAMP was used to detect the DNA of 21 strains of common lactic acid fermentative bacteria in fermented milk. The specificity of the primers used was verified by assessing whether there was an S-type amplification curve and whether the melting curve had a single peak. Distilled water was used as a blank control. All experiments were repeated five times. Positive results are indicated by the "+" symbol, and negative results are indicated by the "−" symbol as shown in Table 1.

2.6. Evaluation of the Effect of Pasteurisation and Storage Time on FQ-LAMP. Fermented milk was prepared by adding *L. bulgaricus* and *S. thermophilus* to raw milk, and the prepared fermented milk was divided into four groups, with three samples in each group. In Group A, the samples were sterilised at 65°C for 10 min; in Group B, samples were sterilised at 75°C for 10 min; in Group C, samples were sterilised at 85°C for 10 min; and in Group D, samples were not sterilised. Samples of the four groups were pretreated, and DNA was extracted for FQ-LAMP detection.

Four randomly selected brands of new date-inactivated fermented milk products were purchased from supermarkets in Shijiazhuang and stored at room temperature in the laboratory (samples of each brand were produced in the same batch). On days 3, 7, 14, 30, 60, 90, 120, and 150 of the shelf life, three samples from each brand were obtained for FQ-LAMP.

2.7. FQ-LAMP Detection Limit for L. bulgaricus and S. thermophilus in Inactivated Fermented Milk and Drawing Standard Curves. L. bulgaricus and *S. thermophilus* were inoculated separately in an MRS liquid medium and incubated at 37°C for 24 h. The *L. bulgaricus* suspension was

Table 2: Information on specific primers.

Target strain	Primer name	Sequence (5′⟶3′)	Primer sequence length (nt)	Amplified region length (bp)
L. bulgaricus (GenBank Acc. No: LC685718)	FIP	TGGAGATCAAGGTGTCCGCGAATCCTGTCTCAGCCAAACAC	41	174
	BIP	CCATCGACATCGTCGTGGTCGTTCACCTTCGATTTCGGCC	40	
	F3	GCGTGGACATCGACCAATT	19	
	B3	TCCAACGTGGGAGTCACC	18	
	BL	TTTGCAGCCCTTCTTCCCCA	20	
S. thermophilus (GenBank Acc. No: AGFN01000211.1)	FIP	TCGAAATTAAGGGTGAAAATGGTCACTTCATCCGACTTACTC TCTG	46	215
	BIP	ACTGATGATTGATAAAGAAGCTCCAGATTCACCGTCGTGATGC	43	
	F3	AGCTAACAATGAGGGCATC	19	
	B3	GTGTTGCTGAGAGTGTGA	18	
	FL	CACTATGCTCATGGGCACGAAAG	23	

Table 3: Optimised FQ-LAMP reaction system for *L. bulgaricus* and *S. thermophilus*.

Reagent name	Volume (μL)		Reagent sources
	L. bulgaricus	*S. thermophilus*	
10× ThermoPol reaction buffer	2.5	2.5	New England Biolabs Inc., USA
$MgSO_4$ (50 mmol/L)	1.0	1.3	
8 U Bst DNA polymerase	1.2	1.0	
dNTPs (10 mmol/L)	1.5	1.5	Sigma-Aldrich, St. Louis, MO, USA
1/400 dilution 10,000 × SYBR green I	0.3	0.3	Coolaber Science and Technology Co., Ltd., Beijing, China
Betaine (5 mol/L)	2.0	2.0	Leagene Co., Ltd., Beijing, China
F3/B3 (10 μmol/L)	0.5	0.7	Tsingke Biotechnology Co., Ltd., Beijing, China
FIP/BIP (10 μmol/L)	3.5	3.5	
Loop (10 μmol/L)	3.5	3.5	
DNA template	1.0	1.0	Bacterial DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China)
Sterile water	4.0	3.5	Tiangen Biotech Co., Ltd., Beijing, China

added to sterilised raw milk in a 5% ratio for fermentation, and then, plate counting of *L. bulgaricus* in the fermented milk was performed, resulting in a count of 8.1×10^8 CFU/g. Subsequently, 0.2 g of fermented milk was obtained, and the DNA was extracted and diluted with a 10-fold gradient to achieve a final concentration of 21 fg/μL to 21 ng/μL. The corresponding concentration of *L. bulgaricus* was 8.1×10^2 to 8.1×10^8 CFU/g. The *S. thermophilus* suspension was added at a 5% ratio to sterilised raw milk for fermentation, and then, plate counting of *S. thermophilus* in the fermented milk was performed, resulting in a count of 6.8×10^8 CFU/g. Subsequently, 0.2 g of fermented milk was obtained, and the DNA was extracted. The extracted DNA was diluted using a 10-fold gradient to achieve a final concentration of 17 fg/μL to 17 ng/μL. The corresponding concentration of *S. thermophilus* was 6.8×10^2 to 6.8×10^8 CFU/g. Subsequently, FQ-LAMP analysis of different concentrations of DNA from *L. bulgaricus* and *S. thermophilus* was performed, with three analyses per concentration gradient, and the average Ct value was calculated. Thereafter, a standard curve was constructed with the logarithm of the corresponding bacterial concentration as the x-axis and the corresponding Ct value as the y-axis.

Five measurements were obtained under the same conditions, and the mean, standard deviation (SD), and coefficient of variance (CV) of the peak time were calculated.

2.8. Comparison of the Accuracy of the FQ-LAMP and Plate Count Methods. *L. bulgaricus* and *S. thermophilus* were inoculated separately in an MRS liquid medium and incubated at 37°C for 24 h, and then different concentrations of each species were inoculated into sterilised milk for fermentation. The prepared fermented milk was divided into two groups; in one group, the number of bacteria was counted using the plate count method, while in the other group, the FQ-LAMP method was used for counting after heat sterilisation treatment.

2.9. Quantitative Detection of L. bulgaricus and S. thermophilus in Inactivated Fermented Milk Samples Using FQ-LAMP. Forty inactivated fermented milk samples claiming to be fortified with *L. bulgaricus* and *S. thermophilus* were randomly selected from three supermarkets in Shijiazhuang, Hebei Province, China, including different batches of the six brands. During the shelf life, each of the six brands was randomly sampled. After sample pretreatment, DNA was extracted, and quantitative detection (two parallels) and analyses of *L. bulgaricus* and *S. thermophilus* in the inactivated fermented milk samples were performed.

2.10. Statistical Analysis. SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft Corporation, Redmond, WA, USA) were used to analyse the mean, SD, CV, and scatter distribution. Comparisons between the two data groups were analysed using an independent sample t-test, and the significance level was set at $p < 0.05$.

3. Results

3.1. FQ-LAMP Specificity. The specificity of the FQ-LAMP primers of *L. bulgaricus* and *S. thermophilus* was evaluated using four strains of *L. bulgaricus* and *S. thermophilus* and thirteen strains of common lactic acid fermentation bacteria in fermented milk. The fluorescence intensity (delta Rn) of the four strains of *L. bulgaricus* (Figure 1(A)) and *S. thermophilus* (Figure 1(B)) showed continuous amplification compared with that of other strains and the blank control. In addition, the melting curves (Figure 1(A, B)) revealed that the melting temperatures of the amplified products were almost identical; they were approximately 88.568°C (*L. bulgaricus*) and 83.704°C (*S. thermophilus*), indicating that the FQ-LAMP assay was highly specific and no nonspecific amplification occurred.

3.2. The Effect of Pasteurisation and Storage Time on FQ-LAMP. FQ-LAMP detection of inactivated fermented milk (groups A, B, and C) and noninactivated fermented milk (group D) was conducted. The results (Figure S1) showed no significant differences in the Ct values between groups A, B, C, and D ($p > 0.05$), indicating that FQ-LAMP detection is not affected by the pasteurisation of fermented milk.

FQ-LAMP detection was conducted on four brands of inactivated fermented milk with different storage times, and the results (Figures S2 and S3) showed no significant difference in the Ct values of the four brands of inactivated fermented milk on days 3, 7, 14, 30, 60, 90, 120, and 150 ($p > 0.05$). Therefore, there was no significant change in the accuracy of FQ-LAMP detection of inactivated fermented milk with different storage times.

3.3. Detection Limit and Standard Curves of FQ-LAMP for L. bulgaricus and S. thermophilus in Fermented Milk. The average Ct values of inactivated fermented milk with seven 10-fold serial dilutions of *L. bulgaricus* were 11.277, 13.428, 15.777, 18.640, 20.651, 22.335, and 26.582. The CV values of the peak emergence time of inactivated *L. bulgaricus* in fermented milk ranged between 2.93 and 5.29% (Table 4). The average Ct values of inactivated fermented milk with 10-fold serial dilutions of *S. thermophilus* were 11.429, 12.917, 15.466, 17.984, 19.693, 21.918, and 26.312. The CV values of the peak emergence time of inactivated *S. thermophilus* in fermented milk ranged between 2.96 and 5.04% (Table 5).

A standard curve was drawn using the average Ct values as the ordinate and the logarithm of the concentration of inactivated *L. bulgaricus* ($\log_{10}$CFU/g) corresponding to the DNA template as the abscissa (Figure 2(a)). The resulting equation is as follows:

$$y = -2.2805x + 31.6318, \quad (1)$$

which describes a linear relationship of the standard curve ($R^2 = 0.9949$) between the Ct values in the 11.277–22.335 min range and the logarithm of the concentrations of inactivated *L. bulgaricus* in the 8.908–3.908 range. This finding indicates that the lowest detection limit

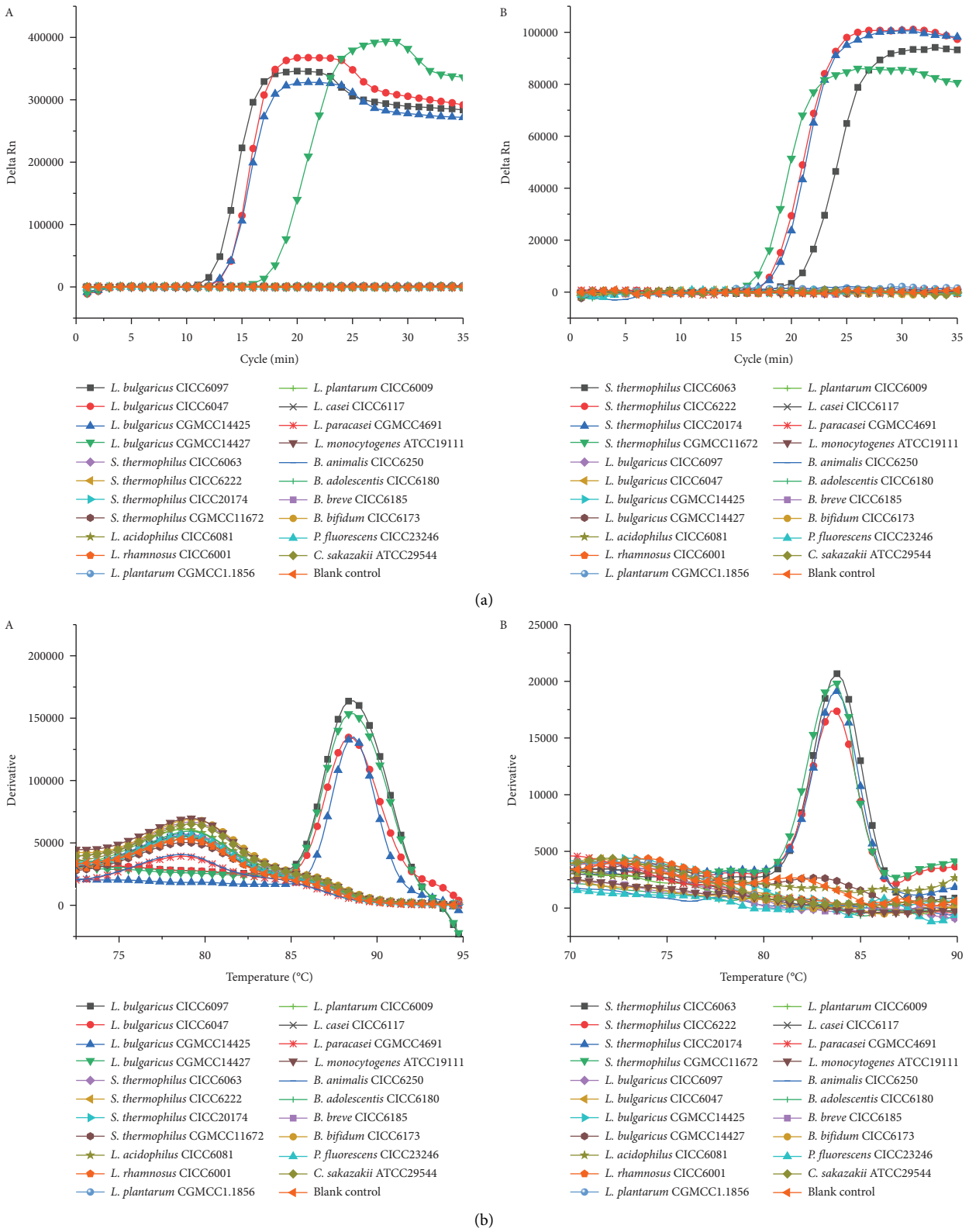

Figure 1: (a) Specific amplification curve for FQ-LAMP. (A) FQ-LAMP-specific amplification curve for *L. bulgaricus*. (B) FQ-LAMP-specific amplification curve of *S. thermophilus*. (b) Melting curve of the FQ-LAMP amplification product. (A) The melting curve of the *L. bulgaricus* product amplified using FQ-LAMP. (B) Melting curve of the *S. thermophilus* product amplified using FQ-LAMP.

Table 4: Reproducibility results of the limit of detection of *L. bulgaricus* in fermented milk using FQ-LAMP.

Order number	Concentration (CFU/g)	Times number	Mean Ct	SD	CV (%)
1	8.1×10^8	5	11.277	0.425	3.78
2	8.1×10^7	5	13.428	0.393	2.93
3	8.1×10^6	5	15.777	0.532	3.38
4	8.1×10^5	5	18.640	0.723	3.88
5	8.1×10^4	5	20.651	0.957	4.64
6	8.1×10^3	5	22.334	1.182	5.29
7	8.1×10^2	5	26.582	1.36	5.12

Note. 1–7: these 10-fold serial dilutions of *L. bulgaricus* were analysed using FQ-LAMP.

Table 5: Reproducibility results of the limit of detection of *S. thermophilus* in fermented milk using FQ-LAMP.

Order number	Concentration (CFU/g)	Times number	Mean Ct	SD	CV (%)
1	6.8×10^8	5	11.428	0.361	3.16
2	6.8×10^7	5	12.917	0.382	2.96
3	6.8×10^6	5	15.466	0.585	3.78
4	6.8×10^5	5	17.984	0.686	3.81
5	6.8×10^4	5	19.693	0.954	4.85
6	6.8×10^3	5	21.918	1.104	5.04
7	6.8×10^2	5	26.312	1.270	4.83

Note. 1–7: these 10-fold serial dilutions of *S. thermophilus* were detected using FQ-LAMP.

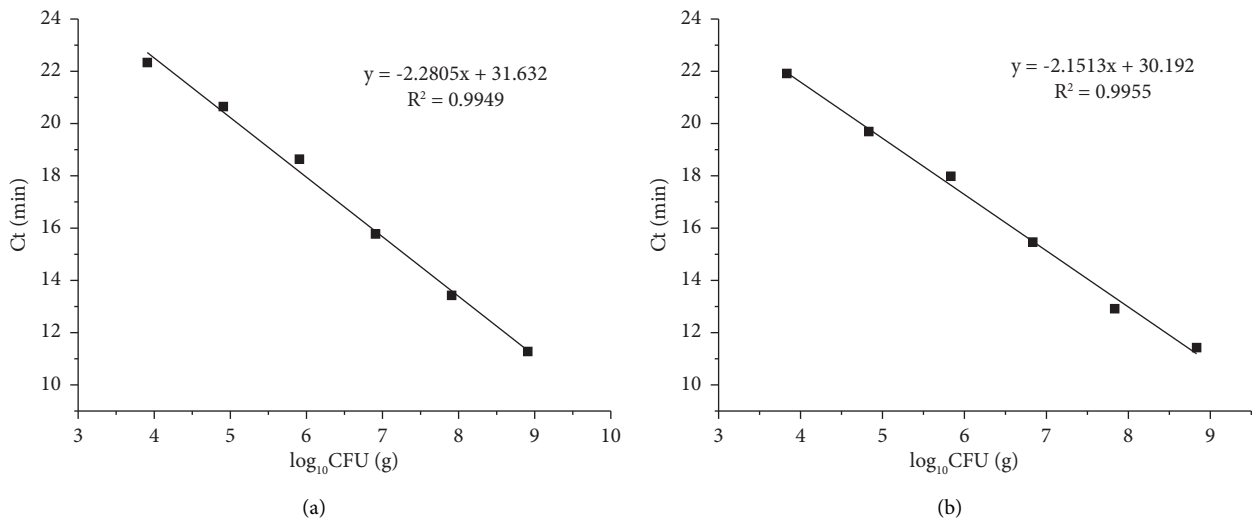

Figure 2: Standard curves of the limit of detection and linear relationship of FQ-LAMP for 10 serial dilutions of inactivated *L. bulgaricus* and *S. thermophilus*. (a) The standard curve shows a linear relationship between the average Ct values of inactivated *L. bulgaricus* and its logarithmic concentrations. (b) The standard curve shows a linear relationship between the average Ct values of inactivated *S. thermophilus* and its logarithmic concentrations.

for FQ-LAMP quantification of *L. bulgaricus* is 8.1×10^3 CFU/g (210 fg/μL)

A standard curve was drawn using the average Ct values of inactivated *S. thermophilus* as the ordinate and the logarithm of the concentrations of inactivated *S. thermophilus* ($\log_{10}$CFU/g) corresponding to the DNA template as the abscissa (Figure 2(b)). The resulting equation is as follows:

$$y = -2.1513x + 30.192, \quad (2)$$

which is a linear relationship of the standard curve ($R^2 = 0.9955$) between the Ct values in the 11.429–21.918 min range and the logarithm of the concentrations of inactivated *S. thermophilus* in the 8.833–3.833 range, indicating that the lowest detection limit for

FQ-LAMP quantification of *L. bulgaricus* is 6.8×10^3 CFU/g (170 fg/μL).

3.4. Comparison of the Accuracy of the FQ-LAMP and Plate Count Methods. The inoculation ratios of *L. bulgaricus* and *S. thermophilus* in fermented milk were 3 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 3. The detection results of the plate count and FQ-LAMP methods are shown in Table 6. The results show that there was no significant difference in the numbers of *L. bulgaricus* and *S. thermophilus* between the two methods. Compared with that of the plate count method ($p > 0.05$), the FQ-LAMP produced a higher quantitative error of 0.16 $\log_{10}$CFU/g. Therefore, the FQ-LAMP can quantify the number of *L. bulgaricus* and *S. thermophilus* in sterilised fermented milk more quickly. Moreover, the counting error of the plate count method was between 0.021 and 0.054, while the counting error of the FQ-LAMP was between 0.085 and 0.178, indicating that the FQ-LAMP method has worse stability and accuracy compared to those of the plate count method.

3.5. Quantitative Detection of L. bulgaricus and S. thermophilus in Inactivated Fermented Milk Samples Using FQ-LAMP. Table 7 shows the FQ-LAMP results for 40 samples of inactivated fermented milk from six brands. Samples beyond the detection range were diluted 10 times and tested again. As shown in Table 7, the total number of *L. bulgaricus* and *S. thermophilus* across all inactivated fermented milk samples was $>10^6$ CFU/g, and the logarithmic concentration of *S. thermophilus* in all products was between 7.482 and 8.936. There was little difference in the concentrations of *S. thermophilus* between the different batches of samples from each brand. The logarithmic concentrations of *L. bulgaricus* ranged from 4.590 to 8.277, and three samples had no detectable *L. bulgaricus*. The number of *L. bulgaricus* in the products was generally lower than that of *S. thermophilus*, and the concentration of *L. bulgaricus* between different batches of brands A and D varied by up to 100 times.

4. Discussion

In recent years, fermented milk consumption and sales have rapidly increased in China [20]. Inactivated fermented milk has many advantages over activated fermented milk and a market growth rate as high as 50% [20]. Owing to the rapid increase in the popularity of inactivated fermented milk and its products, detecting fermentation bacteria is a concern for consumers and poses a crucial quality issue. For such products, the number and strain of the fermentation bacteria are important indicators for determining their quality. Different businesses use different fermentation strains for fermented milk. Fermented milk is mainly prepared through the mixed fermentation of *L. bulgaricus* and *S. thermophilus*, which are highly sensitive to pH and bile; thus, it is difficult for these bacteria to reach the intestinal tract in an active state. Live probiotics are thought to enter the intestine to exert their probiotic effects, but metabolites produced by lactic acid bacteria during fermentation, such as organic acids, bacteriocins, enzymes, extracellular polysaccharides, and short-chain fatty acids, also have beneficial effects [26]. Moreover, bacterial cell components contain peptidoglycan, teichoic acid, lipoteichoic acid, and acetal phospholipid, which have been shown to have beneficial functions [27]. Therefore, *L. bulgaricus* and *S. thermophilus* have beneficial properties in inactivated fermented milk.

The traditional cultivation method is largely used for quantitative analysis of bacteria; however, it is cumbersome and cannot determine specific species and the quantity of dead bacteria, making it impossible to monitor sterilised products in circulation to ensure their quality. In this study, FQ-LAMP specifically amplified *L. bulgaricus* and *S. thermophilus* from 17 common probiotic and pathogenic bacteria in fermented milk, which indicated that the method had strong primer specificity. The FQ-LAMP detection results of fermented milk before and after sterilisation showed no significant difference in Ct values between *L. bulgaricus* and *S. thermophilus* indicating that the FQ-LAMP method can accurately quantify inactivated bacteria. Notably, in the FQ-LAMP analysis of four randomly selected brands of inactivated fermented milk during the 5-month storage period, there was no significant change in the Ct values of *L. bulgaricus* and *S. thermophilus*. This finding suggests that the method is sufficient to analyse product quality during the 5-month shelf life. This may be because short-term pasteurisation reduces the enzyme activity in fermented milk but does not completely destroy the cell structure of Gram-positive bacteria, resulting in little degradation of bacterial DNA. However, this avenue requires further research.

In this study, the FQ-LAMP limit of quantitation of *L. bulgaricus* and *S. thermophilus* in fermented milk was 8.1×10^3 CFU/g and 6.8×10^3 CFU/g, respectively. Similarly, Wang et al. [9] used qPCR to detect *S. thermophilus* with a detection limit of 10^3 CFU/mL, which was in the same magnitude order. The CV range of Ct values for detecting *L. bulgaricus* and *S. thermophilus* using the FQ-LAMP method was 2.93–5.29% and 2.96–5.04%, respectively. In comparison, Achilleos and Berthier [28] used qPCR to quantify lactic acid bacteria in cheese, which had a CV range of 2.16–3.56%. The FQ-LAMP method has a fast amplification speed, and the reaction system has many components that are easily affected by human factors; therefore, the stability of FQ-LAMP and qPCR is relatively poor, especially when the concentration of bacteria is less than 10^5 CFU/g. However, the bacterial count in fermented milk is generally greater than 10^6 CFU/g and does not require precise counting; thus, this method can be used for quantifying inactive *L. bulgaricus* and *S. thermophilus* in milk. Furthermore, FQ-LAMP has a faster amplification speed than qPCR and does not require a thermal cycling device, making it less expensive.

Yamamoto et al. [29] found that *L. bulgaricus* and *S. thermophilus* can be cofermented at certain concentrations, which results in a faster fermentation speed and better flavour. In the process of collaborative fermentation, *S. thermophilus* initially decomposes lactose and produces

Table 6: Detection results of manually prepared samples using the plate count method and FQ-LAMP.

Ratio of *S. thermophilus* and *L. bulgaricus*	Counting results (Log_{10}CFU/g)			
	S. thermophilus		*L. bulgaricus*	
	Plate count	FQ-LAMP	Plate count	FQ-LAMP
3 : 1	8.374 ± 0.054	8.332 ± 0.159	8.069 ± 0.041	8.219 ± 0.159
2 : 1	8.290 ± 0.022	8.422 ± 0.150	8.249 ± 0.023	8.352 ± 0.127
1 : 1	8.348 ± 0.035	8.510 ± 0.120	8.234 ± 0.022	8.300 ± 0.149
1 : 2	8.505 ± 0.038	8.444 ± 0.085	7.277 ± 0.040	8.324 ± 0.097
1 : 3	8.111 ± 0.024	8.208 ± 0.178	8.676 ± 0.049	8.732 ± 0.113

Table 7: FQ-LAMP detection results of *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk that was randomly purchased from the market.

Sample	*L. bulgaricus* (Log_{10}CFU/g)	*S. thermophilus* (Log_{10}CFU/g)
A1	0	8.227
A2	6.193	8.508
A3	6.899	8.036
A4	5.646	8.139
A5	4.876	8.548
A6	5.780	8.892
B1	7.179	8.296
B2	7.486	8.02
B3	6.479	8.266
B4	7.598	7.96
B5	7.321	8.277
B6	7.166	8.437
B7	7.519	8.412
C1	7.412	8.137
C2	7.095	7.756
C3	6.687	8.358
C4	7.935	8.383
C5	6.549	7.865
C6	5.915	8.358
C7	6.241	8.257
D1	4.59	8.936
D2	0	8.528
D3	4.778	8.476
D4	0	8.802
D5	6.691	8.359
E1	8.276	7.482
E2	7.734	8.058
E3	7.335	7.699
E4	7.522	8.125
E5	7.742	7.701
E6	8.182	7.526
E7	7.350	7.985
E8	8.125	8.011
F1	6.211	7.976
F2	6.934	7.898
F3	7.036	8.253
F4	6.147	8.125
F5	6.327	8.256
F6	6.765	8.223
F7	6.2456	7.99

Note. A, B, C, D, E, and F are six brands of inactivated fermented milk.

organic acids, which promotes the growth of *L. bulgaricus*. Subsequently, some amino acids and valine produced by *L. bulgaricus* metabolism contribute to the growth of *S. thermophilus* [2, 30]. A starter with *S. thermophilus* as the dominant strain presented superior fermentation in terms of acid production, butanedione production, and texture characteristics. By contrast, a starter with *L. bulgaricus* as the dominant bacteria showed high acetaldehyde production

and a strong protein hydrolysis ability [31]. *S. thermophilus* is generally more abundant than *L. bulgaricus* in fermented milk products as it produces a good flavour and controls postacidification. In a survey of 40 inactivated fermented milk products on the market, it was found that the concentration of *S. thermophilus* was above 10^7 CFU/g, while the concentration of *L. bulgaricus* varied greatly and even varied by more than 100 times between different batches of the same brand. This may be because the manufacturer has made improvements to the formula of the product, or it may be due to unstable product quality. In this study, *L. bulgaricus* was not detected in three samples, which may be due to the number of bacteria being below the detection limit or the product itself being substandard. Alternatively, some factors may have degraded the DNA of *L. bulgaricus* during storage, but this needs to be further studied.

FQ-LAMP can also be used to quantify *L. bulgaricus* and *S. thermophilus* in other fermented products and other lactic acid bacteria with the generation of appropriate primers. Therefore, with continued development, FQ-LAMP, a reliable and rapid detection method, can be applied to a wider range of fields. Compared with that of the plate count method, FQ-LAMP has a larger error, is, thus, only suitable for rapid counting of products with high concentrations of bacteria, such as fermented milk, and is not suitable for accurate enumeration of bacteria. In the future, further research is needed to improve the accuracy of this method.

5. Conclusions

The FQ-LAMP method has high specificity and sensitivity for detecting *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk. It can accurately quantify *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk in the range of 8.1×10^8 to 8.1×10^3 CFU/g and 6.8×10^8 to 6.8×10^3 CFU/g, respectively. If the bacterial count in fermented milk exceeds the upper limit of quantification, the sample can be diluted before testing. Using the scatter distribution of FQ-LAMP detection, 40 samples of inactivated fermented milk from six brands that were randomly selected from supermarkets were analysed. The concentration logarithm of *L. bulgaricus* was lower than that of *S. thermophilus*, and the concentration of *S. thermophilus* in all samples was above 10^7 CFU/g. By contrast, there was a significant difference in the concentration of *L. bulgaricus*, and three samples did not contain *L. bulgaricus*. Thus, FQ-LAMP is a specific, sensitive, accurate, and reliable detection method for *L. bulgaricus* and *S. thermophilus* in inactivated fermented milk. This method can be used to monitor the number of fermentation bacteria in inactivated fermented milk during storage in real time and provide a basis for evaluating the quality of inactivated fermented milk.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Shuaikang Zhou, Lianxia Hu, and Yuling Xue contributed equally to this work and shared the first authorship.

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Supplementary Materials

Figure S1: Ct values of FQ-LAMP detection in inactivated fermented milk sterilised at different temperatures. A: Samples were sterilised at 65°C for 10 min, B: Samples were sterilised at 75°C for 10 min, C: Samples were sterilised at 85°C for 10 min, D: Samples were not sterilised. The same lowercase letters indicate that there is no significant difference in the FQ-LAMP detection results of *S. thermophilus* between samples at different sterilisation temperatures. The same uppercase letters indicate that there is no significant difference in the FQ-LAMP detection results of *L. bulgaricus* between samples at different sterilisation temperatures. Figure S2: Ct values of FQ-LAMP analysis of *L. bulgaricus* in inactivated fermented milk with different storage times. Samples 1, 2, 3, and 4 are four brands of inactivated fermented milk. The same lowercase or uppercase letters indicate that there is no significant difference in the FQ-LAMP test results of the same sample under different storage times. Figure S3: Ct values of FQ-LAMP analysis of *S. thermophilus* in inactivated fermented milk with different storage times. Samples 1, 2, 3, and 4 are four brands of inactivated fermented milk. The same lowercase or uppercase letters indicate that there is no significant difference in the FQ-LAMP test results of the same sample under different storage times. (*Supplementary Materials*)

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