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Establishment of ddPCR detection technology system for three contaminants in fermented milk

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Abstract

Three typical contaminant bacteria, namely, *Gluconobacter*, *Acetobacter*, and *Lactobacillus*, were selected from fermented milk. The strong specific genes were screened as target sequences, and the droplet digital polymerase chain reaction (ddPCR) amplification system and reaction conditions were optimized. We established a ddPCR detection method for the three kinds of bacteria in fermented milk and then verified the specificity and sensitivity of the method. Absolute quantitative results were also analyzed. Results showed that ddPCR detection method for the three contaminants was specific and sensitive, and that the lowest detectable concentrations of *Gluconobacter*, *Bacteroides*, and *Lactobacillus plantarum* were 8.8×10^0 CFU/mL, 8.9×10^1 CFU/mL, and 9.6×10^1 CFU/mL, respectively. The relationship between the quantitative analysis results and ddPCR test results was good, proving the feasibility of ddPCR for absolute quantitative detection. This manuscript reports on establishment of a ddPCR detection system for three bacterial contaminants in fermented milk. The ddPCR detection system has efficient detection of targeted bacterial contaminants in fermented milks that can be valuable to the dairy industry in terms of preventing economic loss due to microbial spoilage and associated quality defects.

Keywords: droplet digital polymerase chain reaction; *Gluconobacter*; *Acetobacter*; *Lactobacillus*.

Practical Application: The research contributes to the establishment of a standardized ddPCR method for the detection of target bacterial contaminants in fermented milk. The establishment of the ddPCR method will help to improve the identification level of contaminants in fermented milk, standardize the operation of food safety market supervision.

1 Introduction

With the continuous development of society, peoples living standards and quality of life have been continuously improved, and healthy foods have become increasingly desired as consumers' food selection criteria have shifted from taste and price to health-related food information such as nutrition and food materials and ingredients (Bi et al., 2020). Fermented milk is a collective term for milk and other animal dairy products and plays an important role in the formation of microbial fermentation and acidic coagulation products (Farnworth et al., 2007; Muramalla & Aryana, 2011). Fermented dairy foods, owing to their rich nutrient and probiotic content, are associated with a wide range of health benefits (Zhang et al., 2019). Fermented milk contains a lot of live probiotics (Klemm et al., 2020) and some essential nutrients, and it can alleviate lactose intolerance (Barichella et al., 2016), can assist in the treatment of constipation and bacterial diarrhea (González et al., 2019). can regulate the balance of gut microbiome (Galdeano et al., 2011). The latest research found that not only viable Lactobacillus strains but also milk products fermented by *Lactobacillus* can modulate the gut–bone axis (Eor et al., 2020), especially *Lactobacillus plantarum* B719-fermented milk product could serve as a potential candidate for the treatment and prevention of postmenopausal osteoporosis (Lee et al., 2020). Therefore, fermented milk has a certain market demand. However,the product quality of fermented milk extremely susceptible to storage conditions, shelf-life, microbial contamination, and other factors. The effect of microbial contamination on fermented milk is the most serious and difficult to control (Nwamaka & Chike, 2010; Falenski et al., 2011; Poimenidou et al., 2009). Fermented milk production requires various fermenting bacteria, such as *lactic* acid bacteria, yeast and *acetic* acid bacteria in kefir (Saygili et al., 2021), and there are contaminating microorganisms in raw materials such as *staphylococcus* in raw milk (Moghadam et al., 2021). Thus, the fermentation of milk is easily susceptible to bacterial contamination. Once the contaminants began to multiply and metabolize, the fermented milk will produce whey separation, become musty, show discoloration, produce gas, clot, and have alcohol-fermented flavor or sour taste, which affects the function of fermented milk and causes fermented milk to spoil. The quality and safety of dairy products such as fermented milk has attracted more and more attention. Once the contaminated fermented milk is brought into the market, not only it will affect the health of consumers but also generate a huge loss in economic benefits to enterprises, seriously affecting the reputation of the enterprise. Different strains of fermentation had obvious effects on sensory characteristics of fermented milk (Cruz et al., 2021). Therefore, timely and effective detection of bacterial population

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in fermented milk is essential to ensure the quality and safety of fermented milk (Raspor & Goranovič, 2008).

Gluconobacter is an important bacterium belonging to the genus *Acetobacter*, which was discovered and studied previously. *Gluconobacter* belongs to aerobic bacteria and is common in environments with high sugar content. *Acetobacter* is also an important bacterium belonging to the genus *Acetobacter*, which is lately, initially classified as a subgenus of *Acetobacter* (Goodfellow et al., 2012). *Acetobacter* uses mostly glucose, ethanol or acetate as carbon source for its growth with the optimum growth pH of 2.5–6.0. *Lactobacillus plantarum* belongs to the genus *Lactobacillus* and are mostly isolated from plants, suggesting its name. *L. plantarum* belongs to the same type of lactic acid bacteria (Shah, 2000). Growth of *L. plantarum* requires complex nutrient requirements including amino acids, peptides, salts, fatty acids, and fermentable carbohydrates. Acetate and *L. plantarum* in fermentation products, wine industry, and meat industry have also caused great concern in the past few years (Bartowsky & Henschke, 2008; Ouoba et al., 2012). Fermented milk is contaminated with acetic acid bacteria, and internal microbes interact with various mechanisms, which may cause positive, neutral or negative effects directly through physical contact or through signal molecules, ultimately leading to changes in product composition and product quality (Pastink et al., 2008). The detection of acetic acid bacteria as microbial contaminants meets actual production needs and can also improve product quality and safety (Foschino et al., 1993).

The traditional method for detecting acetic acid bacteria is gradient culture analysis method, which is a method of combining traditional purification and culture by using physiological and biochemical identification reactions (Zhou et al., 2017). Phenotypic analysis is simple in principle, simple in operation, and low in cost. However, due to the complex and wide variety of conditions for the separation of acetic acid bacteria, the detection sensitivity is low, the detection period is long, and the separation and screening are easily disturbed by other strains in physiological and biochemical reactions. The characterization of the acetic acid bacteria will affect the accuracy of the test (Gibson et al., 1996; Heid et al., 1996; Zipper et al., 2004). At the end of the 20th century, Vogelstein & Kinzler (1999) proposed the concept of digital polymerase chain reaction (dPCR), which distributes a sample reaction system evenly into a large number of reaction units. Each reaction unit does not contain or contains one to multiple nucleic acid sequences. The number is consistent with the Poisson distribution. The target molecules were independently subjected to polymerase chain reaction (PCR) amplification in each reaction unit. At the end of the amplification, the fluorescence signal of each reaction unit is detected, and the copy number of the target nucleic acid sequence is calculated based on the ratio of the Poisson distribution and the number of reaction units positive for the fluorescence signal to the total number of reaction units. Currently, commercialized dPCR technology can be divided into two categories, namely, microtiter digital PCR or droplet dPCR (ddPCR) and chip-type dPCR (cdPCR) technology. The dPCR exhibits the advantages of high sensitivity, high precision, high tolerance, and absolute quantification. The dPCR is an emerging technology with great potential. The dPCR is widely used in rare mutation detection, copy number variation analysis, and complex sample gene expression detection (Margulies et al., 2005; Sanders et al., 2011; Dube et al., 2008).

In this study, we established a ddPCR detection system for three kinds of contaminant bacteria and carried out the specificity, sensitivity, and absolute quantitative feasibility of the three bacteria, *Gluconobacter*, *Gluconacetobacter* and *L. plantarum*.

2 Materials and methods

2.1 Experimental strains

The strains used in this experiment (Table 1) were stored at -80 °C in the laboratory. All strains were activated with their indicated medium, incubated at the appropriate temperature, and transferred thrice. Except for *Gluconobacter*, *Gluconacetobacter* and *Lactobacillusplantarum*, the rest were control samples.

2.2 DNA extraction

Reagent D treatment of bacterial culture medium was performed as follows. Passage was shaken thrice in a bacterial culture medium, and 100 μL enrichment solution was added for absorption in a 2 mL sterile transparent centrifuge tube. Then, the bacteria from Reagent D were incubated and removed at -20 °C and cooled to room temperature. Liquid of 4-fold volume of Reagent D solution was added to the bacterial solution and incubated at room temperature for 5 min. The medium was placed in a low-temperature centrifuge tube and placed under a 500 W halogen lamp below 15-20 cm. After 5 min, the bacteria were centrifuged at 7400 × g for 5 min at room temperature and carefully removed the supernatant with a pipette. We added 100 μL of sterile deionized water to the centrifuge tube and mixed well for genomic DNA extraction. The extraction of bacterial genomic DNA was carried out according to the method of extracting the bacterial DNA kit by magnetic bead method using the automatic DNA extraction instrument (Thermo Company, USA).

2.3 ddPCR detection method

The factors affecting the results of ddPCR were optimized according to the previous stage, and the optimum amplification conditions of each strain were finally determined.

The total volume of the ddPCR reaction system was 20 μL, consisting of 10 μL 2 × ddPCR Super Mix, 1.2 μL upstream primer, 1.2 μL downstream primer, 0.4 μL probe, 4.4 μL template, and water. The well-mixed PCR reaction system was transferred to the droplet-generating card, and 70 μL of droplet generation oil was added to the droplet-generating card. Then, the droplet-generating card was placed in the droplet generator for reaction. The resulting droplets were subsequently transferred to 96 wells of ddPCR, and the 96-well plate was sealed with a sealing film to prepare a PCR reaction.

The ddPCR reaction procedure was performed as follows: 95 °C predenaturation for 10 min; 94 °C denaturation for 1 min; 56 °C annealing, 45 s; 40 cycles, and 98 °C for 10 min. The annealing temperature of methotrexate was 54 °C. The annealing temperature

Table 1. Experimental strains.

Strain number	Strain name (Latin)	Culture conditions/Gram-character	Culture medium
CGMCC1.0637	*Gluconobacter oxydans*	28 °C, Aerobic, G−	Acetate culture medium
CGMCC1.3748	*Pseudomonas aeruginosa*	28 °C, Aerobic, G−	Acetate culture medium
CICC10357	*Glucose bacteria*	28 °C, Aerobic, G−	Acetate culture medium
CICC21682	*Acetobacter aceti*	30 °C, Aerobic, G−	Acetate culture medium
CICC21684	*Acetobacter aceti*	30 °C, Aerobic, G−	Acetate culture medium
CICC21683	*Acetobacter aceti*	30 °C, Aerobic, G−	Acetate culture medium
CICC22519	*Acetobacter aceti*	30 °C, Aerobic, G−	Acetate culture medium
CGMCC1.24	*Acetobacter liquefaction*	30 °C, Aerobic, G−	Acetate culture medium
CGMCC1.811	*Acetobacter spp*	30 °C, Aerobic, G−	Acetate culture medium
CICC21102	*Sucrose GluconAcetobacter*	30 °C, Aerobic, G−	Acetate culture medium
CICC6076	*Lactobacillus plantarum*	37 °C, Anaerobic, G+	*Lactobacillus* culture medium
CICC6224	*Lactobacillus rhamnosus*	37 °C, Anaerobic, G+	*Lactobacillus* culture medium
CICC6243	*Lactobacillus acidophilus*	37 °C, Anaerobic, G+	*Lactobacillus* culture medium
CICC6024	*Lactobacillus helveticus*	30 °C, Facultative anaerobic, G+	Skim milk powder medium
CICC10389	*Escherichia coli*	37 °C, Facultative anaerobic, G−	Nutrient broth
CICC23471	*Slow Staphylococcus*	37 °C, Facultative anaerobic, G+	Nutrient broth
CICC10398	*Staphylococcus epidermidis*	37 °C, Facultative anaerobic, G+	Nutrient broth
CICC22941	*Staphylococcus saprophyticus*	37 °C, Facultative anaerobic, G+	Nutrient broth
CICC23431	*Staphylococcus aureus*	37 °C, Facultative anaerobic, G+	Nutrient broth
CICC10373	*Hemolytic Streptococcus*	37 °C, Facultative anaerobic, G+	Blood agar medium
CICC22937	*Campylobacter jejuni*	37 °C, Micro-aerobic, G−	Campylobacter medium
CICC6223	*Streptococcus thermophilus*	42 °C, Facultative anaerobic, G+	MRS medium
CICC6077	*Lactobacillus delbrueckii*	37 °C, Facultative anaerobic, G+	MRS medium
CICC6069	*Bifidobacterium infantis*	37 °C, Anaerobic, G+	Bifidobacterium culture medium
CICC21633	*Listeria monocytogenes*	37 °C, Facultative anaerobic, G+	Brain infusion medium
CICC21617	*Vibrio parahaemolyticus*	30 °C, Facultative anaerobic, G−	3.5% sodium chloride broth
CMCC63301	*Bacillus cereus*	30 °C, Facultative anaerobic, G+	MYP medium
NCTC12900	*Escherichia coli O157:H7/NM*	36 °C, Facultative anaerobic, G+	Nutrient broth
ATCC14028	*Salmonella typhimurium*	36 °C, Facultative anaerobic, G+	Nutrient broth
CMCC50071	*Salmonella enterica subsp enterica*	36 °C, Facultative anaerobic, G+	Nutrient broth
CMCC49027	*Proteus vulgaris*	36 °C, Facultative anaerobic, G+	Nutrient broth
CMCC51592	*Shigella sonnei*	36 °C, Facultative anaerobic, G+	Nutrient broth
CICC10041	*Bacillus cereus*	30 °C, Facultative anaerobic, G+	Beef Ingredients Trace Element Medium
CICC21669	*Yersinia enterocolitica*	26 °C, Facultative anaerobic, G−	Nutrient broth

of *Acetobacter* spp. was 52.4 °C, and the annealing temperature of *L. plantarum* was 52.7 °C.

After the end of the ddPCR reaction, the 96-well plate was placed in the QX200 Droplet Reader, and the sample information was sequentially recorded. After the start of the test, the instrument automatically identified the droplets of each sample in sequence, and the droplets were detected by the two-color detection. The positive and negative results were determined based on the intensity of the fluorescence signal emitted by the droplets, and the number of positive and negative droplets per sample was recorded. After the signal acquisition is complete, the software Quantasoft was used to calculate the final result and was imaged.

2.4 Primer and probe design

In combination with the literature, the primers were selected from the sequences published in the GenBank database for the specificity and relatively conserved sequence of the strains of *Gluconobacter*, *Gluconacetobacter*, and *L. plantarum*.. The target genes were glucose bacteria KF896260.1, glucose Bacillus sp. HQ677466.1, and *L. plantarum* AJ579541.1. The primers were designed using Primer Premier 6.0 and verified by Oligo 7.37, followed by BLAST online alignment. Finally, the primers of the above strains were determined by experimentation (Table 2).

2.5 Specificity and sensitivity testing

The DNAs of the strains listed in Table 1 were extracted and diluted to 103 copies. Specificity test was carried out on the optimal reaction conditions for the specific test of *Gluconobacter*, *Glucoseacetobacte* and *L. plantarum*.

Fermented milk purchased from the local supermarkets was not contaminated by *Gluconobacter*, *Glucoseacetobacte* and *L. plantarum* as confirmed by the national standard test. The three kinds of bacteria were added into the fermented milk,

Table 2. Primers and probe sequences.

Target strain	Target gene	Primers and probe sequences (5'→3')
Gluconobacter	ITS	Upstream primers: CAATCCGGAACAAGCTGAAAA
		Downstream primer: TTCGTGCTGCGAGTTTGAGA
		Probe: FAM-CAGTCGTACGTA-BHQI
Glucose-acetobacte	16S	Upstream primers: CAATCCGGAACAAGCTGAAAA
		Downstream primer: TTCGTGCTGCGAGTTTGAGA
		Probe: FAM-CAGTCGTACGTA-BHQI
Lactobacillus plantarum	scrB	Upstream primers: CTACTACGCGCTAATCGG
		Downstream primer: CGTTCAACTCGCCTTGACA
		Probe: FAM-ACGACTACTGAGATCGGTGCT-BHQI

and the concentration of *Gluconobacter*, *Glucoseacetobacter* and *L. plantarum* in the samples were 10^0 CFU/g, 10^1 CFU/g, 10^2 CFU/g, 10^3 CFU/g, 10^4 CFU/g, 10^5 CFU/g, 10^6 CFU/g, 10^7 CFU/g and 10^8 CFU/g. Then, the contaminants were extracted by magnetic beads, and genomic DNA of bacteria was used for the validation test for the sensitivity by using ddPCR assay method.

2.6 Absolute quantification of contaminants

From the NCBI, the theoretical copy numbers of the corresponding target gene of *Gluconobacter* KF896260.1, *Gluconacetobacter* HQ677466.1, and *L. plantarum* AJ579541.1 were found in the entire genome of the strain. According to the ddPCR, the number of copies, the measured sensitivity, and the deviation rate, which are all present in 20 μL of the system, can be calculated as follows:

Measured sensitivity = 20 μL copy number × 18.18 (DNA extraction volume/the volume of the template in the system)/ theoretical copy number,

Deviation rate = (measured sensitivity – count sensitivity)/ count sensitivity × 100%.

To verify the feasibility of ddPCR detection method, ddPCR was detected by different concentrations of *Gluconobacter*, *Gluconacetobacter*, and *L. plantarum*,, and the linear relationship between the counting results and the ddPCR test results was analyzed.

2.7 Actual sample test

53 different fermented dairy products were selected from the market. Three fermented milk samples were randomly selected from 53 kinds of fermented milk to add three target strains with the concentration of 10^2 CFU/mL as the experimental group and the other fermented milk as the control group.

3 Results

3.1 Specificity of PCR method

The genomic DNA of the test strains listed in Table 1 was amplified by PCR with the amplification primers and probes of *Lactobacillus Gluconacetobacter* and *L. plantarum*, followed by detection with a droplet detector. Figure 1 shows that the three strains of glucose bacteria (a), two glucose acids (b), and *L. plantarum* (c) were able to detect positive droplet signals, whereas negative and other nonSalmonella (d) were not amplified, indicating that the study of the establishment of contamination bacterial detection method for the three kinds of bacteria to determine their specificity is good.

3.2 Sensitivity of PCR method

The results showed that the separation of negative and positive droplets occurred from the third reaction, and the results of ddPCR method showed that the concentration of the negative and positive droplets increased from the third reaction to the 8th reaction time. The number of positive microbes that can be detected is very small when the template concentration is 8.8×10^0 CFU/g. The number of copies reported by the droplet analyzer is 0.12 copies/μL. Thus, ddPCR method detected the sensitivity of the bacteria at 8.8×10^0 CFU/g (Figure 2a). The sensitivity of ddPCR detection method showed that when the dilution of the original template was 8.9×10^0 CFU/g, the droplet detector was determined to be negative. The droplet analyzer reported the copy number of 0.20 copies/μL. Thus, ddPCR method was used to detect the glucose-acetate bacterial count sensitivity of 8.9×10^1 CFU/g (Figure 2b). The plate count of *L. plantarum* was 9.6×10^7 CFU/g, and the sensitivity of the ddPCR assay showed that it can be detected in the 8th reaction when the concentration of bacteria was 9.6×10^1 CFU/g. The number of positive microbes was very small, and the copy number was 0.63 copies/μL. Therefore, ddPCR method was used to detect the sensitivity of *L. plantarum* of 9.6×10^1 CFU/g (Figure 2c).

3.3 Sensitivity of PCR method

The measured sensitivity was calculated according to the theoretical copy number of the bacteria and the actual test results of the genus *Lactobacillus acidophilus* and *L. plantarum*. When the measured sensitivity was greater than the counting sensitivity, the deviation rate displayed positive deviation. When the measured sensitivity was less than the counting sensitivity, the deviation rate showed negative deviation. The results showed that the sensitivity of the other two strains was slightly higher than the counting sensitivity (Table 3), and the deviation rate of ddPCR was lower than that of the counting sensitivity of 25% (Table 4). The absolute quantification of ddPCR is feasible in practical applications.

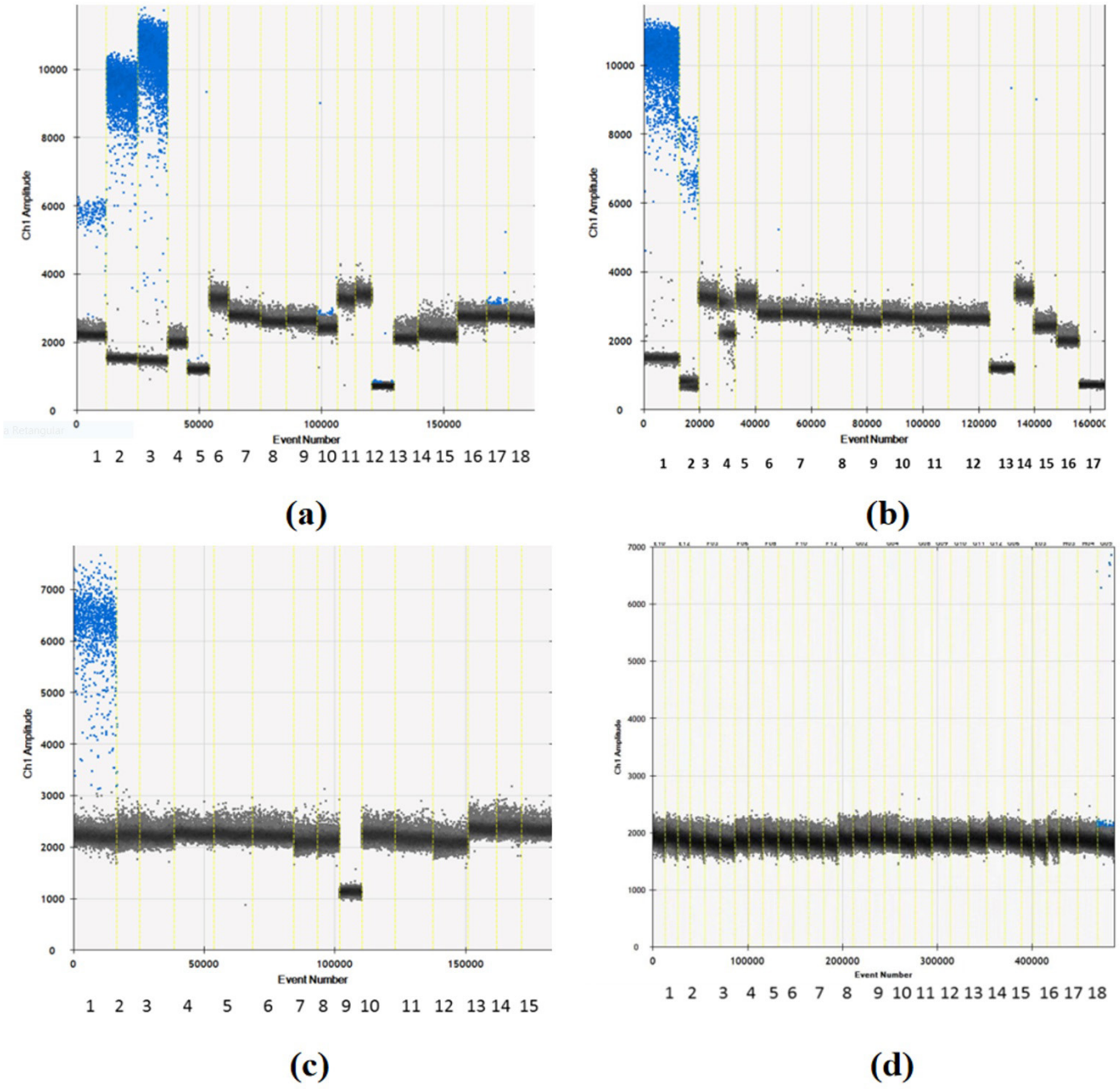

Figure 1. Three kinds of contaminant bacteria determined by ddPCR method and the specific test results. (a) *Glucose bacteria*, (b) *Gluconacetobacter*, (c) *Lactobacillus plantarum*, (d) negative and other nonSalmonella. (a) 1: *Glucose bacteria* (CGMCC1.0637); 2: *Pseudomonas aeruginosa* (CGMCC1.3748); 3: *Glucose bacteria* (CICC10357); 4: *Acetobacter* aceti (CICC21682); 5: *Acetobacter* aceti (CICC22519); 6: *Acetobacter* aceti (CICC21683); 7: *Acetobacter liquefaction* (CGMCC1.24); 8: *Acetobacter* spp. (CGMCC1.811); 9: sucrose Gluconacetobacter (CICC21102); 10: *Salmonella enteritidis* (CICC 21482); 11: *Kentucky brucei* (CICC 21560); 12: *Shigella flexneri* (CICC 21534); 13: *Staphylococcus aureus* (CICC10384); 14: *Bifidobacterium infantis* (CICC6069); 15: *Listeria monocytogenes* (CICC21633); 16: *Escherichia coli* (CICC 10389); 17: *Bacillus cereus* (CICC10041); 18: *Streptococcus thermophilus* (CICC6223). (b) 1: Acetobacter liquefaction (CGMCC1.24); 2: *Acetobacter* spp. (CGMCC1.811); 3: *Glucose bacteria* (CICC10357); 4: *Acetobacter* aceti (CICC21682); 5: *Salmonella enteritidis* (CICC 21482); 6: *Kentucky brucei* (CICC 21560); 7: *Shigella flexneri* (CICC 21534); 8: *Staphylococcus aureus* (CICC10384); 9: *Bifidobacterium infantis* (CICC6069); 10: *Listeria monocytogenes* (CICC21633); 11: *Escherichia coli* (CICC 10389); 12: *Bacillus cereus* (CICC10041); 13: *Streptococcus thermophilus* (CICC6223); 14: *Lactobacillus plantarum* (CICC6076); 15: *Campylobacter jejuni* (CICC22937); 16: *Lactobacillus delbrueckii* (CICC6077); 17: *Lactobacillus helveticus* (CICC6024). (c) 1: *Lactobacillus plantarum* (CICC6076); 2: *Lactobacillus rhamnosus* (CICC6224); 3: *Lactobacillus acidophilus* (CICC6243); 4: *Acetobacter* spp. (CGMCC1.811); 5: *Lactobacillus delbrueckii* (CICC6077); 6: *Streptococcus thermophilus* (CICC6223); 7: *Salmonella enteritidis* (CICC 21482); 8: *Kentucky brucei* (CICC 21560); 9: *Shigella flexneri* (CICC 21534); 10: *Escherichia coli* (CICC 10389); 11: *Bacillus cereus* (CICC10041); 12: *Staphylococcus aureus* (CICC10384); 13: *Yersinia enterocolitica* (CICC 21669); 14: *Bifidobacterium infantis* (CICC 6069); 15: *Acetobacter aceti* (CICC22519).

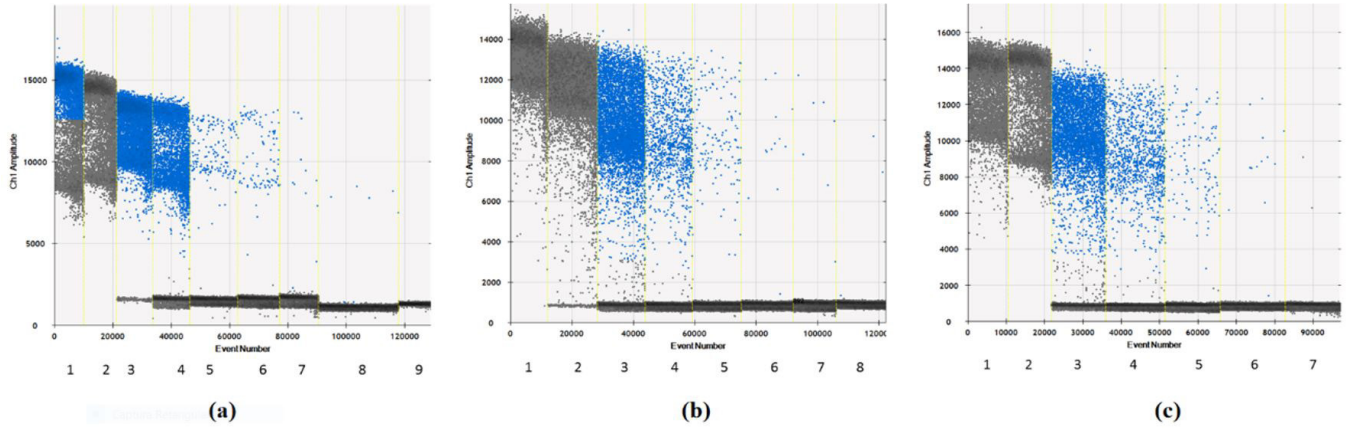

Figure 2. The results of ddPCR method to detect the sensitivity of three contaminants. (a) *Gluconobacter*: 8.8×10^7, 8.8×10^6, 8.8×10^5, 8.8×10^4, 8.8×10^3, 8.8×10^2, 8.8×10^1, 8.8×10^0, 8.8×10^{-1}; (b) *Acetobacter*: 8.9×10^7, 8.9×10^6, 8.9×10^5, 8.9×10^4, 8.9×10^3, 8.9×10^2, 8.9×10^1, 8: 8.9×10^0. (c) *Lactobacillus plantarum*: 9.6×10^7, 9.6×10^6, 9.6×10^5, 9.6×10^4, 9.6×10^3, 9.6×10^2, 7: 9.6×10^1.

Table 3. Relation of copy number and sensitivity.

Name of strain	Theoretical copy number (copies)	Measured copy number (copies/μL)	20 μL copy number (copies)	Measured sensitivity (CFU/mL)
Glucose bacteria	4	0.12	2.4	10.91
Acetobacter spp	1	0.20	4	72.72
Lactobacillus plantarum	2	0.63	12.6	114.53

Table 4. Deviation rate of sensitivity.

Name of strain	Measured sensitivity (CFU/mL)	Count sensitivity (CFU/mL)	Deviation rate (%)
Glucose bacteria	10.91	8.80	23.95
Acetobacter spp	72.72	89.00	−18.29
Lactobacillus plantarum	114.53	96.00	19.31

In this study, ddPCR was detected by different concentrations of Streptomyces griseus, *Acetobacter*, and *L. plantarum*. The linear relationship between the counting results and the ddPCR test results was analyzed. As shown in Figure 3, the standard curve generated by the correspondence between the copy number and the number of colonies of the three strains showed that R^2 was > 0.99, which are 0.9933, 0.9998, 0.9954 respectively, indicating that the linearity of the sensitivity of the ddPCR to the bacteria in yogurt was good. This finding further proved the effectiveness of the method in detecting bacteria.

3.4 Actual sample test

The actual samples were amplified by PCR with amplified primers and probes of *Lactobacillus gluconate* and *Lactobacillus plantarum*, and then detected by drop detector. Figure 4 shows three kinds of fermented milk added with the target strain, all of which show positive results. The remaining samples without target strains showed negative results. The results showed that the food matrix had no significant effect on the detection method of contaminated bacteria established in this study. And the detection method can be expanded to more fermented products for further verification, so as to expand the application scope of the method. Now some companies have begun to look for other non-dairy fermentation alternatives, such as development of vegetal probiotic beverage of passion fruit (Passiflora edulis Sims), yam (Dioscorea cayenensis) and Lacticaseibacillus casei, which can not only promote the survival of probiotics (Guedes et al., 2021), but also reduce the consumption of dairy raw materials. microbial contamination issues.

According to the theoretical copy number of bacteria and the actual detection results of *Lactobacillus acidophilus* and *Lactobacillus plantarum*, the concentration of target bacteria was calculated and measured. When the measured concentration is greater than the added concentration, the deviation rate shows a positive deviation. When the measured concentration is less than the added concentration, the deviation rate is negative. The results (Table 5 & Table 6) show that the deviation of target bacteria concentration is no more than 25% in actual sample detection, which indicates that the method is feasible in practical application. Obviously, it is not just polluting bacteria. In the future, we can choose fermented bacteria that have a positive impact on sensory characteristics of fermented milk, and establish their detection methods, which can more accurately ensure the sensory quality of fermented milk and attract consumers.

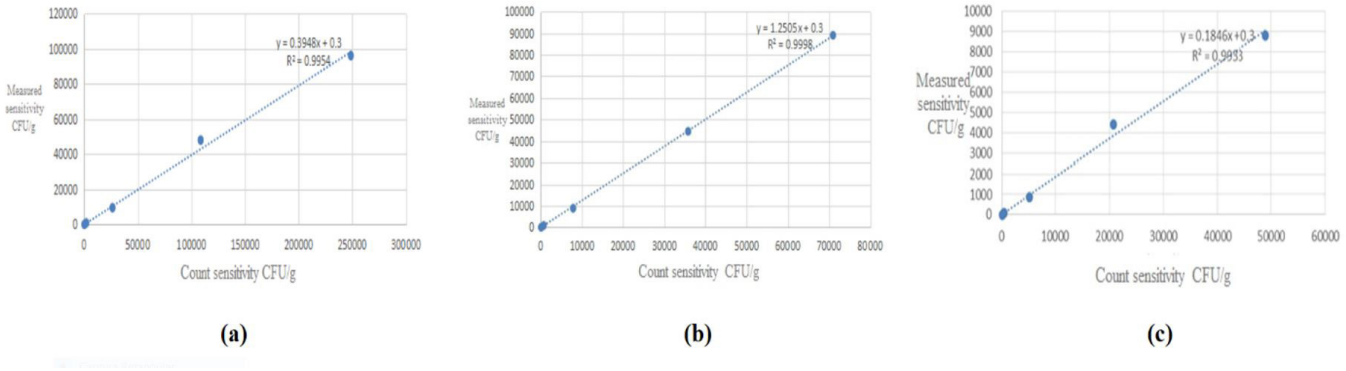

Figure 3. Linear relationship between detection sensitivity of *Glucose* bacteria (a), *Gluconacetobacter* (b) and *Lactobacillus plantarum* (c) by using ddPCR method.

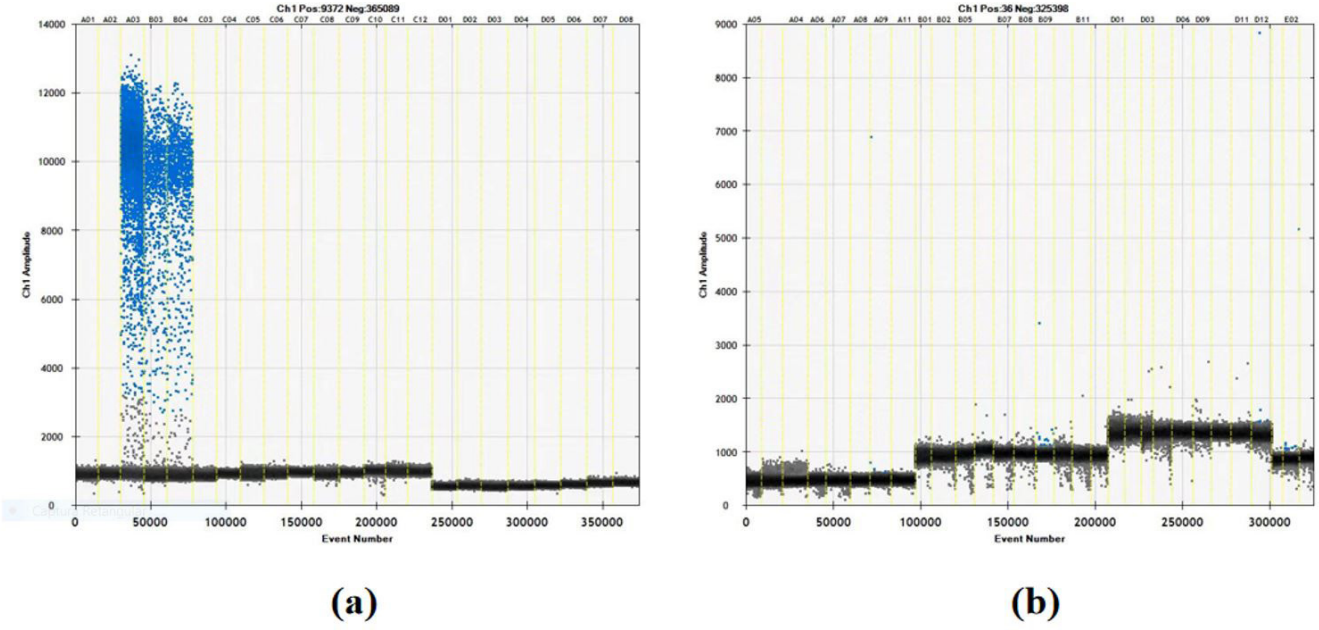

Figure 4. Test results of actual fermented milk with target strain (a) and without target strain (b) by ddPCR method.

Table 5. Relation of copy number and sensitivity of actual samples.

Name of strain	Theoretical copy number (copies)	Measured copy number (copies/μL)	20 μL copy number (copies)	Measured sensitivity (CFU/mL)
Glucose bacteria	4	1.55	31	140.90
Acetobacter spp	1	0.28	5.6	101.81
Lactobacillus plantarum	2	0.80	16	145.44

Table 6. Deviation rate of sensitivity of actual samples.

Name of strain	Measured sensitivity (CFU/mL)	Count sensitivity (CFU/mL)	Deviation rate (%)
Glucose bacteria	140.90	120.00	17.42
Acetobacter spp	101.81	120.00	−15.16
Lactobacillus plantarum	145.44	120.00	21.2

4 Conclusions

Based on the specific primers of *Lactobacillus*, *Gluconobacter*, *Bacteroides*, and *L. plantarum*, primers were used to detect the bacteria and pathogens in fermented milk by the droplet digital polymerase chain reaction, and the genomic deoxyribonucleic acid was extracted by Reagent D. The optimal reaction conditions were optimized, and the droplet digital polymerase chain reaction method for the quantitative detection of three kinds of bacteria and pathogens in fermented milk was established.

The droplet digital polymerase chain reaction method is useful for the detection of bacteria in fermented milk. The detection sensitivity of the method was determined after artificial contamination of the fermented milk. The detection sensitivity of *Bacillus subtilis* was 89 CFU/g, whereas the sensitivity of *L. plantarum* was 96 CFU/g.

The difference between the number of copies and the number of counted colonies of *Lactobacillus*, *Acetobacter*, and *L. plantarum* were within 25%, and 10^4 CFU/g displayed a good linear relationship with the conventional counting results (the standard curve R^2 was greater than 0.99), proving absolute quantitative accuracy of the droplet digital polymerase chain reaction detection in yogurt caused by bacterial contamination and pathogens.

Therefore, this study established an effective and feasible the droplet digital polymerase chain reaction technology system for the quantitative detection of bacteria in fermented milk and provided the theoretical basis and technical guarantee for the wide application of droplet digital polymerase chain reaction in food safety detection.

Conflict of interest

The authors declare no conflict of interest.

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