Development of a novel loop-mediated isothermal amplification assay for the detection of lipolytic *Pseudomonas fluorescens* in raw cow milk from north China

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ABSTRACT

Lipases secreted by psychrotrophic bacteria are known to be heat resistant and can remain active even after the thermal processing of milk products. Such enzymes are able to destabilize the quality of milk products by causing a rancid flavor. Rapid detection of a small amount of heat-resistant lipase-producing psychrotrophic bacteria is crucial for reducing their adverse effects on milk quality. In this study, we established and optimized a novel loop-mediated isothermal amplification (LAMP) assay for the detection of *Pseudomonas fluorescens* in raw cow milk, as the most frequently reported heat-resistant lipase-producing bacterial species. *Pseudomonas fluorescens*-specific DNA primers for LAMP were designed based on the lipase gene sequence. Reaction conditions of the LAMP assay were tested and optimized. The detection limit of the optimized LAMP assay was found to be lower than that of a conventional PCR-based method. In pure culture, the detection limit of the LAMP assay was found to be 4.8×10^1 cfu/reaction of the template DNA, whereas the detection limit of the PCR method was 4.8×10^2 cfu/reaction. Evaluation of the performance of the method in *P. fluorescens*-contaminated pasteurized cow milk revealed a detection limit of 7.4×10^1 cfu/reaction, which was 10^2 lower than that of the PCR-based method. If further developed, the LAMP assay could offer a favorable on-farm alternative to existing technologies for the detection of psychrotrophic bacterial contamination of milk, enabling improved quality control of milk and milk products.

Key words: loop-mediated isothermal amplification, psychrotrophic bacteria, *Pseudomonas fluorescens*, raw cow milk

INTRODUCTION

Cold storage and transportation of raw cow milk creates favorable conditions for the growth of psychrotrophic bacteria (Baur et al., 2015). Psychrotrophic bacteria account for less than 10% of the total microbiota found in fresh raw cow milk, but dominate the microbial community during cold storage (Rasolof et al., 2010; Weber et al., 2014). A large number of psychrotrophic bacteria have been identified as belonging to the genus *Pseudomonas* (Quigley et al., 2013; von Neubeck et al., 2015). These bacteria can grow to high numbers during refrigerated storage, and many can also produce heat-stable extracellular lipases (Dogan and Boor, 2003). Among the genus, *Pseudomonas fluorescens* exhibits the highest metabolic and lipolytic activity in refrigerated milk (Capodifoglio et al., 2016; Stuknytė et al., 2016). The lipolytic enzymes remain active, even after thermal processing steps that can destroy the bacteria themselves (Vithanage et al., 2016). The lipolytic enzymes hydrolyze triglycerides, causing changes in the flavor of the milk, turning it rancid, unclean, soapy, or bitter tasting, and leading to the product being rejected outright (Capodifoglio et al., 2016). The effects are even more of a problem in dairy products with a long shelf life, such as UHT milk (Decimo et al., 2014).

Because it is difficult to inactivate the enzymes secreted by psychrotrophic bacteria in affected milk using existing technologies, it is very important to develop more vigilant test methods for their detection in raw cow milk. The culture-dependent method for the detection of microorganisms in biological samples is both laborious and time consuming, and can thus result in a missed opportunity to control and improve the quality of raw cow milk. Culture-independent methods have been

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shown to be useful for the rapid and specific detection of microorganisms (Cho et al., 2014; Bosward et al., 2016; Cornelissen et al., 2016). As a nucleic acid-based amplification method, loop-mediated isothermal amplification (LAMP) has been developed for the rapid detection of a small amount of microorganisms. Four to 6 specific primers have been designed for use in LAMP, together with a strand-displacing Bst DNA polymerase that can amplify the target DNA by up to 10^9 copies per hour (Notomi et al., 2000). In addition, LAMP proceeds under isothermal conditions and does not require expensive and sophisticated instruments (Cornelissen et al., 2016). Positive LAMP reactions can be judged by the naked eye following DNA amplification with a thermal cycler and gel electrophoresis (Kumar and Mondal, 2015). Additionally, they can be judged by the formation of optic visible magnesium pyrophosphate (turbidity) or by fluorescence using DNA-intercalating dyes, the latter of which enables real-time monitoring of LAMP reactions to be performed by measuring the fluorescence.

In this study, a specific primer set based on the lipase gene sequence of *P. fluorescens* was designed and used to establish a fluorescence-based real-time LAMP assay, with the aim of developing a more specific approach that has a lower detection limit for the detection and quantification of *P. fluorescens* as a potent producer of heat-resistant lipase in raw cow milk.

MATERIALS AND METHODS

Strains and Cultures

Twenty-one different lipolytic *Pseudomonas* isolated strains from 6 different *Pseudomonas* species were identified in raw cow milk originating from north China in our previous study (Xin et al., 2017). These are listed, along with other standard strains that are commonly isolated from raw cow milk, in Table 1. All of these strains were used to evaluate the specificity of the newly developed LAMP assay. *Pseudomonas fluorescens* ATCC 13525 acted as the positive control in this evaluation. *Pseudomonas fluorescens* 38 was used for the optimization of the LAMP assay and to test the detection limit of the assay. The strains were cultured in 10 mL of LB medium for 16 h at 25°C (*Pseudomonas* spp.) or for 24 h at 37°C (non-*Pseudomonas* spp.).

Bacterial DNA Extraction

The DNA content of all strains listed in Table 1, cultured in pure culture, was extracted using the E. Z. N. A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA) for use in LAMP and PCR assays.

Primers for LAMP and PCR

The primer set for use in LAMP was designed based on the conserved gene sequence of the *P. fluorescens* lipase using Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>). The primer set included 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP). The primer sequences are shown in Table 2. The PCR was performed with primers F3 and B3. Primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Optimization of LAMP Reaction Conditions

We initially adopted the conditions reported by Notomi et al. (2000) as the default LAMP reaction conditions. Thereafter, we optimized the reaction conditions by evaluating the effects of adjusting the following reaction variables on the visualized intensity of electrophoretic bands produced by gel electrophoresis of the amplified reaction products: Mg^{2+} concentration (0.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mmol/L), deoxynucleotide triphosphate (dNTP) concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 mmol/L), Bst DNA polymerase (New England Biolab, Ipswich, MA) concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 μ L), inner/outer primer ratio (1:1, 2:1, 4:1, 6:1, 8:1, and 10:1), reaction time (20, 30, 40, 50, 60, and 70 min), and temperature (60, 61, 62, 63, 64, and 65°C). After reaction completion, the reaction mixtures were inactivated at 80°C for 5 min. A negative control, consisting of an identical reaction mixture containing distilled water in place of the DNA template, was included in each reaction run. The LAMP reaction products were analyzed using 2.5% agarose gel electrophoresis at 100 V for 40 min.

A real-time LAMP reaction system was developed by replacing 0.3 μ L of double-distilled H_2O with 0.3 μ L of a 1:200 dilution of 10,000 $\times$ SYBR green I (Sigma-Aldrich Co., St. Louis, MO) in the optimized LAMP reaction system. Real-time LAMP reactions were conducted in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) under the optimized reaction conditions, during which fluorescence of the SYBR Green I was measured every 45 s.

PCR Amplification Conditions

The PCR was carried out in a MyCycler thermal cycler (Bio-Rad, Hercules, CA). Reactions were carried out in a total volume of 25 μ L containing 2 $\times$ PCR Mix, 20 pmol of each primer, 2 μ L of template DNA, and distilled water. Amplification conditions were 94°C for 5 min, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. This sequence was repeated for 35 cycles.

Table 1. Comparative specificity of the real-time loop-mediated isothermal amplification (LAMP) assay, and conventional PCR assay, for the detection of *Pseudomonas fluorescens*

Organism	Source/strain ¹	No. of samples	Specificity (amplification) ²	
			Real-time LAMP	PCR
<i>Pseudomonas fluorescens</i>	Laboratory isolated	5	+	+
<i>Pseudomonas fluorescens</i> 38	Laboratory isolated	1	+	+
<i>Pseudomonas putida</i>	Laboratory isolated	2	–	–
<i>Pseudomonas fragi</i>	Laboratory isolated	10	–	–
<i>Pseudomonas protekii</i>	Laboratory isolated	1	–	–
<i>Pseudomonas psychrophila</i>	Laboratory isolated	2	–	–
<i>Pseudomonas fluorescens</i> (positive control)	ATCC 13525	1	+	+
<i>Pseudomonas fragi</i>	ATCC 4973	1	–	–
<i>Staphylococcus aureus</i>	ATCC 25923	1	–	–
<i>Salmonella typhimurium</i>	CMCC 50018	1	–	–
<i>Escherichia coli</i>	ATCC 700728	1	–	–
<i>Enterobacter sakazakii</i>	ATCC 29544	1	–	–
<i>Listeria monocytogenes</i>	ATCC 19111	1	–	–
<i>Shigella flexneri</i>	ATCC 12022	1	–	–
Double-distilled H ₂ O (negative control)	Laboratory	1	–	–

¹ATCC = American Type Culture Collection, CMCC = China Microbiological Culture Collection, China.

²Where + indicates amplification and – indicates no amplification.

A final extension step was done at 72°C for 10 min. The resulting PCR amplicons were run on a 2.5% agarose gel at 100 V for 40 min. Gels were then stained with ethidium bromide and visualized under a UV trans-illuminator (Gel Doc XR, Bio-Rad).

Detection Limit and Specificity of Real-Time LAMP Assay in Pure Culture

The detection limit of the real-time LAMP reaction system was analyzed and compared with that of the PCR system. The detection limits of the 2 systems in pure culture were determined using 10-fold serial dilutions of cultured *P. fluorescens* 38. Exact cell numbers were determined by standard plate counting. The cultures were 10-fold serially diluted in sterilized normal saline and aliquots (1 mL) of each dilution were used to prepare DNA templates before DNA extraction as described above. Aliquots (2 µL) of each template were tested by both real-time LAMP and PCR assay systems. In the pure culture of *P. fluorescens* 38, the transformation efficiency was calculated using the equation $\text{cfu/mL} \times 3 \times 4 \times 10^{-2}$.

The specificity of the real-time LAMP assay system was examined using the DNA extracted from all the bacteria listed in Table 1 (consisting of 21 different lipolytic strains and other standard strains commonly found in raw cow milk). The concentrations of all bacterial suspensions were adjusted to 10⁶ cfu/mL.

Detection Limits of Real-Time LAMP Assay for Artificial Contamination of Milk with *P. fluorescens*

Pasteurized whole milk obtained from Wondersun Harbin Dairy Co. Ltd. (Harbin, Heilongjiang, China) was artificially inoculated with cultured *P. fluorescens* 38 following the same procedure already described in relation to the analysis of the detection limit of real-time LAMP in pure culture, except that pasteurized milk was used in the dilution. The DNA in the inoculated sample was extracted according to the procedure described by Yang et al. (2014). Noninoculated milk was used as a negative control. The real-time LAMP assay and conventional PCR assay were performed on the extracted DNA samples to estimate and compare their detection limits in pasteurized whole cow milk.

Table 2. Constructed primer sequences for *Pseudomonas fluorescens* lipase gene

Primer ¹	Sequence
F3	5'-CGAAGCGTGCAGAAGGC-3'
B3	5'-GTGCCGCGAAAACCGA-3'
FIP	5'-CTTTTCGCCGAAGAACGTGCCCTTTTCATCAGCGCCAGCACTCTT-3'
BIP	5'-GCTATACCACGGCCAGGTCTTTTGTCCGATTTCCTGCAACTG-3'

¹Two outer primers (F3 and B3) and 2 inner primers (FIP and BIP).

RESULTS

Optimization of LAMP Reaction Conditions

The effects of the reaction variables on the appearance and intensity of electrophoretic bands produced by the reaction products from the LAMP assay system are shown in Figure 1. As shown in Figure 1a, the ladder-like electrophoretic bands appeared when the Mg^{2+} concentration reached 6.0 mmol/L in the reaction system, and were brightest at a concentration of 8.0 mmol/L. Figure 1b illustrates that the bands became apparent at dNTP concentrations of 0.4 to 2.0 mmol/L, and that the brightness of the bands was greatest, and similar, at dNTP concentrations of 1.6 and 2.0 mmol/L. The effects of different levels of Bst DNA polymerase content are shown in Figure 1c. Bands were visible when 0.4 μ L of Bst DNA polymerase was added to the reaction system, and were clear and bright when 0.8 μ L was added. Electrophoretic bands were detectable at a ratio of 4:1 inner:outer primers, and were brightest with an inner:outer primer content ratio of 8:1 (Figure 1d). For reaction temperature, the brightest amplification bands were observed at 64°C (Figure 1e). The brightest electrophoretic bands were produced at a reaction time of 50 min (Figure 1f). According to these observations, we determined the optimal LAMP reaction conditions as being Mg^{2+} concentration, 8.0 mmol/L; Bst DNA polymerase content, 0.8 μ L; dNTP concentration, 1.6 mmol/L; inner:outer primer ratio, 8:1; temperature, 64°C; and reaction time, 50 min.

Detection Limit of Real-Time LAMP Method

The detection limit of the real-time LAMP assay was evaluated by genomic DNA extracted from serial dilution *P. fluorescens* 38, and compared with the detection limit of the PCR method. The results are shown in Figure 2. Concentrations of 4.8×10^5 to 4.8×10^1 cfu per reaction of the template DNA could be amplified by the real-time LAMP assay system, whereas concentrations below this range were not detectable (no amplification curves were observed; Figure 2a). In contrast, the PCR system required at least 4.8×10^2 cfu of bacteria per reaction to generate a visible band on the agarose gel (Figure 2b).

Specificity of Real-Time LAMP Assay

The results of the evaluation of the specificity of the real-time LAMP assay for the detection of *P. fluorescens* as distinct from other related bacterial species, in pure culture, are presented in Table 1. *Pseudomo-*

nas fluorescens ATCC 13525 was used as the positive control. Sterile double-distilled H_2O was used as the negative control. The assay system detected all 6 *P. fluorescens* bacterial strains as well as standard strain *P. fluorescens* ATCC 13525 as positive, including *P. fluorescens* 38. All other strains were correctly detected as negative.

Ability of Real-Time LAMP Assay to Detect *P. fluorescens* Contamination in Milk

To determine the comparative detection limits of the developed real-time LAMP assay and PCR assay for the detection of *P. fluorescens* in milk, pasteurized whole milk was artificially contaminated with different cell densities of *P. fluorescens* 38. The PCR assay positively detected as few as 7.4×10^3 cfu/reaction of the template DNA. In contrast to this, the developed LAMP assay had a lower detection limit of 7.4×10^1 cfu/reaction of template DNA (Figure 3).

DISCUSSION

There is a current need for the development of simple, rapid methods for the detection of psychrotrophic bacteria producing heat-resistant lipase in raw cow milk. The availability of such methods would enable better control of such bacteria in milk, improving the quality of dairy products and decreasing the economic costs associated with rejection of spoiled milk. Bacteriological methods, immunological methods, and molecular methods, such as multiplex PCR and real-time PCR, have all been applied to the detection of microorganisms in food (Alhogail et al., 2016; Nagaraj et al., 2016; Xu et al., 2017). However, these methods are not always feasible for use on farm or in modest-sized microorganism testing laboratories because they are often expensive and require the use of bulky apparatus. Moreover, such methods typically take in the region of 2 h to produce results. Recently, LAMP has been developed as a nucleic acid amplification technique and is now widely used for the detection of foodborne bacteria, viruses, and parasites (Abirami et al., 2016; Tian et al., 2016; Yang et al., 2016). This method possesses many advantages over the aforementioned conventional methods, such as a requirement for a simple heating block rather than an expensive thermal cycler. In addition, the LAMP method is typically has a detection limit that is 1 to 2 orders of magnitude lower than the PCR method. It also has a shorter turnaround time (30–60 min) and supports a variety of methods for the visualization and interpretation of results (Fischbach et al., 2015).

The aim of the present study was to explore the possibilities of LAMP as a method for the detection of psychrotrophic bacteria that produced heat-resistant lipase in raw cow milk. *Pseudomonas fluorescens* is one of the major psychrotrophic bacteria producing heat-resistant lipase in raw cow milk. *Pseudomonas fluorescens* 38 has been isolated from raw cow milk obtained from north China and has a high heat-resistant lipase-producing

ability (Xin et al., 2017). On this basis, *P. fluorescens* 38 was selected and used to optimize the LAMP assay and to determine its limit of detection. We designed a real-time LAMP assay based on the lipase gene of *P. fluorescens* 38.

To develop a rapid method that has a lower detection limit for the detection of *P. fluorescens* that produce heat-resistant lipase, we optimized the LAMP reaction

Figure 1. Effects of Mg^{2+} concentration, deoxynucleotide triphosphate (dNTP) concentration, Bst DNA polymerase content, inner/outer primer ratio, temperature, and reaction time on electrophoretic visualization of bands produced from loop-mediated isothermal amplification (LAMP) reaction products. (a) Effects of Mg^{2+} concentration. 1: negative control; 2: 0.0 mmol/L; 3: 4.0 mmol/L; 4: 6.0 mmol/L; 5: 8.0 mmol/L; 6: 10.0 mmol/L; and 7: 12.0 mmol/L. (b) Effects of dNTP concentration. 1: negative control; 2: 0.0 mmol/L; 3: 0.4 mmol/L; 4: 0.8 mmol/L; 5: 1.2 mmol/L; 6: 1.6 mmol/L; and 7: 2.0 mmol/L. (c) Effect of Bst DNA polymerase content. 1: negative control; 2: 0.0 μ L; 3: 0.4 μ L; 4: 0.8 μ L; 5: 1.2 μ L; 6: 1.6 μ L; and 7: 2.0 μ L. (d) Effects of inner/outer primer ratio. 1: negative control; 2: 1:1; 3: 2:1; 4: 4:1; 5: 6:1; 6: 8:1; and 7: 10:1. (e) Effects of temperature. 1: negative control, 2: 60°C, 3: 61°C, 4: 62°C, 5: 63°C, 6: 64°C, and 7: 65°C. (f) Effects of reaction time. 1: negative control; 2: 20 min; 3: 30 min; 4: 40 min; 5: 50 min; 6: 60 min, and 7: 70 min. In all panels, M represents the molecular weight marker.

Figure 2. Detection limit of the real-time loop-mediated isothermal amplification (LAMP) assay (a) in comparison with the PCR assay and (b) for the detection of *Pseudomonas fluorescens* 38 amplified DNA. (a) Detection limit of the real-time LAMP assay at different colony-forming units/reaction concentrations. Real-time LAMP assays were performed using 4.8×10^5 to 4.8×10^{-1} cfu/reaction of the template DNA. Sterile double-distilled H_2O in place of the template DNA served as a negative control. (b) Detection limit of the PCR assay at different colony-forming units/reaction concentrations. Lane M: molecular weight marker; lanes 1, 2, 3, 4, 5, 6, and 7 represent 4.8×10^5 , 4.8×10^4 , 4.8×10^3 , 4.8×10^2 , 4.8×10^1 , 4.8×10^0 , and 4.8×10^{-1} cfu/reaction, respectively.

conditions via a series of single-factor experiments. In our future planned research, the influence of interactions among the variables on the reaction products will be considered. In the present study, the real-time LAMP assay was established in combination with fluorescence quantification using a real-time PCR instrument. Under this system, the fluorescence signals could be continuously recorded and analyzed, which enables a significant time saving to be made (compared with conventional methods) in the confirmation of results

(40 min saved). Portable fluorescence detectors have already been applied to on-site detection of microorganisms and have been shown to deliver the same results as fluorescence quantification PCR instruments (Fu et al., 2016). Moreover, a battery-operated portable fluorescence detector can be used in conjunction with the LAMP assay system (Ye et al., 2015; Lee et al., 2016). Furthermore, because the LAMP reaction tubes are not opened until the reaction has completed, the potential risks of cross-contamination and false-

Figure 3. Comparative detection limit of the real-time loop-mediated isothermal amplification (LAMP) assay (a) and the PCR assay system (b), for the detection of *Pseudomonas fluorescens* contamination in milk. (a) Detection limit of the real-time LAMP assay at different colony-forming units/reaction of the template DNA. The LAMP assays were performed using 7.4×10^5 to 7.4×10^{-1} cfu/reaction of the template DNA. Sterile double-distilled H₂O in place of the template DNA served as a negative control. (b) Detection limit of the PCR assay at different colony-forming units/reaction of the template DNA. Lane M: molecular weight marker; lanes 1, 2, 3, 4, 5, 6, and 7: 7.4×10^5 , 7.4×10^4 , 7.4×10^3 , 7.4×10^2 , 7.4×10^1 , 7.4×10^0 , and 7.4×10^{-1} cfu/reaction, respectively.

positives are significantly reduced compared with existing methods. A further benefit of the real-time assay system is that it has the ability to be scaled up for high-throughput detection and to be used for accurate target quantification (Tang et al., 2016). The detection limit of the developed LAMP assay was compared with a conventional PCR assay system in the present study. Our results revealed that the detection limit of the

LAMP assay was 10 times lower than that of the PCR assay in pure culture (detection limit of 4.8×10^1 cfu/reaction of template DNA vs. 4.8×10^2 cfu/reaction, respectively). Although our preliminary findings have shown that the LAMP primers we used (when applied under the specified reaction conditions) are specific for *P. fluorescens*, we plan to conduct further studies with a greater number of species and strains of *P. fluorescens*

that produce heat-resistant lipase and other bacteria to further evaluate the detection limit and specificity of the method.

An important aspect of our study was to test the developed LAMP assay protocol for the detection of lipolytic *P. fluorescens* in pasteurized whole cow milk, where its application could be used to control the quality of milk in practice. The method of genomic DNA extraction is key to the successful application of the LAMP method in this context, and must be simple, efficient, and low cost. Milk consists of several macromolecules, such as proteins and fats, which could potentially interfere with the DNA amplification efficiency. Thus, removal of these macromolecules is required. Around 80% of total milk proteins are caseins, which are incorporated into the micelle structures together with a high proportion of the available calcium and inorganic phosphate. The use of EDTA can chelate the calcium ions and eventually disrupt the micelles, resulting in the casein and whey moving into the supernatant. Triton X-100 has also been used successfully to remove residual lipids (Kumar and Mondal, 2015). Centrifugation can also remove a proportion of the protein and fat present in milk. Previous studies comparable to ours have combined centrifugation with the DNA extraction kit to extract the genomic DNA in milk (Yang et al., 2011; Song et al., 2012). Although these modified extraction kits result in a satisfactory detection limit, the detection cost is elevated by the addition of the extraction reagents and the detection time is longer. A LAMP-compatible rapid and simple method to increase the DNA yield is therefore desirable. The LAMP assay was revealed to have a similar limit of detection in pasteurized cow milk and pure culture. Comparing this to the existing literature, 2 studies have previously reported that the results produced from the LAMP assay in detecting *P. fluorescens* in pure culture and in artificially contaminated milk samples were of a similar order of magnitude (Cho et al., 2014; Ye et al., 2015). Three out of 5 published studies have suggested that the detection limit of the method is lower in pure culture than in milk (Yang et al., 2011; Wang et al., 2015; Cornelissen et al., 2016). The DNA extraction method used is an important factor that may affect the results obtained (Sowmya et al., 2012). In the present study, when compared with the conventional PCR method, the detection limit of LAMP assay was found to be 10^2 times lower than the PCR method in milk. This difference between the 2 methods was not as evident when they were applied to analysis of *P. fluorescens* in pure cultures, suggesting that the PCR method may have been inhibited by certain compounds in the milk. This is consistent with a previous report that

LAMP is less affected by inhibitory substances present in biological samples (such as milk) than conventional PCR (Sowmya et al., 2012; Cho et al., 2014; Bosward et al., 2016). According to Machado et al. (2013), fat is a PCR inhibitor. The average reaction time values of real-time LAMP were, respectively, 10 to 28 cycles and 17 to 32 cycles when the DNA template concentrations in the pure bacterial cultures and artificially contaminated milk samples ranged from 4.8×10^5 to 4.8×10^1 cfu/reaction and 7.4×10^5 to 7.4×10^1 cfu/reaction of the template DNA, respectively. This suggests that the real-time LAMP assay was somewhat influenced by the presence of compounds in the milk.

As a DNA-based detection method, LAMP cannot distinguish between DNA arising from viable cells and that arising from dead cells (Chen et al., 2011; Wan et al., 2012). In the present research, this is an advantage in terms of being able to accurately assess the potential contamination of heat-resistant lipase in milk because heat-resistant lipase are still active in contaminated dairy products even if the parent bacteria (*P. fluorescens*) has been destroyed. In this sense, a LAMP detection method is more accurate than a culture-dependent method.

In the present study, lipolytic *P. fluorescens* were successfully detected in pasteurized whole milk. It has previously been shown that extracellular enzymes secreted by psychrotrophic bacteria, mostly *Pseudomonas* species, are well-known causes of spoilage in other matrices including dairy products and meat (Rajmohan et al., 2002; Caldera et al., 2016). On this basis, we plan to further evaluate the LAMP method for the detection of psychrotrophic bacteria and their heat-resistant enzymes on additional food matrices.

CONCLUSIONS

This study has developed and optimized a LAMP assay for the detection of lipolytic *P. fluorescens* in raw cow milk that compares favorably with a conventional PCR-based method. The procedure offers a simple, rapid, species-specific method that has a lower detection limit compared with existing methodologies and has the potential to improve the control of heat-resistant lipase in commercially produced raw cow milk. The challenges that remain before the LAMP assay is suitable for use in the field include the availability of a compatible rapid isolation procedure for extraction of DNA from bacteria in raw cow milk. Nevertheless, once fully developed, the LAMP assay method has the potential to make the detection of heat-resistant lipase-producing *P. fluorescens* both easier and faster and, furthermore, could be applied across the whole

of China, thereby delivering significant economic and societal benefits.

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