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Citation	Food Research International, 42(8), 893-898 https://doi.org/10.1016/j.foodres.2009.04.010
Issue Date	2009-10
Doc URL	https://hdl.handle.net/2115/39281
Type	journal article
File Information	FRI42-8_p893-898.pdf



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Title

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Running Title

Real-time PCR detection of *S. thermophilus*

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Abstract

A real-time PCR method targeting a gene sequence encoding 16S rRNA processing protein, *rimM*, for specific detection of *Streptococcus thermophilus* was developed. The designed real-time PCR primers and probe were specific for *S. thermophilus* JCM20026, LMG6896, LMG18311, OJT101, OJT102 but not *Enterococcus* spp., *Lactococcus lactis* subsp. *lactis*, and *Streptococcus salivarius* which are phylogenetically closely related to *S. thermophilus* and are difficult to identify using culture-based methods. The linear range of the developed real-time PCR method was from 2.7 to 8.6 log CFU ml⁻¹ with an amplification efficiency of 96%. Minor differences (about 0.4 log CFU ml⁻¹) were observed between counts of *S. thermophilus* obtained by culture and real-time PCR method in plain yoghurt and yoghurt containing fruits. Therefore, the developed real-time PCR method could be of potential application in specific detection and accurate enumeration of *S. thermophilus* in a wide range of dairy products.

Keywords: Dairy products, Real-time PCR, *rimM* gene, *Streptococcus thermophilus*

1. Introduction

S. thermophilus is a thermophilic lactic acid bacterium (LAB) widely used as a starter culture in the manufacture of dairy products and regarded as the second most important industrial dairy starter after *Lactococcus lactis* (Hols et al., 2005). The role of *S. thermophilus* in milk fermentation is due to its rapid conversion of lactose into lactic acid, causing a rapid decrease in pH and the production of important metabolites such as exopolysaccharides (EPS) that contribute to the desirable viscous texture and rheological properties of fermented milk products (Delorme, 2008). In addition, consumption of dairy products containing *S. thermophilus* has been reported to be beneficial for human health (Canani, et al., 2007; Trois, et al., 2008). It is essential that products sold with any health claims meet the criterion of a minimum of 10^6 CFU ml⁻¹ probiotic bacteria; because the minimum therapeutic dose per day is suggested to be 10^8 - 10^9 cells (Kurman & Rasic, 1991). Besides, most dairy fermentations involving *S. thermophilus* consist of multiple strains of LAB either as part of the starter culture or as added probiotics. The enumeration of LAB species is important to study their role and their dynamics in various niches (Furet, Qu  n  e, & Tailliez, 2004). Therefore it is essential to accurately monitor the counts of *S. thermophilus* from the fermentation stage up to the point of consumption. However, it is extremely difficult to accurately detect and enumerate *S. thermophilus* particularly in food samples that may contain phylogenetically closely related bacterial species with similar physiological and biochemical properties.

The traditional culture-based methods often used for enumeration of *S. thermophilus* have disadvantages such as, long incubation time, difficulty in differentiation of *S. thermophilus* from *S. salivarius* and *Enterococcus* spp., difficulty in choosing enumeration conditions and medium (Delorme, 2008). For instance, to improve the discrimination of *S. thermophilus* from other streptococci or enterococci and to prevent overgrowth of Gram-negative rods in analysis of fecal samples, *S. thermophilus* (ST) medium (Dave & Shah, 1996) has been modified by addition of bromocresole purple, bromocresole green and nalidixic acid (Brigidi, Swennen, Vitali, Rossi, & Metteuzzi, 2003). The colonies presumed to be *S. thermophilus* were then confirmed by PCR-based techniques.

Development of a molecular culture-independent method such as quantitative real-time PCR offers advantages such as sensitivity, accuracy, simplicity, and the possibility of robotic automation (Powell, Ferguson, Bowman, & Snape, 2006). Despite the importance of *S. thermophilus* in dairy fermentation, only one real-time PCR method that relies on primers derived from the 16S rDNA gene sequence of *S. thermophilus* has so far been developed (Furet, Quénée, & Tailliez, 2004). The major drawback is that about 80% of the genes in *S. thermophilus* are similar to other streptococcal genes highlighting its relatedness to pathogenic species which makes design of species-specific real-time PCR primers difficult (Bolotin et al., 2004). The 16S rDNA sequence of *S. thermophilus* is closely related to those of *S. salivarius*, *S. vestibularis* and *Enterococcus faecium*. Previously primers ThI

and ThII designed on the basis of 16S-23S rDNA internal transcribed spacer (ITS) region were reported to distinguish between *S. thermophilus* and *S. salivarius* (Tilsala-Timisjarvi & Alatossava, 1997) but later on it was found out that these primers could not discriminate between the two species (Elli et al., 2006). Furthermore 16S-23S rDNA ITS region of *S. thermophilus* and *S. salivarius* share 78.5%, 62.2%, and 61.8% of identity with the corresponding spacer regions of *S. pneumoniae*, *L. lactis* and *E. hirae* respectively (Nour, Niami, Beck, & Branlant, 1995).

Due to the low discriminatory power of primers designed based on *S. thermophilus* 16S rDNA and 16S-23S rDNA ITS region, there is a need to identify other gene sequences with higher discriminatory power for designing *S. thermophilus* real-time PCR primers and probes. Therefore the objective of this study was to develop accurate, sensitive and specific real-time PCR method that can be used for detection and enumeration *S. thermophilus* in dairy products.

2. Materials and Methods

2.1. Bacteria and growth conditions

The bacterial species that were used for testing the specificity of the real-time PCR method were chosen on the basis of both their close phylogenetic relatedness to *S. thermophilus* and the difficulty to distinguish from *S. thermophilus* using culture-based techniques (Table 1). Enterococci, lactococci and streptococci were cultured in M17 broth (Becton, Dickinson

Co., Sparks, Md., USA) whereas lactobacilli were cultured in MRS broth (Becton, Dickinson Co., Sparks, Md., USA). *Bifidobacterium breve* and *R. productus* were cultured in Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical, Tokyo, Japan). Except *S. equinus*, *L. lactis* subsp. *lactis*/*L. raffinolactis*, which were cultured at 28 and 30 ° C respectively, all other strains, were incubated at 37°C. Incubation was carried out under anaerobic conditions consisting of N₂, H₂ and CO₂ in the ratio of 8:1:1 respectively.

2.2. Design of real-time PCR primers and probe

A gene sequence (Gene ID: 3167473) of *S. thermophilus* CNRZ1066 encoding 16S rRNA processing protein (*rimM*) was retrieved from the National Center for Biotechnology Information (NCBI) Microbial Genome database (<http://www.ncbi.nlm.gov>) and used for designing primers and probe. The *rimM* protein is essential for efficient assembly of the 30S subunit prior to maturation of 16S rRNA (Bylund, Wipemo, Lundberg, & Wikström, 1998). A search for gene sequences similar to *rimM* of *S. thermophilus* using basic local alignment search tool (BLAST; <http://blast.ncbi.nih.gov/blast.cgi>) of the GenBank database showed that the *rimM* sequence significantly differs from that other bacterial species (the Expectation (E) value of the most similar bacterial DNA sequence was 2.0×10^{-16}). The real-time PCR primers and probes were designed using Primer Express v2.0 software (Applied Biosystems, Foster City, CA) and synthesized by Invitrogen, Japan. The specificity of designed primers and probe were checked against all the

available data in the GenBank database. The probe was labelled with the florescent 6-carboxyfluorescein (FAM) at the 5' and with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. The sequences were 5'-CGCTAATCGGTCAGGTAAAGAG-3', 5'-CCCTTTACGTTTCACTACCCAAA-3', 5'-TCTCCAACCGGGTGCTAATGA-3' for forward primer 341F, reverse primer 411R and the TaqMan probe 365T, respectively.

2.3. Extraction of DNA from bacterial cells

DNA was extracted from bacterial cultures, milk and yoghurt samples using UltraClean™ Microbial DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA) according to the manufacturer's instructions with minor modifications. Briefly, 180 µl of sample was transferred to a tube containing beads. About 120 µl of bead solution and an enzyme cocktail containing 5 mg ml⁻¹ of lysozyme (Wako Pure Chemical industries, Osaka Japan), 2.5 mg ml⁻¹ of labiase (Seikagaku, Tokyo, Japan), and 30 µg ml⁻¹ of *N*-acetylmuramidase (Seikagaku, Tokyo, Japan) was added to the bead tube, and incubated at 37°C for 2 h. The tubes were then spun for 20 s at 4 m s⁻¹ on Fastprep™ FP120 (Qbiogene, Carlsbad, CA, USA). The concentration of isolated DNA was measured by absorbance at 260 nm with a Beckman DU 640 spectrophotometer (Beckman Coulter Inc, Fullerton, CA).

2.4 Real-time PCR conditions

The amplification reactions were carried out in a total volume of 50 μ l. The real-time PCR mixture consisted of 1X TaqMan universal PCR mixture (Applied Biosystems, Foster City, CA, USA) containing 900 nM of each primer, probe (250 nM) and genomic DNA. TaqMan[®] exogenous internal positive control reagents (Applied Biosystems, Foster City, CA, USA) were spiked into the reactions mixtures to distinguish true negatives from PCR inhibition according to the manufacturer's instructions. The real-time PCR amplifications were performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The reactions were carried out by incubation for 2 min at 50°C (for activation of uracil N-glycosylase) and for 10 min at 95°C (for activation of AmpliTaq Gold DNA polymerase), followed by 15 s at 95°C (for denaturation) and 1 min at 60°C (for annealing and extension) for 40 cycles. Each sample was analyzed in triplicate. Data analysis was carried out using the ABI PRISM 7000 sequence detection software v1.0 (Applied Biosystems, Foster City, CA, USA).

2.5 Specificity of the PCR assay

DNAs extracted from pure culture of bacterial strains (Table 1) were used to test the specificity of the designed real-time PCR assay. Non-*S. thermophilus* strains were discriminated from *S. thermophilus* by evaluation of the threshold cycle (C_t) value. The C_t value was defined as the cycle at which the fluorescence was significantly different from the background.

Samples of purified genomic DNA (20 ng) were used as templates for real-time PCR assay. Sterilized milliQ grade water was used as none template control.

2.6. Primer specificity test using end-point PCR

The specificity of primers designed was further tested under end-point PCR conditions. The PCR reaction for primer specificity test was performed in 50 µl reaction mixture containing 1 µl of template DNA, 50 pmol of each primer, 5 µl of dNTP mixture (2.0 mM each), 1X PCR buffer, 3.5 µl of MgCl₂ solution (25 mM), 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). The amplification program was 95°C for 2.3 min, 30 cycles of 95°C for 0.3 min, 60°C for 1 min and a final extension of 72°C for 6 min. The presence of amplified DNA products were detected on 3% agarose gel (NuSieve® 3:1 agarose, Cambrex Bio Science Rockland, Inc. Rockland USA) after staining for 15 min with SYBR Green 1 in a UV illuminator (Atto, Tokyo, Japan).

2.7 Construction of standard curve

Serial dilutions of DNA extracted from pure cultures of *S. thermophilus* AHU1838 was used for real-time PCR assay. The standard curve was derived by plotting the C_t values against corresponding cell counts at each dilution.

Accuracy and sensitivity of the developed real-time PCR assay

Skim milk powder was reconstituted (12.6% w/v) in distilled and sterilized water. The reconstituted skim milk was sterilized at 90°C for 10 min. Cells of *S. thermophilus* AHU1838 was serially diluted 8 fold in M17 broth and sterilized milk. DNA was extracted from each of the serially diluted samples as described above and subjected to real-time PCR amplification. The number of *S. thermophilus* cells in each dilution was plotted against the corresponding C_t values.

2.9 Yoghurt samples

Yoghurt products either purchased from a store or supplied by Ohayo Milk Company (Okayama, Japan) were transported to the laboratory at 4°C and analysed within 24 hours so as to obtain a high degree of correlation between viable counts and real-time PCR results. The kinds of yoghurt used in this study were; plain yoghurt, and yoghurts containing strawberry, blueberry, and mango as ingredients. Samples of yoghurt were opened under aseptic conditions and divided into two portions. One portion was used for enumeration of *S. thermophilus* in yoghurt by culture-based method whereas the other portion was used for enumeration of *S. thermophilus* using the developed real-time PCR method.

2.10. Enumeration of *S. thermophilus* using plate count method

Samples were homogenised, serially diluted in sterile saline (0.85% NaCl solution) and spread plated on M17 agar plates (Becton, Dickinson Co., Sparks, Md., USA). Inoculated M17 agar plates were incubated anaerobically at 37°C for 48 h in an air tight jar (AnaeroPack Rectangular Jar™) containing O₂ absorbing and CO₂ releasing pack (AnaeroPack™, Mitsubishi Gas Chemical Co., Tokyo, Japan).

3. Results

3.1 Specificity of primers and probe

The specificity of primers used in this study was tested using genomic DNA obtained from 47 strains of bacteria. The primers amplified DNA obtained from *S. thermophilus* AHU1838, *S. thermophilus* JCM20026, *S. thermophilus* LMG6896, *S. thermophilus* LMG18311, *S. thermophilus* OJT101 and *S. thermophilus* OJT102 (Table 1). However, the primers did not amplify DNA obtained from *S. thermophilus* NBRC13957, *S. thermophilus* 21072. In addition, no amplification signal was detected in DNA obtained from *E. faecium*, *E. faecalis*, *L. lactis* subsp. *lactis* and *S. salivarius* which have been reported to be phylogenetically closely related to *S. thermophilus*. The designed real-time PCR primers did not amplify DNA obtained from strains of LAB commonly used in production of fermented dairy products indicating that the developed real-time PCR method can be used for specific detection of *S. thermophilus* in fermented products containing multiple

strains of LAB. Electrophoresis of PCR products obtained using the designed primers was done and bands were observed only in lanes corresponding to *S. thermophilus* JCM20026, *S. thermophilus* AHU1838, *S. thermophilus* LMG18311, *S. thermophilus* LMG6896, *S. thermophilus* OJT101 and *S. thermophilus* OJT102. These results indicate that the real-time PCR primers and probe designed was specific for *S. thermophilus*.

3.2 Construction of standard Curve

In order to construct a calibration curve, serial dilutions of DNA prepared from pure culture of *S. thermophilus* AHU1838 were used for the PCR assay. The efficiency of the curve was excellent as the correlation coefficient was 0.998 (Fig 1). The linear range of standard curve was from 2.7 to 8.6 log CFU ml⁻¹. The amplification efficiency of DNA used for developing the standard curve was 96% indicating that the developed PCR method has the capacity to duplicate amplicons in each PCR cycle.

3.3 Accuracy and sensitivity of the developed real-time PCR assay

To determine the accuracy and sensitivity of the real-time PCR, *S. thermophilus* cells were serially diluted 8 fold in M17 broth and sterilized skim milk. DNA was extracted from the serially diluted samples. Sensitivity was defined as highest dilution able to give a positive amplification. This was determined to be 2.7log CFU ml⁻¹ both in pure culture and in milk. The correlation coefficient and slope of curves derived from diluted M17 broth

and milk were very similar (Fig. 2). Compared with the standard curve there was no marked difference in steepness of the slope. This indicates that the developed real-time PCR assay can accurately enumerate *S. thermophilus* cells in milk and fermented milk products. In a preliminary trial, the effectiveness of three types of kits (UltraClean™ Microbial DNA isolation kit, UltraClean™ Soil DNA isolation kit and UltraClean™ Fecal DNA Isolation Kit) to extract DNA from pure culture, plain yoghurt, strawberry yoghurt and blueberry yoghurt was tested. A higher yield of DNA was obtained from yoghurt samples extracted using UltraClean Microbial DNA kit (data not shown). Further more there were no significant differences between the C_t values of pure culture, plain yoghurt and yoghurt containing fruits having similar counts of *S. thermophilus* (data not shown). These results indicated that UltraClean™ Microbial DNA isolation kit has a higher DNA extraction efficiency compared to UltraClean™ Soil DNA isolation kit and UltraClean™ Fecal DNA isolation kit. In addition, pre-incubation of *S. thermophilus* cells with bacterial cell wall degrading enzymes resulted into a higher DNA yield and improved real-time PCR amplification efficiency.

3.4 Enumeration of S. thermophilus in yoghurt samples by culture and real-time PCR

The applicability of the developed real-time PCR method to enumerate *S. thermophilus* cells in yoghurt samples was tested. The results obtained by real-time PCR and culture methods were very similar to plate count results

(Table 2). The counts of *S. thermophilus* cells obtained by culture method were on average about 0.4log CFU ml⁻¹ higher than those obtained by real-time PCR. In addition not much difference was observed in *S. thermophilus* counts obtained between plain yoghurt and yoghurt containing fruits using the developed real-time PCR method. Therefore the differences in composition of the yoghurts did not affect the reliability of the real-time PCR results.

4.0 Discussion

In this study a real-time PCR method for detection and enumeration of *S. thermophilus* in milk and fermented dairy products was developed. The designed primer set was found to clearly distinguish between *S. thermophilus* and other phylogenetically closely related bacterial species such as *Enterococcus* spp., *L. lactis* and *S. salivarius*. Initially, we attempted to develop real-time PCR primers based on the 16S rDNA sequence of *S. thermophilus* CNRZ1066. However, *S. thermophilus* has 6 copies of 16S rDNA gene which can affect the sensitivity and accuracy of the real-time PCR. Furthermore, a search of the NCBI Microbial Genome database using BLAST revealed that primers and probes designed on the basis of 16S rDNA gene of *S. thermophilus* had similar matching sequences to 16S rDNA gene sequence of *S. pyogenes*, *S. salivarius*, and uncultured *Streptococcus* spp. Previously, Furet, et al., 2004, designed real-time PCR primers St1 and St2 based on 16S rDNA sequence of *S. thermophilus*. The sequence of St1 primer

displayed significant homology with the corresponding target region of *S. agalactiae*, *S. didelphis*, *S. pyogenes* and *S. suis* (Furet et al., 2004). Bentley, Leigh, & Collins, 1991, also reported that *S. salivarius* and *S. vestibularis* exhibited very high levels of 16S rDNA homology with each other and the type strain of *S. thermophilus*. These findings highlight the difficulty in designing real-time PCR primers for specific detection and enumeration of *S. thermophilus* on the basis of 16S rDNA sequence. The *rimM* gene exists as a single copy enabling design of real-time PCR primers and probes that are much more specific and sensitive for *S. thermophilus*. Among lactic acid bacteria, the *rimM* sequence of *L. lactis* subsp. *lactis*, *L. johnsonii*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. salivarius* subsp. *salivarius*, *S. mutans*, *L. reuteri*, *L. sake*, *L. gasseri*, *L. plantarum*, *B. adolescentis*, and *L. casei* could be retrieved from the NCBI Microbial Genome database. Other bacterial strains in which the *rimM* sequence has been reported are *E. coli*, *S. pneumoniae*, *S. pyogenes*, and *C. perfringens*. Thus the *rimM* sequence may serve as an alternative target for designing primers and probes for bacterial species that have closely related 16S rDNA sequences in addition to the 16S-23S intergenic spacer region.

The primers and probes designed based on the *rimM* sequence of *S. thermophilus* CNRZ1066 could detect *S. thermophilus* AHU1838, *S. thermophilus* JCM20026, *S. thermophilus* LMG18311, *S. thermophilus* LMG6896, *S. thermophilus* OJT101 and *S. thermophilus* OJT102 but not *S. thermophilus* 21072 and *S. thermophilus* NBRC13957. This could be

attributed to differences between *rimM* sequence of *S. thermophilus* strains detected and strains not detected using the developed real-time PCR method. Likewise, the *SodA* sequence (Poyart, Quesne, Coulon, Berche, & Trieu-Cout, 1998) which can be used for specific detection of *S. thermophilus* has closely matching (99% homology) DNA sequences with *S. thermophilus* LMG18311, *S. thermophilus* LMG6896 and *S. thermophilus* CNRZ1066 amongst others, but not all strains of *S. thermophilus*. Furthermore, heterogeneity in 16S-23S rDNA ITS, and *lacSZ* sequence has been reported in *S. thermophilus* strains (Mora, Ricci, Guglielmetti, Daffoncho, & Fortina, 2003; Ercolini, Fusco, Blaiotta, & Coppola, 2005). Evaluation of genetic diversity in *S. thermophilus* using randomly amplified polymorphic DNA fingerprinting (RAPD) and amplified *epsC-D* restriction analysis led to identification of 21 different genotypes (Mora, Fortina, Parini, Ricci, Gatti, Giraffa, & Manachini, 2002). It is likely that there could be a significant variation in the *rimM* sequence of various strains of *S. thermophilus* making it not possible to detect all strains of *S. thermophilus* using the designed real-time PCR primers and probe.

PCR efficiency was identified as the primary parameter that determines the suitability of DNA for quantitative analysis (Cankar, Štebih, Dreö, Žel, & Gruden, 2006). Factors that inhibit the amplification of nucleic acids by PCR act at one or more of the three essential points in reaction in the following ways: they interfere with cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation or capture, and they inhibit polymerase

activity for amplification of target DNA (Wilson, 1997). Moreover, Wilson, (1997) reported that, in PCR, milk components may block DNA and shield it from access by polymerase and the Ca^{2+} ions in milk may be a cause of its inhibitory properties. In this study measures were taken to eliminate effects of PCR inhibitors right from the cell lysis step up to the PCR amplification stage. Preliminary studies revealed that pre-incubation of *S. thermophilus* cells with bacterial cell wall degrading enzymes increased the yield and real-time PCR amplification efficiency of the extracted DNA. Although the UltraClean™ Microbial DNA kit protocol stated that up to 1.8 ml of bacterial culture could be extracted, only 0.18 ml of each sample were used for DNA extraction so as to reduce the amount of PCR inhibitors in the extracted DNA (Josefen, Krause, Hansen, & Hoorfar, 2007). The high degree of correlation between the standard curve and curves for serially diluted M17 broth and milk indicates that the extraction protocol used was effective in purification of DNA and the addition of TaqMan® exogenous internal positive control reagents minimized the effect of PCR inhibitors. In this study the amplified DNA sequence was 71 bp and this could have contributed to the high real-time PCR efficiency. A short length of the amplified DNA sequence was suggested to contribute to the robustness and high real-time PCR efficiency (Rönnner, & Lindmark, 2007).

The reliability of the developed real-time PCR method for detection of *S. thermophilus* was further tested in yoghurt containing fruits. Fruits have been reported to contain polyphenols and other compounds that can hinder

real-time PCR amplification. Inhibition severity is directly related to the amount of tannins present, with complete inhibition of Taqman[®] real-time PCR occurring at concentrations greater than 1.4 ng per 25 µl reaction and tannin concentrations below 1.4 ng per 25 µl will also impede amplification and confound real-time PCR estimates (Kontanis & Reed, 2006). However, real-time PCR results obtained from DNA extracted from pure culture, plain yoghurt, strawberry yoghurt and blueberry yoghurt were similar. The high degree of similarity in real-time PCR results obtained from DNA extracted from *S. thermophilus* culture, plain yoghurt, and yoghurt containing fruits suggest that the DNA extraction process in combination with the addition of Taqman[®] exogenous internal positive control reagents to the reaction mixtures prevented PCR inhibition. The average percentage difference between the plate count and the real-time PCR results was 3.96 indicating that the developed real-time PCR can be used for accurate enumeration of *S. thermophilus* in fermented dairy products.

5.0 Conclusion

The developed real-time PCR method can be applied for rapid detection and accurate enumeration of specific strains of *S. thermophilus* in a wide range of dairy products. There is a possibility that the developed real-time PCR method can be applied for enumeration of *S. thermophilus* in much more complex matrices such as cheese, dairy products fermented using multi-strain starter cultures and faecal samples. Further studies are required to

determine if the *S. thermophilus* strains that were not detected using the designed real-time PCR posses the *rimM* gene and the possibility of developing real-time PCR primers and probe for such strains.

Acknowledgments

We would like to thank Japan Small Scale Dairy Processors Association, Snow Brand Milk Products Co. Ltd, Japan and Hokkaido Food Processing Research Centre, Sapporo, Japan for providing some of the bacterial strains used in this study.

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