Technical note: Development of a quantitative PCR method for monitoring strain dynamics during yogurt manufacture

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ABSTRACT

Yogurt starter cultures may consist of multiple strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST). Conventional plating methods for monitoring LB and ST levels during yogurt manufacture do not allow for quantification of individual strains. The objective of the present work was to develop a quantitative PCR method for quantification of individual strains in a commercial yogurt starter culture. Strain-specific primers were designed for 2 ST strains (ST DGCC7796 and ST DGCC7710), 1 LB strain (DGCC4078), and 1 *Lactobacillus delbrueckii* ssp. *lactis* strain (LL; DGCC4550). Primers for the individual ST and LB strains were designed to target unique DNA sequences in clustered regularly interspersed short palindromic repeats. Primers for LL were designed to target a putative mannitol-specific IIBc component of the phosphotransferase system. Following evaluation of primer specificity, standard curves relating cell number to cycle threshold were prepared for each strain individually and in combination in yogurt mix, and no significant differences in the slopes were observed. Strain balance data was collected for yogurt prepared at 41 and 43°C to demonstrate the potential application of this method.

Key words: starter culture, *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, real-time quantitative polymerase chain reaction (qPCR)

Technical Note

Yogurt starter cultures typically consist of multiple strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST; Auclair and Accolas, 1983). *Lactobacillus delbrueckii* ssp. *bulgaricus* and ST strains can differ with regard to optimum growth temperature and the production of acid, aroma, flavor compounds, and exopolysaccharide produced during fermentation (Imhof et al., 1995; Tamime and Rob-

inson, 1999; Broadbent et al., 2003). Thus, particular strains are selected for commercial starter cultures so that under a specific set of conditions (e.g., yogurt mix formulation and temperature), the strain balance achieved promotes rapid acidification while producing a product with the desired organoleptic qualities (Chandan and O'Rell, 2006).

At times, it may be necessary or desirable to change the yogurt mix formulation or fermentation conditions, but such modifications can alter the growth profile and balance of individual strains (Radke-Mitchell and Sandine, 1986). Conventional plating methods for following ST and LB levels during yogurt manufacture require 48 to 72 h and do not allow for enumeration of individual strains because strains of the same species or subspecies are phenotypically very similar (IDF, 1988). In such cases, molecular techniques such as quantitative PCR (qPCR), which can discriminate between individual strains, would improve our understanding of the relationship between strain balance and product quality. The objective of this study was to develop a qPCR method for monitoring individual strains in a commercial starter culture during yogurt manufacture.

Development of a qPCR method for monitoring individual strains is contingent on identification of unique sequences, which can be used as targets for primers. Clustered regularly interspersed short palindromic repeat (CRISPR) sequences, which have been shown to be prevalent in the genomes of lactic acid bacteria including *Lactobacillus* spp. and *Streptococcus* spp., have been shown to be highly variable, allowing differentiation at the strain level (Horvath et al., 2008, 2009). The CRISPR sequences consist of alternating repeat sequences, which are short and highly conserved, and spacers, which are highly variable. In ST strains, repeat sequences are typically 36 bp in length, whereas repeats for LB are usually between 28 and 30 bp in length (Horvath et al., 2008, 2009). Repeats are separated by spacers, whose sequences are commonly derived from bacteriophage and other foreign DNA and confer resistance to phage attack (Barrangou et al., 2007). Differences in the number of repeats and spacers as well as the arrangement and sequence of the spacers have been observed in ST strains (Horvath et al., 2008).

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In this study, 2 commercial ST strains (DGCC7796 and 7710), 1 LB strain, (DGCC4078), and 1 *Lactobacillus delbrueckii* ssp. *lactis* strain (LL; DGCC4550) were obtained from Danisco USA Inc. (Madison, WI). The ST strains were propagated at 37°C in M17 broth with lactose [Becton Dickinson and Co. (BD), Franklin Lakes, NJ]. The LB and LL strains were cultivated in acidified de Man, Rogosa, and Sharpe (MRS) broth (pH 5.4; BD) and incubated at 37°C under anaerobic conditions (10% carbon dioxide, 5% hydrogen, and 85% nitrogen). Stock cultures were prepared from overnight cultures by combining equal quantities of broth and 20% glycerol (wt/vol; Sigma-Aldrich Corp., St. Louis, MO) and were stored at -80°C.

Primers for the individual ST strains were designed to target CRISPR loci previously identified in these strains (GenBank accession no. EF434468 and EF434469; <http://www.ncbi.nlm.nih.gov/genbank/>; Table 1; Barrangou et al., 2007). The primer set for LB was designed to target a CRISPR locus based on sequence information provided by the culture supplier. Because a CRISPR sequence was not available for LL, primers were designed to target a sequence in the phosphotransferase system, mannitol-specific IIBc component gene (GenBank accession no. AF496224.1). Primers were designed with Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and primer specificity was evaluated in silico and by conventional PCR. Agarose gel electrophoresis was used to confirm that a single amplicon of the correct size was produced for the target strain and no cross-reaction occurred with each strain-specific primer set and the nontarget strains in that particular starter culture (data not shown).

Quantitative PCR experiments were conducted to identify the optimal annealing temperature and primer

concentration for each primer set (data not shown). The optimized qPCR reaction mix consisted of iQ SYBR Green Super Mix (50 µL; Bio-Rad Laboratories Inc., Hercules, CA), primers at a final concentration of 50 nM, and nuclease-free water for a final reaction volume of 100 µL. Aliquots (25 µL) of the reaction mix were dispensed in triplicate wells, and qPCR was conducted with an iQ5 real-time PCR detection system (Bio-Rad Laboratories Inc.). Optimized qPCR conditions consisted of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 64.6°C for 30 s, and 72°C for 10 s. Following qPCR, melting curve analysis was conducted at temperatures ranging from 55 to 95°C, with a 0.5°C increase every 30 s. Agarose gel electrophoresis was used to verify that a single reaction product of the expected size was produced, and cross-reaction with other strains in the starter culture was not observed (data not shown).

Standard curves were prepared with individual strains spiked in the yogurt mix. The yogurt mix was prepared by combining 7:1 water and nonfat, dry milk powder in a large stainless steel canister (final concentration 12.5% milk solids-not-fat). The mix was heated to 200°F (93.3°C) for 45 min with constant agitation and then cooled and stored at 4°C.

Aliquots of individual strains grown in M17 with lactose or acidified MRS broth (45 mL) were transferred to sterile, 50-mL centrifuge tubes and centrifuged for 5 min at 1,250 × *g* (Beckman GPR centrifuge; Beckman Instruments Inc., Fullerton, CA). The supernatant was discarded and the pelleted cells were resuspended in 0.1% Polypeptone (BD) to yield cell concentrations of approximately 10⁹ cfu/mL. Serial dilutions were prepared in 0.1% Polypeptone (BD) and cell concentrations were determined by plating. Serial dilutions of ST strains were plated in duplicate on M17 agar

Table 1. Strain-specific primers for commercial starter culture strains and *Lactobacillus farciminis* (LF) ATCC 29644^T

Target microorganism ¹	Primer name ²	Sequence (5'→3')	GC content (%)	T _m ³ (°C)	Expected product (bp)
ST DGCC7796	7796f	GGTCTGTAATTTTCATTCCTCGT	43.4	54.8	87
	7796r	CCGCATGTGTTTAGAGAAACC	47.6	54.1	
ST DGCC7710	7710f	CGAGGAGCTATTGGCACAAC	55.0	56.1	85
	7710r	CAGTGGTCAGCAGATTGTCA	50.0	54.9	
LB	LBf	TGGGGGTGATCCTTTGTTATGG	50.0	57.0	86
	LB r	GGCGCTGGCCAAAAGGAT	61.1	59.2	
LL	LLf	TGGTTTCAGCCAAGGATGCC	55.0	58.3	81
	LL r	ACAGCAGCAACGACAAAGGA	50.0	57.4	
LF ⁴	LFf	GAGAGGTCT ACTCTCAAACCTCGT	47.8	55.4	84
	LF r	TATCGCGCAGTTTTCGGTGT	50.0	57.3	

¹ST DGCC7796 and ST DGCC7710 = *Streptococcus thermophilus* (ST) strains; LB = *Lactobacillus delbrueckii* ssp. *bulgaricus*; LL = *Lactobacillus delbrueckii* ssp. *lactis*.

²f = forward primer; r = reverse primer.

³Melting temperature.

⁴This strain was used as an external control and was not part of the commercial starter culture.

Table 2. Standard curve data for individual starter culture strains spiked in bulk starter base

Target microorganism ¹	Equation for line ²	Efficiency ³ (%)	R ² value ⁴	CI ⁵		
				Count (log cfu/g)	Lower	Upper
ST DGCC7796	= 14.9 – 0.243 mean Ct	93.6	0.89	5.0	4.4	5.6
				6.0	5.6	6.4
				7.0	6.7	7.3
				8.0	7.7	8.3
ST DGCC7710	= 14.3 – 0.256 mean Ct	93.3	0.94	5.0	4.7	5.3
				6.0	5.8	6.3
				7.0	6.8	7.2
				8.0	7.6	8.2
LB	= 15.8 – 0.320 mean Ct	118	0.86	5.0	4.8	5.8
				6.0	5.7	6.4
				7.0	6.5	7.2
				8.0	7.1	8.2
LL	= 14.5 – 0.277 mean Ct	98.4	0.94	5.0	4.8	5.6
				6.0	5.8	6.3
				7.0	6.7	7.2
				8.0	7.5	8.2

¹ST DGCC7796 and ST DGCC7710 = *Streptococcus thermophilus* (ST) strains; LB = *Lactobacillus delbrueckii* ssp. *bulgaricus*; LL = *Lactobacillus delbrueckii* ssp. *lactis*.

²Standard curves relating cycle threshold (Ct) to cell counts (cfu/g) were determined using Minitab 16 software (Minitab Inc., State College, PA). Results are based on combined data for 3 replicates.

³The efficiency of PCR reactions was calculated as the average of 3 replicates.

⁴Results are based on combined data for 3 replicates.

⁵Confidence intervals were based on $\alpha = 0.05$. Confidence intervals provide the range for counts obtained using the standard curves relating Ct to cell counts (cfu/g).

with lactose (BD) and incubated at 37°C for 48 h (IDF, 1988). Serial dilutions of LB and LL strains were plated in duplicate on acidified MRS agar (BD) and incubated anaerobically at 37°C for 72 h (IDF, 1988). Serial dilutions were used to inoculate prewarmed yogurt mix (41°C) at concentrations ranging from 10⁵ to 10⁸ cfu/g. Prior to DNA extraction, 1:10 dilutions were prepared using 0.1% Polypeptone. Aliquots (1 mL) were transferred to sterile, screw-cap microcentrifuge tubes (Sorensen Bioscience Inc., West Salt Lake City, UT) and centrifuged for 5 min at 16,000 × *g* to pellet the cells. The supernatant was discarded, and genomic DNA was isolated from the cells with the PowerFood Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) using the manufacturer's procedure for difficult-to-lyse cells, which includes heat treatment at 70°C for 10 min. Isolated genomic DNA (2 µL) was added to the qPCR reaction mix, and qPCR was conducted as described above. Results were analyzed using iQ5 Optical System Software (version 2.0; Bio-Rad Laboratories Inc.). This experiment was conducted in triplicate.

Regression calculations and confidence intervals (95%) were determined using Minitab 16 software (Minitab Inc., State College, PA). Statistical analysis of the standard curves was conducted with ANOVA (general linear model). Primer efficiency (E) was calcu-

lated using the following equation: $E = 10^{(-1/\text{slope})} - 1$ (Kubista et al., 2006).

Data for combined replicates of individual starter culture strains spiked in yogurt mix are presented in Table 2. Coefficient of determination values of plots of mean cycle threshold (Ct) versus log cfu/g for individual replicates ranged from 0.96 to 1.0, and plots of combined data yielded lower values (0.86 to 0.94; Table 2). Efficiencies for combined data ranged from 93.3 (ST DGCC7710) to 118% (LB).

To simulate yogurt manufacture, which involves multiple strains, standard curves prepared with combinations of the starter culture strains spiked in yogurt mix were determined. For standard curves with strains spiked in yogurt mix, each strain (target) was added at concentrations ranging from approximately 10⁴ to 10⁸ cfu/g along with the other 3 starter culture strains (nontarget), each present at a concentration of approximately 10⁶ cfu/g. Cell counts and qPCR analysis were conducted as described above. Three replicates of the experiment were performed. For all of the strains, no significant difference ($\alpha < 0.05$) was observed in the slopes of standard curves prepared with individual strains and 4-strain combinations spiked in yogurt mix.

An external control was used to monitor the DNA extraction process and qPCR. Criteria for the external control included an organism that is not commonly

Figure 1. pH data and quantification of individual strains in commercial starter culture (Danisco USA Inc., Madison, WI) inoculated in bulk starter base and incubated at (A) 41°C and (B) 43°C. DGCC7796 and DGCC7710 = *Streptococcus thermophilus* strains; LB = *Lactobacillus delbrueckii* ssp. *bulgaricus*; LL = *Lactobacillus delbrueckii* ssp. *lactis*. Color version available in the online PDF.

present in a dairy processing environment and would exhibit similar lysis characteristics as the starter culture strains. *Lactobacillus farciminis* ATCC 29644^T (LF; American Type Strain Culture Collection, Manassas, VA) was selected, as this microorganism possesses a similar peptidoglycan structure (Lys-D-Asp) as LB and LL (Reuter, 1983; Schillinger and Lücke, 1987; Settanni et al., 2005; Felis and Dellaglio, 2007), and is more commonly associated with marinated meats and sour-dough bread. A primer set targeting LF 16S-23S rDNA ITS (accession number AF500490) was designed using Primer-Blast (Table 1; Rachman et al., 2003). Specificity was evaluated in silico and by qPCR using isolated genomic DNA (25 ng/25-μL reaction) from LF and the 4 starter culture strains. No cross-reactivity was observed with the LF primers and the starter culture strains or the starter culture-specific primers and LF.

For the external control stock, an overnight culture in acidified MRS broth was enumerated by plating and the broth was aliquoted in sterile screw-cap vials and stored at -80°C. To determine the mean Ct and standard deviation for the external control stock, stock was thawed on ice, aliquots (0.1 mL) were added to diluted yogurt mix (1:10 dilution) immediately before DNA isolation, and Ct values were determined by qPCR.

When used as the external control, stock from a previously frozen vial was thawed on ice and vortexed thoroughly. A 0.1-mL aliquot was added to 1 mL of a 1:10 dilution of yogurt mix that had been pregrown with LB and ST immediately before DNA isolation. After qPCR, Ct values from the quantification of LF spiked into yogurt samples were compared with the mean Ct value previously determined for the external control stock prepared in MRS broth. If these Ct values

differed by greater than 3 standard deviations, DNA isolation and qPCR would be repeated using a backup sample. However, the DNA extraction process used in this work was highly reproducible.

To demonstrate the application of the qPCR method for following strain balance, we monitored growth of 2 ST strains, 1 LB strain, and 1 LL strain during yogurt manufacture at 2 different temperatures. Yogurt mix inoculated with the freeze-dried commercial starter culture (50 g, Danisco USA Inc.) was dispensed into 2 sterile bottles and incubated at 41 and 43°C until pH 4.6 was reached. Samples for qPCR were collected at 1-h intervals. Deoxyribonucleic acid was isolated and qPCR analysis was conducted as previously described. Standard curves were used to relate Ct values to cell counts. Two replicates of the experiment were performed.

Estimated counts of the 4 strains are presented in Figure 1. At both 41 and 43°C, ST DGCC7796 was the predominant strain, followed by ST DGCC7710. Acidification profiles were similar at both temperatures. Whereas conventional plating methods do not allow for quantification of individual ST strains, this method provided a useful way of following strain dynamics under different growth conditions. The method described here suggests that CRISPR sequences may contain sufficient variation to permit rapid development of a specific assay for strains where limited genomic information is available. We will apply this method in the future to study the influence of different formulations and processing conditions on yogurt strain balance, and to develop assays targeting other strains of ST, LB, and LL.

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