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Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk

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Abstract

This study describes selective plating methodologies for enumeration of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk based on selective antibiotic-free media. Enumeration of *S. thermophilus* was performed using M17-lactose. MRS-fructose was suitable for enumeration of *L. bulgaricus* and MRS-maltose for differentiation between *L. acidophilus* and *L. paracasei*. The selective enumeration of *B. lactis* was obtained using MRS-raffinose containing 0.05% LiCl. The bacterial counts obtained using selective methods were equivalent to those under optimum culture conditions at a probability level of 95%. Performance of the methods was verified in fermented milk products where identification of the enumerated species was confirmed by species-specific polymerase chain reaction. This study shows that combination of species-specific polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis has great detection and identification potential for verification of accurate species labelling in fermented milk without prior isolation of the bacteria.

Keywords: Selective methods; Lactobacilli; Bifidobacteria; Fermented milk; PCR-DGGE

1. Introduction

The CODEX standard for fermented milk products (CODEX STAN 243-2003) establishes them as the products obtained by fermentation of milk by the action of suitable starter microorganisms that should be viable, active and abundant in the product to the date of minimum durability (Codex Alimentarius Commission, 2003). The name yoghurt should be used when the milk is only fermented by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Regarding viability, the norm specifies that the sum of microorganisms constituting the starter culture should be at least 10^7 cfu g^{-1}, and that minimum counts of other labelled microorganisms should be 10^6 cfu g^{-1}. Therefore, microbial viability and authenticity are prominent criteria to be analytically verified for the compliance of fermented milk with the required product specifications. Likewise, probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit in the host (FAO/WHO, 2002). However, the minimum amount of probiotics needed to obtain a clinical effect has not been established. As more information on probiotics is available, it seems likely that numbers will vary as a function of the strain and the health effect desired (Roy, 2005).

Fermented milk products are the most popular means of delivering probiotic bacteria in food. Among them, strains of *L. acidophilus*, *L. casei* complex and *Bifidobacterium lactis* predominate in commercial probiotic products (Fasoli et al., 2003; Gueimonde et al., 2004; Masco, Huys, De Brandt, Temmerman, & Swings, 2005; Yeung, Sanders,

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Kitts, Cano, & Tong, 2002). The presence of multiple and closely related species in these products makes the differential enumeration of probiotic and yoghurt starter bacteria difficult due to similarity in growth requirements and overlapping biochemical profiles of the species. Numerous media have been proposed for selective and differential enumeration of lactobacilli and bifidobacteria in mixed bacterial populations, and some have been the subject of specific reviews (Charteris, Kelly, Morelli, & Collins, 1997; Shah, 2000; Roy, 2001; Coeuret, Dubernet, Bernardeau, Gueguen, & Vernoux, 2003) and of comparative performance analyses (Payne, Morris, & Beers, 1999; Talwalkar & Kailasapathy, 2004; Masco et al., 2005; Van de Casteele et al., 2006). In order to cover a high spectrum of species, most media for selective enumeration of mixed cultures have complex compositions that include antibiotics as selective ingredients, which could impact on the response of not only the sensitive strains but also of target bacteria and result in inaccurate or irreproducible quantitative results. A comparison of methods described in literature (Talwalkar & Kailasapathy, 2004) concluded that no reliable techniques are yet developed to accurately enumerate *L. acidophilus*, *L. casei* and *Bifidobacterium* in different commercial yoghurts. Overall, it seems rational that the choice of selective methods should focus on the type of food and the species, even strains, to enumerate in each particular situation (Lourens-Hattingh & Viljoen, 2001; Sartory, 2005).

As previously stated, identification of species is another important issue to be verified for the compliance of fermented milk with the required product specifications in terms of accurate species labelling and, if appropriate, to support health claims that could be associated with added probiotics. Phenotypic methods alone are inadequate for identification of lactobacilli and bifidobacteria species (Dellaglio & Felis, 2005). To achieve a rapid and reliable identification of species, polymerase chain reaction (PCR)-based methods using species-specific primers targeting the 16S rRNA gene sequence diversity have become very popular (Coeuret et al., 2003). In addition, culture-independent methods for bacterial identification based on genetic analysis have become a valuable tool, since these techniques have the advantage to analyze the product as a whole. Separation of genus or species-specific PCR products by denaturing gradient gel electrophoresis (DGGE) has become the most commonly used technique among the culture-independent methods for detection and identification of lactobacilli and bifidobacteria from fermented products (Ercolini, 2004).

The aim of this study was to develop selective plating methodologies for enumeration and identification of mixed cultures of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in fermented milk products based on selective antibiotic-free media and different incubation conditions. To evaluate the performance of selective media for complete recovery of viable bacteria, methods were validated on the basis of their precision, accuracy, reproducibility, selectivity and specificity characteristics, in relation to culture conditions, which were used as reference methods. Efficacy of the selective methods was verified by identification of the presumptive colonies using species-specific PCR. The study is also complemented with the application of a culture-independent procedure based on PCR–DGGE analysis to the rapid detection and identification of the mixed species in fermented milk products.

2. Material and methods

2.1. Microorganisms and culture conditions

Strains used in the assay were *S. thermophilus* STY-31, *L. delbrueckii* subsp. *bulgaricus* LBY-27, *L. acidophilus* LA-5, *L. paracasei* subsp. *paracasei* LC-01, and *B. lactis* BB-12. The strains were purified from a commercial synbiotic product (Simbiotic Drink; Priégola, Madrid, Spain). To allow the correct identification of strains, 16S rRNA gene nucleotide sequencing was carried out from pure cultures. The entire gene was amplified using the primers *Sac*I-POmod and *Sal*I-T7-PC5 (Table 1) and the PCR conditions described previously by Rodtong and Tannock (1993). Additional primers used to assist in sequencing were 16Smidfor and P3rev (Table 1). Sequencing of PCR fragments was carried out for both strands at the DNA Sequence Service of the Centro de Investigaciones Biológicas-CSIC (Madrid, Spain). *S. thermophilus* was grown in M-17 broth (Pronadisa, Madrid, Spain) containing 2% lactose. *Lactobacillus* subsp. and *B. lactis* were grown under anaerobic conditions (Gas-Pack, Anaerogen; Oxoid Ltd., Hampshire, England) in MRS broth (Pronadisa) supplemented with 0.05% L-cysteine hydrochloride, excepting *L. paracasei* subsp. *paracasei* that was grown aerobically in MRS broth. Incubations were carried out for 18–24 h at 37 °C and at 30 °C for *L. paracasei* subsp. *paracasei*.

2.2. Selective methods

Culture conditions described above were selected as reference methods. Media were supplemented with 1.5% bacteriological agar (Scharlab, Barcelona, Spain) and incubation extended to 48 h for *S. thermophilus* and 72 h for *B. lactis* and lactobacilli.

The selective conditions for the enumeration of *S. thermophilus* included inoculation of appropriate dilutions by the pour-plate technique into M-17 agar containing 1% lactose (M17-lactose) and incubation at 45 °C for 24 h. For enumeration of *L. delbrueckii* subsp. *bulgaricus*, appropriate dilutions were pour-plated into MRS fermentation broth (Pronadisa), which does not contain either glucose or meat extract (De Man, Rogosa, & Sharpe, 1960), enriched with 0.2% Tween 80 and supplemented with 1% fructose, 0.8% casein acid hydrolysate, 0.05% cysteine, and 1.5% agar (MRS-fructose). Plates were incubated in anaerobic

Table 1
Polymerase chain reaction primers used in this study for the identification of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis*

Species	Name	Sequence 5′→3′	Product (bp)
S. thermophilus	Thermfor	ACGCTGAAGAGAGGAGCTTG	157
	Thermrev	GCAATTGCCCCTTTCAAATA	
L. bulgaricus	Bulgfor	TCAAAGATTCCTTCGGGATG	232
	Bulgrev	TACGCATCATTGCCTTGGTA	
L. acidophilus	Acidfor	AGCGAGCTGAACCAACAGAT	227
	Acidrev	AGGCCGTTACCCTACCAACT	
L. paracasei	Casfor	GCACCGAGATTCAACATGGAA	142
	Casrev	GCCATCTTTCAGCCAAGAACC	
B. lactis	Forlac	GCGCTGGGCTGCTCTGGAAGC	116
	Revlac	TGGCGACGAGCTCATCGACATACT	
All species	*Sac*I-POmod	CCGAGCTCAACAGAGTTTGATCCTGGCTCAG	792–825[a]
	P3rev	GGACTACCAGGGTATCTAAT	
	16Smidfor	GGCCGTTACTGACGCTGAG	767–771[a]
	*Sal*I-T7-PC5	GGTCGACCGTTAATACGACTCACTATAGGGATACCTTGTTACGACTT	

[a]Size range of products obtained from the five species.

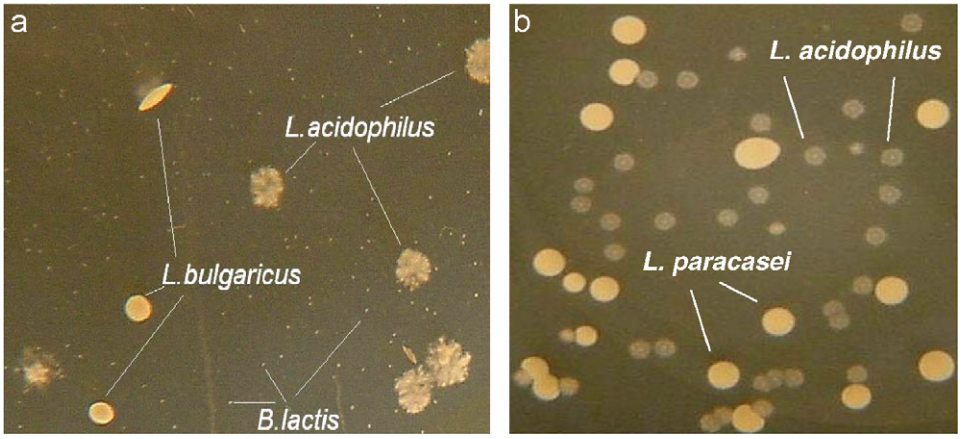

Fig. 1. Differentiation of *L. acidophilus* from *L. delbrueckii* subsp. *bulgaricus* and *B. lactis* on MRS-fructose agar (a) and from *L. paracasei* subps. *paracasei* on MRS-maltose agar (b).

jars at 45 °C for 72 h and lenticular colonies with 1–2 mm diameter were enumerated as *L. delbrueckii* subsp. *bulgaricus*, whereas cottony–fluffy colonies of 2–3 mm diameter corresponded to *L. acidophilus* (Fig. 1a). Enumeration of *L. acidophilus* was performed by spreading out appropriate dilutions onto MRS fermentation broth enriched with 0.2% Tween 80 and supplemented with 1% maltose, 0.05% cysteine, and 1.5% agar (MRS-maltose). Plates were incubated in a 20% CO_2 atmosphere incubator at 37 °C for 72 h. Flat, rough colonies with irregular edges and 1–2 mm diameter corresponded to *L. acidophilus*, whereas *L. paracasei* subsp. *paracasei* developed as white, smooth and circular colonies of 2–3 mm diameter (Fig. 1b). The method was also selected for the enumeration of *L. paracasei* subsp. *paracasei*. Enumeration of *B. lactis* was carried out by pour-plating appropriate dilutions into MRS fermentation broth supplemented with 1% raffinose, 0.05% LiCl, 0.05% cysteine, and 1.5% agar (MRS-raffinose). Plates were incubated in anaerobic jars at 45 °C for 72 h.

2.3. Efficiency tests

To evaluate performance of the selective methods to enumerate lactic acid bacteria (LAB) and *B. lactis*, recommendations of the ISO/TR 13843 (ISO, 2000) and ISO/IEC 17025 (ISO, 2005) standards on validation of microbiological methods were followed. Parameters evaluated were precision, accuracy, reproducibility, selectivity and specificity. The precision and accuracy of the methods were determined by the comparison between the bacterial counts obtained with selective and reference methods. Overnight pure cultures were diluted and inoculated into both selective and reference media. After logarithmic transformation of the results to normalize the distribution, counts obtained in both media were compared using paired Student's *t*-test to obtain $t_{exp} = d_m/(s_d/n^{1/2})$, where d_m is the mean of differences (d) between counts on selective and reference methods, s_d is the standard deviation of d, and n the number of samples. Other parameters calculated were relative recovery $= 10^{-d_m}$ and relative standard deviation

of differences $= 1 - 10^{-s_d}$. Reproducibility of the methods was tested by the analysis of identical samples by two different operators and using different equipments. At the same time, the matrix effect for bacterial enumeration in fermented milk was evaluated. Samples consisted of 10% reconstituted skim milk powder (Scharlab) acidified to pH 4.6 with 5 M lactic acid and inoculated with each species at both, high levels (10^7 cfu mL^{-1}) and low levels (10^5 cfu mL^{-1}). Pairs of counts were compared and the relative standard deviation of reproducibility was calculated as $1 - 10^{-s_{dr}}$, where s_{dr} is the standard deviation of differences between counts from the two operators. For selectivity and specificity analysis, cultures from all five bacterial species were mixed at the level of 10^7 cfu mL^{-1} for each strain and appropriate dilutions inoculated into the selective media. Results were expressed as the percentage of the presumptive target counts in relation to theoretical counts. In addition, 10% reconstituted skim milk, pH 4.6, was inoculated with cultures of each target strain at low level (10^5 cfu mL^{-1}) and mixed with the other four strains at high level (10^7 cfu mL^{-1}). Appropriate dilutions were plated and analysed for the presence of presumptive false positive and negative colonies. All analyses were performed at least in triplicate and differences were compared at a significance level of 0.05 by a Student's *t*-test using Excel software (Microsoft, Redmond, WA, USA).

2.4. Analysis of fermented milk products

2.4.1. Enumeration of bacterial viable counts

The fermented milk Simbiotic Drink containing the yoghurt and probiotic strains and the product with only the yoghurt bacteria, both from Priégola, were analysed through their shelf life (28 d) for performance of the selective methods. Viable counts were determined in samples (1 mL) by using serial decimal dilutions prepared in Ringer's solution (Scharlab) supplemented with 0.05% cysteine. Appropriate dilutions were plated in duplicate and analysed using the selective methods described above.

2.4.2. Identification of presumptive target colonies

Presumptive positive colonies (10%) grown with selective methods in the highest dilution plate were checked by species-specific PCR to verify the efficacy of the media for specific enumeration. Species-specific primers were designed within variable regions in the 16S rRNA encoding genes of *S. thermophilus*, *L. paracasei* subsp. *paracasei*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* using the Lasergene PrimerSelect module of the Lasergene software package (DNAStar Inc., Madison, WI, USA). Species-specific primers for identification of *B. lactis* were designed on the basis of the transaldolase gene variable regions (Requena et al., 2002). Primer pairs (Table 1) were selected upon confirmation that only targeted species would give a PCR product. Colonies were picked up using sterile toothpicks, suspended in 20 μL milliQ water, boiled at 100 °C for 5 min and frozen at −20 °C. Diagnostic PCR reactions were carried out with 2 μL of thawed cell suspensions and the primers described in Table 1. The amplification programme was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s, and a final extension time of 72 °C for 5 min.The products (5 μL) were separated on a 2% agarose gel and analysed for the yield of amplicons with the expected sizes (Table 1).

2.4.3. PCR–DGGE analysis

In order to obtain bacterial DNA from fermented milk, the samples (3 mL) were neutralized to pH 6.5 with 1 M NaOH and cleared by adding 10 mL of 0.2% EDTA, pH 12, to cause casein micelle dispersion. The bacterial cells were collected by centrifugation at 10,000 × *g* for 15 min and mixed (1:1) with glass beads (diameter, 150–212 μm; Sigma Chemical Co., St. Louis, MO, USA) for mechanical disruption by vortexing the ice-cooled suspensions four times over 4 min. Cell debris and glass beads were collected by centrifugation (12,000 × *g* for 5 min) and genomic DNA was obtained from the cell free extract as described by Meile, Rohr, Geissman, Herensperger, and Teuber (2001). The DNA was used as template (500 ng) for PCR amplification using the conditions described above. A 40-bp GC clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3′) was attached to either the forward or reverse primer (Table 1) to obtain PCR products suitable for separation by DGGE. Thus, the following species-specific primers were employed: Therm-for-GC and Thermrev for the identification of *S. thermophilus*, Bulgfor and Bulgrev-GC for *L. delbrueckii* subsp. *bulgaricus*, Acidfor-GC and Acidrev for *L. acidophilus*, Casfor-GC and Casrev for *L. paracasei* subsp. *paracasei*, and Forlac and Revlac-GC for *B. lactis*. An identification ladder containing equal amounts of PCR products from pure cultures was prepared.

DGGE was performed with a DCode system (Bio-Rad Laboratories, Hercules, CA, USA), using a 9% polyacrylamide gel with a 40–60% gradient of 7 M urea and 40% formamide that increased in the electrophoresis running direction. Electrophoresis was carried out in 20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA (0.5 × TAE) buffer at 130 V and 60 °C for 4.5 h. Gels were stained with $AgNO_3$ as described by Sanguinetti, Dias-Neto, and Simpson (1994).

3. Results and discussion

3.1. Comparison of bacterial counts using selective and reference methods

This study has focused on the development of selective methods suited to recover the maximum population of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in fermented milk as compared with that of supporting optimal growth (reference methods) to avoid underestimation of the bacterial counts. Therefore, formulation of the methods was based on antibiotic-free media, carbohydrate

fermentation patterns and different incubation conditions. Incubation in aerobiosis at 45 °C during 24 h in M17-lactose agar was found suitable for selective enumeration of *S. thermophilus*, since it prevented the growth of *L. paracasei* subsp. *paracasei* found at 37 °C and that of *L. delbrueckii* subsp. *bulgaricus* and *B. lactis* that developed under anaerobic conditions. Extension of the incubation period for 48 h allowed the appearance of pinpoint colonies of *L. acidophilus*. Therefore, these incubation conditions provided selective characteristics for *S. thermophilus* enumeration, although the medium does not inhibit the growth of the other bacteria.

The finding of a non-antibiotic medium for selective enumeration of *L. delbrueckii* subsp. *bulgaricus* could not be based on the acidified MRS medium recommended by ISO/FDIS 7889 IDF 117 standard on enumeration of yoghurt characteristic microorganisms (ISO, 2002), since it also allowed growth of *L. acidophilus*, *L. paracasei* subsp. *paracasei*, and *B. lactis*. Increasing the incubation at 45 °C and replacement of glucose by fructose were conditions selective against *L. paracasei* subsp. *paracasei* and *B. lactis*, respectively. The method was differential against *L. acidophilus* when the medium was enriched with 0.2% Tween 80, showing a clear morphological differentiation between lenticular colonies corresponding to *L. delbrueckii* subsp. *bulgaricus* and cottony-fluffy colonies of *L. acidophilus* (Fig. 1a). The role of the compound to cause such peculiar colony morphology in *L. acidophilus* was not elucidated. Selective enumeration of *L. acidophilus* against *S. thermophilus* and *B. lactis* in fermented milk using MRS-maltose and incubation in a 20% CO_2 atmosphere was shown in a previous report (Martín-Diana, Janer, Peláez, & Requena, 2003). In the present study, the method also demonstrated to be selective against *L. delbrueckii* subsp. *bulgaricus* and differential against *L. paracasei* subsp. *paracasei*, since a clear difference in colony morphology could be assessed in the plates (Fig. 1b). Counts of *L. acidophilus* were similar when the two methods, growth in MRS-maltose and MRS-fructose, were compared (results not shown). MRS-maltose was also selected as suitable for enumeration of *L. paracasei* subsp. *paracasei* since it gave excellent results compared with the reference method (Table 2).

MRS containing cysteine–HCl can provide optimal overall growth conditions for the non-selective enumeration of bifidobacteria (Roy, 2001; Leuschner, Bew, Simpson, Ross, & Stanton, 2003), and it was therefore selected as reference medium for *B. lactis*. Selective conditions for enumeration of this species were incubation at 45 °C, use of raffinose as a carbohydrate source and addition of 0.05% LiCl to suppress lactobacilli growth. The method was selective for *B. lactis* against the LAB strains studied. Overall characteristics of the method allowed reduction of the concentration of the antimicrobial compound LiCl to 0.05% instead of 0.2–0.3%, the amount usually added in selective media for enumeration of *Bifidobacteria* (Hartemink, Kok, Weenk, & Rombouts, 1996; Payne et al., 1999; Roy, 2001).

The Student's *t*-test was used to compare each species enumeration on both methods (reference and selective). The resulting *t* values (Table 2) were lower than the tabulated value of *t* ($t_{tab} = 2.29$) in all cases, which indicates that there were no significant differences between the two methods in bacterial enumeration at a probability level of 95%. The highest relative standard deviation of differences in counts between methods was observed for *L. delbrueckii* subsp. *bulgaricus*. In general, higher *L. delbrueckii* subsp. *bulgaricus* counts were found at 45 °C than at 37 °C (results not shown). Optimum temperature growth at 44 °C for *L. delbrueckii* subsp. *bulgaricus* has been previously reported (Beal, Louvet, & Corrieu, 1989), and a recommendation to increase temperature incubation for *L. delbrueckii* subsp. *bulgaricus* slowly growing strains is included in the ISO/FDIS 7889 IDF 117 standard on yoghurt colony count technique at 37 °C (ISO, 2002). Reproducibility of the methods was tested by the analysis of identical samples, acidified milk (pH 4.6) inoculated with each species at both high level (10^7 cfu mL^{-1}) and low level (10^5 cfu mL^{-1}), by two different operators and using different equipment. As shown in Table 2, the relative standard deviation of reproducibility was equal or lower than 0.14 log units.

Table 2
Counts (log cfu mL^{-1}) and evaluation of performance of the selective methods to enumerate *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis*

Parameter	*S. thermophilus*	*L. bulgaricus*	*L. acidophilus*	*L. paracasei*	*B. lactis*
Mean[a] (log cfu mL^{-1}) of reference method	9.02	7.74	7.35	9.39	8.75
Mean[a] (log cfu mL^{-1}) of selective method	9.01	8.04	7.34	9.41	8.78
Relative standard deviation of differences	0.08	0.47	0.08	0.10	0.12
Student's t[b]	0.70	1.58	0.53	0.52	0.76
Relative recovery	0.97	2.02	0.97	1.03	1.07
Relative standard deviation of reproducibility	0.14	0.13	0.13	0.07	0.08
Selectivity (%)	101.8	103.9	100.7	101.1	97.6

[a]Means are average from three independent analyses.
[b]$t_{tab} = 2.29$.

To verify the selectivity of the methods, cultures of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* were mixed at approximately 10^7 cfu mL^{-1} in the ratio of 0.2, 1, 5, 0.2, and 1, respectively, and appropriate dilutions plated into the selective media. The results, expressed as the percentage of presumptive target counts in relation to theoretical counts, are shown in Table 2. Percentages close to 100% indicate that the selective methods yielded counts that were nearly equal to the theoretical counts, which indicates that the efficacy of the methods for selective enumeration of the five species in mixed cultures could be considered acceptable. Specificity and selectivity of the methods were also analysed in acidified milk, pH 4.6, that was inoculated with cultures of each target strain at low level (10^5 cfu mL^{-1}) and the other four strains at high level (10^7 cfu mL^{-1}). There was no interference between species for the enumeration of *S. thermophilus* and *B. lactis* in the corresponding selective media, and for *L. paracasei* subsp. *paracasei* when it was incubated in aerobiosis at 30 °C for 48 h in MRS agar. However, differential enumeration of *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* in MRS-fructose (Fig. 1a) and of *L. acidophilus* and *L. paracasei* subsp. *paracasei* in MRS-maltose (Fig. 1b) could only be made when differences of counts between the two species were lower than 2 log units (results not shown).

3.2. Enumeration of bacteria in fermented milk products using the selective methods

The performance of the methods for selective enumeration of yoghurt and probiotic bacteria was carried out in the commercial probiotic product Simbiotic Drink (Priégola), containing *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* as stated in the label, and the product manufactured with only the yoghurt bacteria. Results in Table 3 are averages from eight batches of each product analysed over 4 weeks. The results obtained indicated that there was low variation between production batches, reliable counts for the probiotic and yoghurt strains and acceptable viability of the species throughout the shelf life of the products. The selective methods were therefore suitable for enumeration of the species, mostly because they were evaluated for the specific microorganisms present in the product. The results strengthen the rising opinion that selective or differential media should be evaluated for the specific strains of species of interest in each particular product (Lourens-Hattingh & Viljoen, 2001). As stated by Talwalkar and Kailasapathy (2004), the search for a single media in the literature that would provide reliable cell counts of *L. acidophilus*, *Bifidobacterium* spp., and *L. casei* in several different products could be unsuccessful.

Table 3
Viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in probiotic fermented milk and yoghurt through storage at 4 °C during four weeks

Species	Counts (cfu mL^{-1}) in fermented milk stored at 4 °C			
	1 wk	2 wk	3 wk	4 wk
Probiotic fermented milk				
S. thermophilus	9.32 (0.09)	9.15 (0.18)	9.22 (0.14)	9.14 (0.37)
L. bulgaricus	7.58 (0.28)	7.36 (0.53)	7.63 (0.07)	6.05 (1.03)
L. acidophilus	7.12 (0.30)	7.11 (0.33)	6.81 (0.33)	6.44 (0.36)
L. paracasei	6.49 (0.12)	6.48 (0.27)	6.49 (0.13)	6.47 (0.21)
B. lactis	8.08 (0.13)	8.03 (0.22)	7.97 (0.22)	8.15 (0.15)
Yoghurt				
S. thermophilus	8.88 (0.12)	8.91 (0.46)	8.98 (0.22)	8.94 (0.05)
L. bulgaricus	8.26 (0.11)	8.35 (0.13)	8.23 (0.13)	8.21 (0.07)

[a]Means are average from eight batches. Standard deviation in parenthesis.

Developed colonies (10%) from each selective media were subjected to confirmation test by species-specific PCR identification using primers based on the 16S rRNA gene sequence obtained from the LAB strains and on the partial sequence of the transaldolase gene sequence from *B. lactis* previously described (Requena et al., 2002). Specificity of the primers and PCR conditions to identify the analysed species were tested with DNA from pure cultures. Formation of specific amplicons was exclusively observed from the corresponding species (results not shown). All the presumptive target colonies analysed from selective and differential plates confirmed their identity by yielding PCR products of the expected sizes (Fig. 2). In addition, the species-specific primer pairs designed and the PCR conditions developed in this study proved to be a very rapid and effective method for the identification of the species.

3.3. Identification by PCR–DGGE of species present in the fermented milk

The primers used for species-specific PCR identification of colonies were also suitable for identification of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in culture-independent analysis of the fermented milk Simbiotic Drink using PCR–DGGE. The efficiency of separation was assayed by comparing amplicons obtained from pure cultures with the GC clamp attached to the forward or the reverse primer (see Material and methods). The appropriate products were combined to obtain the reference ladder that allowed for the identification of species in the fermented milk without prior isolation (Fig. 3). In spite of the length homogeneity of amplicons (Table 1 and Fig. 2), the technique allowed a distinguishable separation of fragments, showing a great detection and identification potential for analysis of these products. The efficiency of PCR–DGGE for lactobacilli and bifidobacteria identification of commercial probiotic products has been recently demonstrated (Fasoli et al., 2003; Temmerman, Scheirlinck, Huys, & Swings, 2003). In the present work, the high annealing temperature (60 °C) of the species-specific PCR applicable to the five species would have the additional advantage of carrying out one-step species identification by

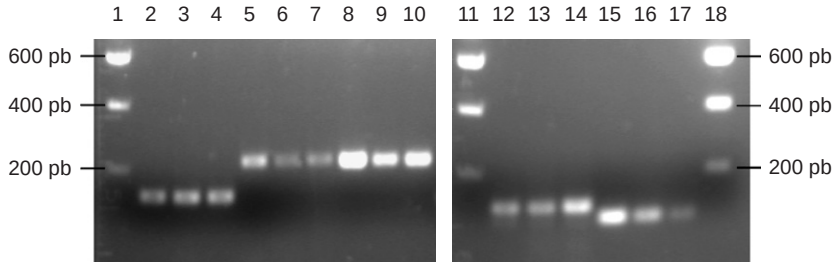

Fig. 2. Polymerase chain reaction products obtained from pure culture DNA and two colonies enumerated as *S. thermophilus* (lanes 2, 3 and 4), *L. delbrueckii* subsp. *bulgaricus* (lanes 5, 6 and 7), *L. acidophilus* (lanes 8, 9 and 10), *L. paracasei* subps. *paracasei* (lanes 12, 13 and 14) and *B. lactis* (lanes 15, 16 and 17).

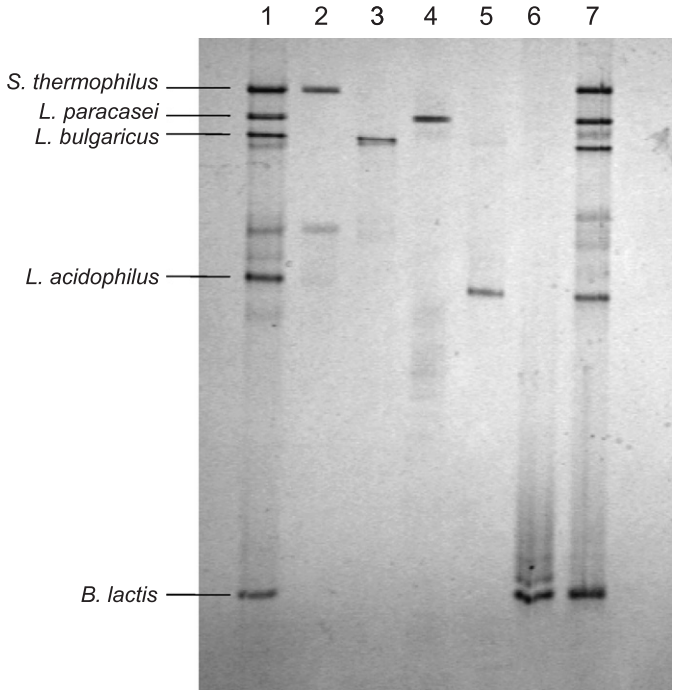

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) analysis of the polymerase chain reaction products obtained from probiotic fermented milk using the species-specific primers for *S. thermophilus* (lane 2), *L. paracasei* subsp. *paracasei* (lane 3), *L. delbrueckii* subsp. *bulgaricus* (lane 4), *L. acidophilus* (lane 5) and *B. lactis* (lanes 6). Lanes 1 and 7: DGGE identification ladder.

multiplex PCR combined with separation of fragments by DGGE, a study that is currently underway.

4. Conclusions

The present study shows that the combined use of selective plating media and different incubation conditions provide an effective antibiotic-free approach to the selective enumeration of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in mixed cultures present in fermented milk products. The choice of methods was based on carbohydrate fermentation patterns and incubation at different temperatures and atmospheric conditions that were targeted to the species present in the product. Efficiency of the selective methods was verified by evaluation of performance using statistical parameters such as precision, accuracy, reproducibility, selectivity and specificity, and by identification of the enumerated species by species-specific PCR. As a complementary advantage, this study also demonstrates that the combination of species-specific PCR and DGGE analysis shows a great detection and identification potential for verification of accurate species-labelling in fermented milk without previous isolation of the bacteria.

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References

Beal, C., Louvet, P., & Corrieu, G. (1989). Influence of controlled pH and temperature on the growth and acidification of pure cultures of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398. *Applied Microbiology and Biotechnology*, *32*, 148–154.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1997). Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *International Journal of Food Microbiology*, *35*, 1–27.

CODEX Alimentarius Commission. (2003). CODEX standard for fermented milks. Codex Stan 243-2003. Retrieved September 1, 2006, from 〈http://www.codexalimentarius.net/download/standards/400/CXS_243e.pdf〉.

Coeuret, V., Dubernet, S., Bernardeau, M., Gueguen, M., & Vernoux, J. P. (2003). Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Le Lait*, *83*, 269–306.

Dellaglio, F., & Felis, G. E. (2005). Taxonomy of lactobacilli and bifidobacteria. In G. W. Tannock (Ed.), *Probiotics and prebiotics: Scientific aspects* (pp. 25–49). Norfolk, UK: Caister Academic Press.

De Man, J. C., Rogosa, M., & Sharpe, E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, *23*, 130–135.

Ercolini, D. (2004). PCR–DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, *56*, 297–314.

FAO/WHO. (2002). Guidelines for the evaluation of probiotics in food. Retrieved August 23, 2006, from 〈ftp://ftp.fao.org/es/esn/food/wgreport2.pdf〉.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., & Torriani, S. (2003). Bacterial composition of commercial probiotic products as evaluated by PCR–DGGE analysis. *International Journal of Food Microbiology*, *82*, 59–70.

Gueimonde, M., Delgado, S., Mayo, B., Ruas-Madiedo, P., Margolles, A., & De los Reyes-Gavilán, C. G. (2004). Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks. *Food Research International*, *37*, 839–850.

Hartemink, R., Kok, B. J., Weenk, G. H., & Rombouts, F. M. (1996). Raffinose–Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. *Journal of Microbiological Methods*, *27*, 33–43.

ISO. (2000). *Water quality—Guidance on validation of microbiological methods. ISO/TR 13843:2000*. Geneva, Switzerland: International Organisation for Standardization.

ISO. (2002). *Yogurt—Enumeration of characteristics microorganisms—Colony count technique at 37 °C. ISO/FDIS 7889 IDF 117*. Geneva, Switzerland: International Organisation for Standardization.

ISO. (2005). *General requirements for the competence of testing and calibration laboratories. ISO/IEC 17025:2005*. Geneva, Switzerland: International Organisation for Standardization.

Leuschner, R. G. K., Bew, J., Simpson, P., Ross, P. R., & Stanton, C. (2003). A collaborative study of method for the enumeration of probiotic bifidobacteria in animal feed. *International Journal of Food Microbiology*, *83*, 161–170.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yoghurt as probiotic carrier food. *International Dairy Journal*, *11*, 1–17.

Martín-Diana, A. B., Janer, C., Peláez, C., & Requena, T. (2003). Development of a fermented goat's milk containing probiotic bacteria. *International Dairy Journal*, *13*, 827–833.

Masco, L., Huys, G., De Brandt, E., Temmerman, R., & Swings, J. (2005). Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. *International Journal of Food Microbiology*, *102*, 221–230.

Meile, L., Rohr, L. M., Geissman, T. A., Herensperger, M., & Teuber, M. (2001). Characterization of the D-xylulose 5-phosphate/D-Fructose 6-phosphate phosphoketolase gene (xfp) from *Bifidobacterium lactis*. *Journal of Bacteriology*, *183*, 2929–2936.

Payne, J. F., Morris, A. E. J., & Beers, P. (1999). Evaluation of selective media for the enumeration of *Bifidobacterium* sp. in milk. *Journal of Applied Microbiology*, *86*, 353–358.

Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M. A., Tanaka, R., et al. (2002). Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. *Applied and Environmental Microbiology*, *68*, 2420–2427.

Rodtong, S., & Tannock, G. W. (1993). Differentiation of *Lactobacillus* strains by ribotyping. *Applied and Environmental Microbiology*, *59*, 3480–3484.

Roy, D. (2001). Media for the isolation and enumeration of bifidobacteria in dairy products. *International Journal of Food Microbiology*, *69*, 167–182.

Roy, D. (2005). Technological aspects related to the use of bifidobacteria in dairy products. *Le Lait*, *85*, 39–56.

Sanguinetti, C. J., Dias-Neto, E., & Simpson, A. J. G. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques*, *17*, 914–921.

Sartory, D. P. (2005). Validation, verification and comparison: Adopting new methods in water microbiology. *Water SA*, *31*, 393–396.

Shah, N. P. (2000). Probiotic bacteria: Selective enumeration and survival in dairy foods. *Journal of Dairy Science*, *83*, 894–907.

Talwalkar, A., & Kailasapathy, K. (2004). Comparison of selective and differential media for the accurate enumeration of strains of *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Lactobacillus casei* complex from commercial yoghurts. *International Dairy Journal*, *14*, 143–149.

Temmerman, R., Scheirlinck, I., Huys, G., & Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, *69*, 220–226.

Van de Casteele, S., Vanheuverzwijn, T., Ruyssen, T., Van Assche, P., Swings, J., & Huys, G. (2006). Evaluation of culture media for selective enumeration of probiotic strains of *lactobacilli* and bifidobacteria in combination with yoghurt or cheese starters. *International Dairy Journal*, *16*, 1470–1476.

Yeung, P. S. M., Sanders, M. E., Kitts, C. L., Cano, R., & Tong, P. S. (2002). Species-specific identification of commercial probiotic strains. *Journal of Dairy Science*, *85*, 1039–1051.