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Evaluation of culture media for selective enumeration of *Bifidobacterium* spp. in combination with different strains of *Streptococcus thermophilus* isolated from commercial yogurt starter cultures

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Streptococcus thermophilus and _Lactobacillus delbrueckii_ subsp. _bulgaricus_ are involved in the manufacture of yogurt, and _Bifidobacterium_ spp. can be supplemented if probiotic properties are expected. In such products, enumeration of each bacterial component is a hard task since they have similar nutritional requirements. For this reason, there is still no official and reliable methodology for this purpose. The objectives of the present study were to evaluate the most appropriate culture medium for the enumeration of four different _Bifidobacterium_ species in the presence of _S. thermophilus_ and _L. delbrueckii_ subsp. _bulgaricus_ isolated from commercial cultures and investigate the influence of different strains of _S. thermophilus_ on the choice of medium. Among 11 isolates of _S. thermophilus_ obtained from these cultures, and which were submitted to repetitive element sequence-based PCR (rep-PCR), nine were found to be distinct strains. For selective enumeration of the _Bifidobacterium_ species, results were influenced by the _S. thermophilus_ strain used, but De Man, Rogosa and Sharpe (MRS) agar containing bile salts showed the best results for all of the _Bifidobacterium_ species.

Key words: *Bifidobacterium* spp., *Streptococcus thermophilus*, culture media, selectivity.

INTRODUCTION

In recent decades, the search for health promotion has increased significantly. The market for functional foods is dominated by products that influence intestinal health, and within these, probiotics represent a large portion of

this market, especially when bacteria of the genera *Bifidobacterium* and *Lactobacillus* are used. Fermented milks containing probiotics, particularly yogurt, are the main products commercialized worldwide that claim to promote health (Siró et al., 2008). Although the number of microorganisms necessary to guarantee a probiotic effect is not definitely established (Raeisi et al., 2013), several studies and regulatory agencies recommend a minimum of 10^6 CFU ml^{-1} viable cells in the final product (Anvisa, 2008; Tripathi and Giri, 2014). Therefore, the determination of the number of probiotic microorganisms in commercial products is necessary.

A wide variety of culture media have been proposed in the literature for the selective enumeration of bifidobacteria in the presence of yogurt bacterial components (Rybka and Kailasapathy, 1996; Vinderola and Reinheimer, 1999; Talwalkar and Kailasapathy, 2004; Casteele et al., 2006; Lima et al., 2009). Some studies have used different commercial starter cultures, but the identification of the strains that comprise these cultures and their effects on the selection of the selective culture medium have not been established.

Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium longum and *Bifidobacterium animalis* are among the *Bifidobacterium* species used for the production of probiotic fermented milks (Ashraf and Shah, 2011). It is noteworthy that the use of the strain *B. animalis subsp lactis* BB 12 presents desirable technological characteristics such as an unchanged appearance, taste and flavour in foods and remaining viable during the product shelf life (Möller and De Vrese, 2004). Another very important aspect is the search for new *Bifidobacterium* strains with probiotic potential that may contribute to the development of new functional products. According to Vinderola et al. (2011), the challenge today is increasingly specific and consists in developing a particular strain in a specific food administered over a period of time that is capable of conferring a specific beneficial effect on health.

Starter cultures that are commercially available are used to provide various technological properties to the fermented products, such as texture, flavor and viscosity. Distinct strains of these microorganisms, especially *Streptococcus thermophilus*, may be present in a single commercial culture or in different cultures from the same manufacturer to promote different features of the products. Giraffa et al. (2001) identified a wide variety of *S. thermophilus* strains in various dairy products. This fact must be considered in the choice of the culture medium because the response of the medium is dependent on the probiotic strain (Vinderola et al., 2011) associated with the medium's ability to inhibit the starter culture. The main objective of the present study was to compare the efficacy of selective culture media recommended in the literature for the differential enumeration of *B. longum* 5^{1A}, *B. breve* 110^{1A}, *B. pseudolongum* 119^{1A} and *B. bifidum* 162^{2A} in the presence of distinct starter culture of yogurt. An additional objective was to evaluate the influence of the different *S. thermophilus* strains which can be found in commercial starter cultures, as determined by rep-PCR, particularly for *Bifidobacterium* counting.

MATERIALS AND METHODS

Microbial cultures

The *B. longum* 5^{1A}, *B. breve* 110^{1A}, *B. pseudolongum* 119^{1A} and *B. bifidum* 162^{2A} species used in this study were isolated and characterized at the Laboratory of Ecology and Physiology of Microorganisms of the Institute of Biological Sciences, Federal University of Minas Gerais (ICB-UFMG). These microorganisms were isolated from the feces of healthy children from the city of Salvador (Bahia, BR), and were identified by Gram stain, respiratory and biochemical tests followed by multiplex PCR, according to Kwon et al. (2005). The bacteria were maintained at -80ºC in De Man, Rogosa and Sharpe broth (MRS, Acumedia, Lansing, USA) supplemented with 0.25 g ml^{-1} sterile glycerol until use. The bifidobacteria cultures were activated in MRS broth at 37°C for 24-48 h, maintaining a minimum headspace in the tube.

The starter cultures used were classified into three groups as shown in Table 1: lyophilized mixed commercial cultures containing the microorganisms *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; lyophilized pure commercial cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*; and frozen pure cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The frozen pure cultures of ST NCDO 1968 (SAT) and LB NCDO 1489 (LAT) were acquired from the Culture Collection and the other were isolated from commercial cultures.The frozen pure cultures of *S. thermophilus* were kept at -80ºC in an ultra-freezer in M17 broth (Difco, USA) supplemented with 5 g l^{-1} lactose, and those of *L. delbrueckii* subsp. *bulgaricus* were kept in MRS broth (Acumedia). Both cultures were supplemented with 0.25 g ml^{-1} sterile glycerol until use, when bacterial strains were thawed and using the broths described above. For this, 1 ml of each culture was transferred to 10 ml of the respective broth and incubated at 40ºC for 24 h. The cultures classified as lyophilized commercial cultures, mixed or pure, of the types DVS (direct vat system) or DVI (direct-to-vat inoculation) were prepared by aseptically diluting the contents of the sachet in 1 L of 120 g l^{-1} reconstituted milk (Molico, Nestlé, Brazil), pretreated at 100ºC/25 min, cooled to 5ºC, and then distributed in sterile test tubes with screw caps and frozen at -18ºC until use. The tubes were thawed and then kept at 40ºC for 30 min.

S. thermophilus strains differentiation

To differentiate the *S. thermophilus* strains, five colonies were mixed culture YoFlex L 812 from the same manufacturer (Christian Hansen). Four other colonies named SC1, SC2, SC4 and SC5 were isolated from the pure culture TA 40 (Danisco/Dupont). The SAT colony was isolated from the pure culture provided by the

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Table 1. Source and bacterial composition of the lyophilized and frozen cultures used in the study.

Cultures	Strain	Source
Lyophilized mixed cultures		
YoFlex L 812 (DVS)	*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*	Christian Hansen, Denmark
Yoflex Harmony 1.0 (DVS)	*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*	Christian Hansen, Denmark
Lyophilized pure cultures		
LB 340 (DVI)	*L. delbrueckii* subsp. *bulgaricus*	DaniscoDuPont, France
TA 40 (DVI)	*S. thermophilus*	DaniscoDuPont, France
Frrozen pure cultures		
ST NCDO 1968	*S. thermophilus*	André Tosello Foundation, Brazil
LB NCDO 1489	*L. delbrueckii* subsp. *bulgaricus*	André Tosello Foundation, Brazil
Isolated from pure culture TA 40	*S. thermophilus*	DaniscoDuPont, France
Isolated from mixed culture YoFlex 702	*S. thermophilus*	Christian Hansen, Denmark
Isolated from mixed culture YoFlex L 812	*S. thermophilus*	Christian Hansen, Denmark

André Tosello Foundation. The cultures associated with each manufacturer are listed in Table 1. Identification of Christian Hansen strains was confirmed by Gram Stain and 16S rRNA gene sequencing using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´) according to Lane (1991).

The genotypic differentiation between *S. thermophilus* strains belonging to the same species was performed using the methodology described by Gevers et al. (2001). Genomic DNA was extracted from pure cultures after 24 hof growth in 10 mL of MRS broth. Before the extraction of genomic DNA using the Wizard SV Genomic kit (Promega), the bacteria were submitted to a pretreatment in which the bacterial isolates were centrifuged, washed with 1 mL of deionized water and resuspended in 1 mL of 5 M LiCl under agitation for 1 h. Then, another centrifugation was performed, the supernatant was discarded, and the pellet was washed with 1 mL of deionized water and resuspended in TES buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 25 mM sucrose) containing lysozyme (10 mg mL^{-1}) and maintained at 37ºC for 1 h.

The isolates were subjected to repetitive element sequence-based PCR (rep-PCR) using the oligonucleotide $(GTG)_5$ as a primer ($(GTG)_5$-PCR). After amplification, the products were resolved on an agarose gel (2%) and visualized on an Easydoc 100 UV transilluminator (BioAgency, São Paulo, Brazil) after staining with ethidium bromide. The DNA profiles obtained by $(GTG)_5$-PCR were analyzed using the BioNumerics V6.5 software (Applied Maths, Kortrijk, Belgium). The genetic similarity values calculated through the Pearson's correlation coefficient were used to generate a similarity dendrogram by the UPGMA method.

After this procedure, the isolates ST3 and ST6, SC4 and SC5, SB1 and SAT were selected to perform the tests in selective culture media for the enumeration of the *Bifidobacterium*. The lyophilized commercial cultures were also included in the test culture medium. Two of *L. delbrueckii* subsp*. bulgaricus* strains, LB 340 (Danisco/Dupont) and LAT (LB NCDO 1489, André Tosello Foundation), were introduced for complementation of the culture medium being tested, since these microorganisms are used in association with *S. thermophilus* in the production of fermented milk.

Selective culture media

Table 2 shows the culture media and dosing of inhibitors used in this study for the selective enumeration of the species *B. longum* 5^{1A}, *B. breve* 110^{1A}, *B. pseudolongum* 119^{1A} and *B. bifidum* 162^{2A}. For the preparation of selective media, MRS agar was used as the basis and was supplemented with the inhibitors described in Table 2. The inhibitors, with the exception of lithium chloride and sodium propionate, were dissolved in distilled water and sterilized by filtration (Millipore filter, type HA, 0.45 µm, Millipore Corporation, Bedford, USA). Lithium chloride and sodium propionate were added to MRS agar and sterilized at 121ºC for 15 min. The modified Maltose medium was prepared using all of the MRS agar ingredients, except that glucose was replaced by equivalent amounts of maltose (Merck Darmstadt, Germany). This sugar was dissolved in distilled water, filter sterilized and added aseptically to the autoclaved medium previously cooled to 50ºC at a concentration of 20 g l^{-1}. MRS agar was used as a reference for counting *L. delbrueckii* subsp. *bulgaricu*s and the M17 agar supplemented with 5 g l^{-1} lactose was used for counting *S. thermophilus*. MRS agar was also used for counting the total lactic acid bacteria (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricu*s) in the mixed cultures (Lima et al*.,* 2009).

Quantitative analysis

For counting *Bifidobacterium*, the activated cultures were serially diluted and plated in the reference medium. For this purpose, 1 ml of each activated culture was added to 9 ml of sterile peptone water (1 mg ml^{-1}), and decimal serial dilutions were performed. The Petri dishes were incubated at 37ºC for 72 h under anaerobic conditions using an anaerobic generator (Anaerobac, Probac of Brazil). The same procedure was followed for determining the count of the bifidobacteria in the selective media.

The bacteria of the starter culture were enumerated in the reference media and also in selective media. To count the viable cells in the reference media, a serial dilution was prepared, and Petri dishes were incubated at 40ºC for 48 h under microaerophilic conditions.

Table 2. Selective media used in this study for viable cell counts of *B.longum* 5[1A], *B. breve* 110[1A], *B. pseudolongum* 119[1A] and *B. bifidum* 162[2A].

Medium	Selective agent in media (g l^{-1})	References
B-MRS	Bile salts (0.5) Cystein (1)	*Vinderola and Reinheimer (2000) *Talwalkar and Kailasapathy (2004)
G-MRS	Gentamicin (0.02)	*Lima et al. (2009)
D-MRS	Dicloxacilin (0.00025) Lithium chloride (1.1) Cystein (0.5)	*Lima et al. (2009)
GB-MRS	Bile salts (0.2) Gentamicin (0.03) Cystein (0.5)	Lima et al. (2009) Vinderola and Reinheimer (1999)
NPNL-MRS	Nalidixic acid (0.75) Paromomycin sulfate (0.01) Neomycin sulfate (0.005) Lithium chloride (0.15)	Vinderola and Reinheimer (1999)
LP-MRS	Lithium chloride (2.0) Sodium propionate (3.0) Cystein (1)	Vinderola and Reinheimer (2000) Talwalkar and Kailasapathy (2004)
M-MRS	Maltose (20)	Rybka and Kailasapathy (1996)

*The concentration of inhibitory agent was adapted from this reference.

For counting in the selective media, those media with a high percent recovery of bifidobacteria were chosen. Thus, the selective media in which there was no significant difference (P>0.05) between the count of bifidobacteria in the given media and the reference medium MRS were evaluated. Subsequently, a serial dilution of the activated cultures was performed, followed by plating in the selected media and incubation at 37ºC for 72 h under anaerobic conditions.

The percent recovery of the microorganisms was determined according to Equation 1:

$$percent\ recovery = \frac{CMS}{CMR}\ X\ 100$$

Where, CMS is the microorganism count, in CFU ml^{-1}, in the selective medium, and CMR is the count in the reference medium.

Statistical analysis

The experiments were performed in triplicate. The results were submitted to analysis of variance (ANOVA) using Excel (version 2013), and the difference between the mean counts (log_{10} CFU ml^{-1}) were obtained by Tukey's test considering P<0.05.

RESULTS

Genotypic discrimination of the *Streptococcus thermophilus* strains

The analysis of the dendrogram generated from the banding pattern obtained by DNA fingerprinting clearly demonstrated the formation of two distinct clusters: one containing the samples ST4, SAT, ST5 and SC4 and another containing the samples ST1, ST6, SC2, SC5, SC1, ST3 and SB1 (Figure 1). The samples in the first group exhibited significant variation in the number and position of the bands and were thus considered to be different strains. In the second cluster, the samples ST1, ST6; ST3 and SB1 were also considered strains that differ from each other. Conversely, the samples SC2, SC5 and SC1 displayed minimal qualitative differences and no quantitative variation in the banding pattern and, therefore, can be considered to represent a single strain. Thus, of the eleven samples initially obtained, nine different *S. thermophilus* strains were detected: ST4, SAT, ST5, SC4, ST1, ST6, ST3, and SB1 and the group SC1, SC2 and SC5.

The samples (SC2, SC1 and SC5) obtained from the same commercial culture TA 40 (Danisco/DuPont) were grouped as similar strains, and the sample SC4, although obtained from the same product, was positioned in another cluster. The samples ST5, ST1, ST6 and ST3 were also isolated from the same culture and considered strains that differ from each other. These results confirm the presence of different strains of *S. thermophilus* that are part of the same commercial culture. The manufacturer describes this product as a mixture of selected strains that are carefully chosen and combined to meet specific requirements.

The samples ST3 and SB1 were isolated from commercial cultures YoFlex 702 and YoFlex L812, respectively, both from the same manufacturer (Christian Hansen). These samples were identified as distinct strains. These data confirm the presence of different strains of *S. thermophilus* in distinct commercial cultures, even though they are from same manufacturer. The SAT sample

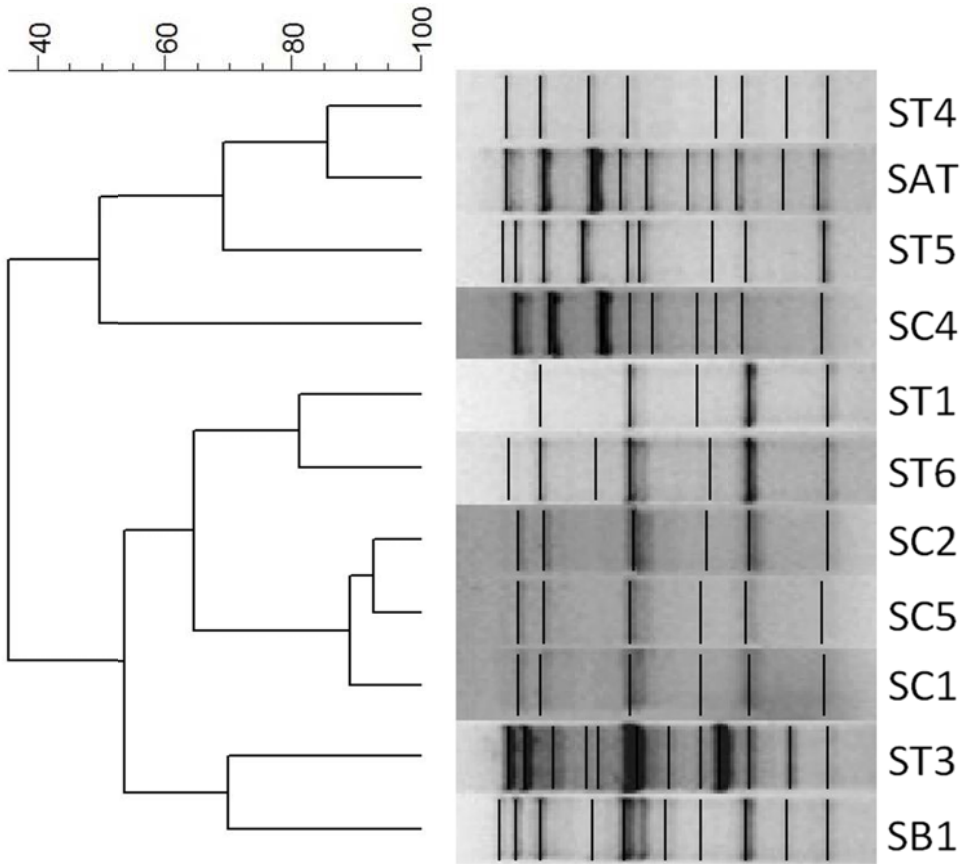

Figure 1. Dendrogram of *S. thermophilus* samples isolated from commercial cultures. Samples ST1, ST3, ST4 and ST5 isolated from the mixed culture YoFlex 702; sample SB1 isolated from the mixed culture YoFlex L812; samples SC1, SC2, SC4 and SC5 isolated from the pure culture TA 40; and sample SAT isolated from the pure culture provided by the André Tosello Foundation

was identified as unique among all the strains studied.

Recovery of bifidobacteria

The count (log_{10} CFU ml^{-1}) and the percent recovery of the *Bifidobacterium* species in the different selective culture media in comparison with the reference MRS medium are presented in Table 3. The bifidobacteria count in the B-MRS, LP-MRS, M-MRS and G-MRS media did not differ significantly ($p>0.05$) from that observed in MRS medium, which was of approximately 8 log_{10} CFU ml^{-1}. For the preparation of B-MRS, the concentration of bile salts was reduced three-fold in relation to the protocol proposed by Vinderola and Reinheimer (2000), since in the present study high concentrations of bile salts (0.15 g l^{-1}) inhibited the growth of the bifidobacteria tested (data not shown). In the GB-MRS, D-MRS, and NPNL-MRS media, there was no growth of the *Bifidobacterium* species, except for a low percent recovery (30%) observed for *B. bifidum* 162^{2A} in the D-MRS medium, indicating reduced resistance of the bifidobacteria studied to inhibitors present in these media.

Recovery of the microorganisms from the starter culture

Differential media, based on the difference in the morphology of bacterial colonies to count the microorganism of interest, are already described in the literature. However, this method is very subjective and the use of selective media is highly recommended (Casteele et al., 2006). Therefore, in this study, the B-MRS, LP-MRS, M-MRS and G-MRS media were evaluated for the inhibition of bacteria from the yogurt starter culture because these media showed the highest percent recovery of bifidobacteria. The counts (log_{10} CFU ml^{-1}) of the species *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, either in pure or mixed starter cultures, in this these different selective media are presented in Table 4. The data show that the number of viable cells in pure or mixed cultures varies depending on the medium used and the strain evaluated. The media that showed the best results for the inhibition of *S. thermophilus* were B-MRS and M-MRS, while for *L. bulgaricus* inhibition, the best results were obtained with the B-MRS, LP-MRS and G-MRS media. For the bacteria of the L 812 mixed

Table 3. Cell count (log_{10} CFU ml^{-1}) and Percent Recovery (%) of *B. longum* 5^{1A}, *B. bifidum* 162^{2A}, *B. breve* 110^{1A}, and *B. pseudolongum* 119^{1A} on the different selective media.

Parameter	Cell count/Percent Recovery of *Bifidobacterium* spp							
	***B. longum* 5^{1A}**		***B. bifidum* 162^{2A}**		***B.breve* 110^{1A}**		***B. pseudolongum* 119^{1A}**	
Culture media	**Cell count**	**Percent Recovery**	**Cell count**	**Percent Recovery**	**Cell count**	**Percent Recovery**	**Cell count**	**Percent Recovery**
MRS	8.69[a]		8.39[a]		8.44[a]		8.52[a]	
B-MRS	8.38[a]	96.43	7.92[a]	94.32	8.38[a]	99.33	8.31[a]	97.57
LP-MRS	8.99[a]	103.41	8.39[a]	100	8.95[a]	106.04	8.54[a]	100.27
M-MRS	8.72[a]	100.31	8.46[a]	100.79	8.92[a]	105.69	8.42[a]	98.83
G-MRS	7.95[a]	91.52	8.41[a]	100.20	7.65[a]	90.68	7.66[a]	89.94
GB-MRS	-	-	-	-	-	-	-	-
D-MRS	-	-	2.50[b]	29.83	-	-	-	-
NPNL-MRS	-	-	-	-	-	-	-	-

B, bile salts and cystein; G, gentamicin; D, dicloxacilin, lithium chloride and cystein; GB, gentamicin, bile salts and cystein; NNLP, lithium chloride, nalidixic acid, neomycin sulfate and paromomycin sulfate; LP, lithium chloride, sodium propionate and cystein; M: maltose.
[a, b] Means in the same column without common letter differ significantly ($p<0.05$)
- No growth detected

culture, there was a decrease in the count of up to 4 $logs_{10}$ in the B-MRS and G-MRS media and for the Harmony culture, whereas a decrease of only 1.4 $logs_{10}$ was observed in the B-MRS medium, both cultures of which were from the manufacturer Christian Hansen.

The counts of the SC4 and SC5 strains of *S. thermophilus* from the same culture (TA 40) were similar, while the counts of the ST3 and ST6 strains of the same culture (YoFlex 702) differed. All of the selective media evaluated inhibited the ST3 sample, but only the B-MRS medium inhibited the ST6 sample. For the strains of the culture YoFlex L 812, the results also differed. In the B-MRS medium, there was only a 1 log_{10} reduction for the sample SB1 compared to the reference medium and up to an approximately 4 log_{10} reduction for the L 812 culture.

In the manufacture of yogurt, a combination of the lactic acid bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricu*s is used for fermentation. Therefore, it is essential to evaluate the percent recovery of both microorganisms in the same culture medium. As shown in Table 4, the B-MRS medium can be used for the selective enumeration of the bifidobacteria studied when using the combination of the pure culture of *S. thermophilus* TA 40, SC4, SC5, ST3 and ST6 with the pure culture of *L. delbrueckii* subsp. *bulgaricu*s LB 340 or also with the mixed culture L 812 because the microorganisms of the starter cultures showed a low recovery in this medium. However, the inhibition of starter bacteria was not complete in B-MRS medium, and the colonies exhibited a “pin point” morphology with a reduction of 4 $logs_{10}$ for TA 40 and L 812.

The G-MRS medium can be used in fermented products using a combination of *S. thermophilus* ST3 with *L. delbrueckii* subsp. *bulgaricu*s LB 340 and/or LAT. For the mixed culture Harmony, the choice of a selective medium was not possible because this starter culture was not satisfactorily inhibited.

DISCUSSION

Genotypic discrimination of the *Streptococcus thermophilus* strains

The analysis of the dendrogram (Figure 1) showed the presence of different strains of *S. thermophilus* that are part of the same commercial culture. The presence of distinct strains of this microorganism was also found in different cultures, even though they are from the same manufacturer. A wide variety of *S. thermophilus* strains were also isolated from samples of artisan yogurts (Erkus et al., 2014) and fermented milk products (Yu et al., 2011).

The presence of various *S. thermophilus* strains in commercial cultures makes these fermented products more attractive because each of them has different technological advantages for the preparation of these foods. Many *S. thermophilus* strains produce exopolysaccharides, which provide texture and enhance the sensory characteristics of the products (Prasanna et al., 2013). Volatile compounds that give flavor to fermented products are also due in large part to the type of strains present in the starter culture used. However, the selective medium is specific to each strain of the microorganisms of the starter culture, which hinders the selection of a medium for the enumeration of the probiotic.

Recovery of bifidobacteria

The *Bifidobacterium* species studied were resistant to inhibitory agents such as bile salts, the antibiotic

Table 4. Cell count (log_{10} CFU ml^{-1}) and percent recovery (%) of *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* on the different selective media.

Strain	Culture	Cell count/Percent Recovery of *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus*									
		Selective media culture									
		M17	MRS	LP-MRS		B-MRS		M-MRS		G-MRS	
		Cell count		Cell count	Percent Recovery	Cell count	Percent Recovery	Cell count	Percent Recovery	Cell count	Percent Recovery
S. thermophilus	TA 40	8.19[a]		7.02[a,b]	86	*3.30[b]	40	-	0	7.60[a]	93
	SC4	7.80[a]		7.32[b]	94	-	0	-	0	6.49[c]	83
	SC5	8.03[a]		7.54[b]	94	-	0	-	0	6.95[c]	87
	ST3	7.46		-	0	-	0	-	0	-	0
	ST6	8.43[a]		6.57[b]	78	-	0	8.81[a]	105	7.61[a,b]	90
	SB1	9.16[a]		7.19[b]	78	7.18[b]	78	9.10[a,b]	99	7.30[a,b]	80
	SAT	8.59[a]		8.46[a]	98	8.53[a]	99	8.94[a]	104	8.66[a]	101
L. delbrueckii subsp. *bulgaricus*	LB 340		6.72[a]	-	0	-	0	6.03[a]	90	-	0
Mixed culture	LAT		9.09[a]	7.29[b]	80	7.80[a,b]	86	7.70[a,b]	85	-	0
	L 812		9.04[a]	8.22[a]	91	4.53[b]	80	7.59[a]	84	4.88[b]	54
	Harmony		7.32[a]	7.39[a]	101	5.85[a]	50	6.91[a]	94	6.14[a]	84

LP, lithium chloride, sodium propionate and cystein; B, bile salts and cystein; M, maltose; G, gentamicin. [a, b] Means in the same row without common letter differ significantly ($p<0.05$). -, No growth detected; * "Pin point" colonies.

gentamicin and combination of lithium chloride and sodium propionate. Similar results reported by Lima et al. (2009) confirm the good growth capacity of bifidobacteria in these media.

In the present study, there was no growth of the *Bifidobacterium* species in the NPNL-MRS media. This is different from the high percent recovery of near 100% reported by Vinderola and Reinheimer (1999) for *B. bifidum* in this medium. The components of the NPNL medium are widely used for the selective enumeration of bifidobacteria and are employed to count these microorganisms in yogurt (Donkor et al., 2006; Ashraf and Shah, 2011; Prasanna et al., 2013), since the concentration is carefully evaluated. Casteele et al. (2006) reported high counts of the *B. lactis* strains BL and B-420 when they used high concentrations of the inhibitors according to the protocol proposed by Dave and Shah (1996). Conversely, some species have shown low recovery in that medium. For this reason, in this study, the NPNL medium was used with reduced concentrations of inhibitors, as proposed by Vinderola and Reinheimer (1999), but there was no improvement in the percent recovery of bifidobacteria. Casteele et al. (2006) also evaluated the recovery of other strains of *B. lactis* (LMG 18314 and LMG 11430) in NPNL medium with reduced concentrations of inhibitors and obtained also unsatisfactory results. Overall, the results of the present study and other studies emphasize the close relationship between different *Bifidobacterium* strains and the ability to resist to inhibitors.

Recovery of the microorganisms from the starter culture

The results of the percent recovery of the bacteria from the starter culture in selective media reinforce the dependence of the medium in relation to the strains of the microorganisms from the culture. The growth of distinct strains (ST3 and ST6) isolated from the same culture (YoFlex 702) exhibited different percent recovery in the same culture medium (Table 4). Additionally, the count of the sample (SB1) isolated from the mixed culture (YoFlex L 812) and the count of its own mixed culture were different in the same medium (bile).

Besides containing distinct strains of microorganisms, the commercial yogurt cultures also have a proportion of *S. thermophilus* to *L. delbrueckii* subsp. *bulgaricus* that can vary between different products. Lower rates of post-acidification are obtained in cultures with higher *S. thermophilus* counts. Cultures that provide lower rates of post-acidification are preferred for *Bifidobacterium* containing products because these probiotics are sensitive to acidity. However, each bacterial strain in a starter culture can present different nutritional requirements, which hinders the selection of a universal medium for the enumeration of probiotic supplemented in a fermented food.

In the present study the B-MRS medium showed the best result among the media evaluated, with low percent recovery for both bacteria of the starter culture, be it pure or mixed. The same finding was reported by Sohrabvandi

et al. (2012) when they evaluated the effectiveness of the B-MRS medium for the enumeration of bifidobacteria in the presence of a yogurt culture. However, here the concentration of bile salts was reduced to maintain the high rate of recovery of the bifidobacteria strains analyzed. In contrast, Lima et al. (2009) found that the concentration of bile salts did not need to be reduced when the strain *B. animalis* Bb12 was used. Thus, for the preparation of B-MRS medium a preliminary study to determine the adequate concentration of bile salts must be performed for each probiotic strain

Several studies indicate that LP-MRS medium is selective for bifidobacteria in the presence of the starter culture. Pinto et al. (2012) used this medium for counting the viable cells of *Bifidobacterium* BB 12 in yogurt, and Castro et al. (2009) did the same in fermented dairy beverages. Using this same medium, other species, such as *B. bifidum* (Krasaekoopt et al., 2006), *B. longum* (Souza et al., 2012) and *B. lactis* (Kailasapathy, 2006), were enumerated in yogurt. However, in this study, LP-MRS medium was able to inhibit the LB 340 and ST3 strains

The MRS medium supplemented with cysteine (0.5 g l^{-1}) and the antibiotic mupirocin (50 g l^{-1}) has been shown to be suitable for selective enumeration of different species of bifidobacteria in the presence of distinct strains of bacilli, lactococci, lactobacilli and streptococci normally found in animal feed (Simpson et al., 2004). However, this antibiotic is expensive compared to other inhibitors.

Conclusion

According to the literature, the choice of a selective medium for the enumeration of *Bifidobacterium* in the presence of a starter culture is dependent on the probiotic species present in the product. However, the present study shows that this choice is also dependent on the strains of the bacteria in the starter culture. Additionally, commercial starter cultures may contain various *S. thermophilus* strains in their formulations, with different nutritional requirement and different sensitivity pattern to inhibitory compound, thus hindering the selection of the selective culture medium. For these reasons, once the medium for the selective enumeration of a certain strain of *Bifidobacterium* in the presence of a particular yogurt culture is defined, this result cannot be extrapolated to other cultures, even if they are from the same manufacturer. It is suggested that probiotic product manufacturers indicate the specific culture medium for the enumeration of the microorganism of interest for each type of product marketed, since changes in starter culture and / or probiotic may alter the defined medium for selective enumeration of the probiotic. In the present study, the B-MRS culture medium showed the best result for the counts of *Bifidobacterium* species in the presence of the different strains of the starter cultures studied, but only after adjusting the concentration of bile salts.

Conflict of interest

The authors did not declare any conflict of interest.

REFERENCES

Anvisa (National Health Surveillance Agency) (2008). Foods with claims or functional properties and health, novel foods / ingredients, bioactive substances and probiotics: list of claims approved functional property. Available at: http://www.anvisa.gov.br/alimentos/comissoes/tecno_lista_alega.htm. Accessed on: May 12, 2014.

Ashraf R, Shah N (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. Int. J. Food Microbiol. 149(3):194-208.

Casteele SV, Vanheuverzwijn T, Ruyssen T, Assche PV, 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. Int. Dairy J. 16(12): 1470–1476.

Castro FP, Cunha TM, Ogliari PJ, Teofilo RF, Ferreira MMC, Prudencio ES (2009). Influence of different content of cheese whey and oligofructose on the properties of fermented lactic beverages: Study using response surface methodology. LWT - Food Sci. Technol. 42(5):993–997.

Dave RI, Shah NP (1996). Evaluation of Media for Selective Enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus* and Bifidobacteria. J. Dairy Sci. 79(9):1529-1536.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. Int. Dairy J. 16(10): 1181–1189.

Erkus O, Okuklu B, Yenidunya AF, Harsa S (2014). High genetic and phenotypic variability of *Streptococcus thermophilus* strains isolated from artisanal Yuruk yoghurts. LWT - Food Sci. Technol. 58: 348-354.

Gevers D, Huys G, Swings J (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol. Lett. 205(1):31-36.

Giraffa G, Paris A, Valcavi L, Gatti M, Neviani E (2001). Genotypic and phenotypic heterogeneity of *Streptococcus thermophilus* strains isolated from dairy products. J. Appl. Microbiol. 91(5): 937-943.

Kailasapathy K (2006). Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT 39(10): 1221–1227.

Krasaekoopt W, Bhandari B, Deeth HC (2006). Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. LWT 39(2):177–183.

Kwon HS, Yang EH, Lee SH, Yeon SW, Kang BH, Kim TV (2005). Rapid identification of potentially probiotic *Bifidobacterium* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol. Lett. 250(1): 55-62.

Lane DJ (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (ed.) Nucleic Acid Techniques in Bacterial Systematics, Wiley, New York, pp 115-175.

Lima KGC, Kruger MF, Behrens J, Destro MT, Landgraf M, Franco BDGM (2009). Evaluation of culture media for enumeration of *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium animalis* in the presence of *Lactobacillus delbrueckii subsp bulgaricus and Streptococcus thermophilus*. LWT –Food Sci. Technol. 42(2): 491-495.

Möller C, De Vrese M (2004). Review: probiotic effects of selected acid bacteria. Milchwissenschaft 59: 597–601.

Pinto SS, Fritzen-Freire CB, Munoz IB, Barreto PLM, Prudencio ES, Amboni RDMC (2012). Effects of the addition of microencapsulated *Bifidobacterium* BB-12 on the properties of frozen yogurt. J. Food Eng. 111(4):563–569.

Prasanna PHP, Grandison AS, Charalampopoulos D (2013). Microbio-

logical, chemical and rheological properties of low fat set yoghurt produced with exopolysaccharide (EPS) producing *Bifidobacterium* strains. Food Res. Int. 51(1):15-22.
Raeisi SN, Ouoba LII, Farahmand N, Sutherland J, Ghoddusi HB (2013). Variation, viability and validity of bifidobacteria in fermented milk products. Food Control 34(2): 691-697.
Rybka S, Kailasapathy K (1996). Media for the enumeration of yoghurt bacteria. Int. Dairy J. 6(8-9):839-850.
Simpson PJ, Fitzgerald GF, Stanton C, Ross RP (2004). The evaluation of a mupirocin-based selective medium for the enumeration of bifidobacteria from probiotic animal feed. J. Microbiol. Methods 57:9-16.
Siró I, Kápolna E, Béata K, Lugasi A (2008). Functional food. Product development, marketing and consumer acceptance – A review. Appetite 51(3):456-467.
Sohrabvandi S, Mortazavian AM, Dolatkhahnejad MR, Monfared AB (2012). Suitability of MRS-bile agar for the selective enumeration of mixed probiotic bacteria in presence of mesophilic lactic acid cultures and yoghurt bacteria. Iran J. Biotechnol. 10:16-21.
Souza TC, Zacarias MF, Silva AM, Binetti A, Reinheimer J, Nicoli JR, Vinderola G (2012). Cell viability and immunostimulating and protective capacities of *Bifidobacterium longum*5^{1A} are differentially affected by technological variables in fermented milks. J. Appl. Microbiol. 112(6):1184-1192.
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. Int. Dairy J. 14(2):143-149.
Tripathi MK, Giri SK (2014). Probiotic functional foods: Survival of probiotics during processing and storage. J. Functional Foods 9:225-241.
Vinderola CG, Reinheimer JA (1999). Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria. Int. Dairy J. 9(8):497-505.
Vinderola CG, Reinheimer JA (2000). Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. Int. Dairy J. 10(4):271-275.
Vinderola CG, Binetti A, Burns P, Reinheimer JA (2011). Cell viability and functionality of probiotic bacteria in dairy products. Frontiers in Microbiol. 2:1-6.
Yu J, Wang WH, Menghe BLG, Jiri MT, Wang HM, Liu WJ, Bao QH, Lu Q, Zhang JC, Wang F, Xu HY, Sun TS, Zhang HP (2011). Diversity of lactic acid bacteria associated with traditional fermented dairy products in mongolia. J. Dairy Sci. 94:3229-3241.