

Research Paper

## Evaluation of culture media for selective enumeration of bifidobacteria and lactic acid bacteria

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### Abstract

The purpose of this study was to test the suitability of Transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) and MRS-clindamycin-ciprofloxacin (MRS-CC) agars, along with several other culture media, for selectively enumerating bifidobacteria and lactic acid bacteria (LAB) species commonly used to make fermented milks. Pure culture suspensions of a total of 13 dairy bacteria strains, belonging to eight species and five genera, were tested for growth capability under various incubation conditions. TOS-MUP agar was successfully used for the selective enumeration of both *Bifidobacterium animalis* subsp. *lactis* BB-12 and *B. breve* M-16 V. MRS-CC agar showed relatively good selectivity for *Lactobacillus acidophilus*, however, it also promoted the growth of *Lb. casei* strains. For this reason, MRS-CC agar can only be used as a selective medium for the enumeration of *Lb. acidophilus* if *Lb. casei* is not present in a product at levels similar to or exceeding those of *Lb. acidophilus*. Unlike bifidobacteria and coccus-shaped LAB, all the lactobacilli strains involved in this work were found to grow well in MRS pH 5.4 agar incubated under anaerobiosis at 37 °C for 72 h. Therefore, this method proved to be particularly suitable for the selective enumeration of *Lactobacillus* spp.

**Key words:** mupirocin, clindamycin, ciprofloxacin, *Bifidobacterium*, *Lactobacillus acidophilus*.

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### Introduction

Nobel laureate Ilya Ilyich Mechnikov proposed more than a century ago that the regular consumption of lactic acid bacteria (LAB) in fermented milks was responsible for improved health and longevity in Bulgarian farmer populations (Anukam and Reid, 2007). Since then, a considerable interest has been shown in the microorganisms of cultured dairy foods because of their capacity to decrease the risk of certain diseases (Zacarchenco and Massaguer-Roig, 2004). During the past three decades, various bifidobacteria and lactobacilli species have received attention as probiotic organisms (Zacarchenco and Massaguer-Roig, 2006). They have been associated with health-promoting effects and, thus, have been incorporated into a wide range of dairy foods worldwide (Ashraf and Shah, 2011; Karimi *et al.*, 2012). It is estimated that at least 500 newly developed

probiotic products have been brought to the market globally over the last 10 years (Ashraf and Shah, 2011).

The numerous health benefits reported for *Bifidobacterium* spp. and *Lactobacillus* spp. include: contribution to a faster recolonization of the intestinal microbiota after administration of antibiotics, treatment and prevention of diarrhoea, alleviation of constipation, possible treatment of inflammatory bowel disease, reduction in lactose intolerance in some individuals, reduction in serum cholesterol level, increased resistance to microbial infections, impact on immune function, and potential role in cancer prevention (Leahy *et al.*, 2005; Möller and De Vrese, 2004; Moriya *et al.*, 2006; Varga, 1999; Zavisic *et al.*, 2012).

The therapeutic effects exerted by probiotic bacteria are dependent on the number of viable microbial cells reaching the human gut (Ghoddusi and Hassan, 2011;

Leahy *et al.*, 2005). Regulatory authorities around the world are looking for assurance that a probiotic product can deliver viable microorganisms at sufficient numbers to the large intestine in order to provide a benefit to the consumer. Concentrations of at least  $10^6$  colony forming units (cfu) per gram should be present at the time of consumption if a health claim is to be made (Ashraf and Shah, 2011; Codex Alimentarius Hungaricus Commission, 2004; Gläser, 1992; Karimi *et al.*, 2012; Sanders and Huis in't Veld, 1999; Shah, 2000; Van de Castele *et al.*, 2006).

Unlike classic yogurt bacteria, probiotic organisms grow slowly in milk and, therefore, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are often added to cultured milks to speed up the fermentation process (Ashraf and Shah, 2011; Tharmaraj and Shah, 2003). For technological or other reasons, lactobacilli and bifidobacteria are sometimes also used in combination with mesophilic lactic/aromatic cultures, such as lactococci (*i.e.*, *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*) and leuconostocs (*e.g.*, *Leuconostoc mesenteroides* subsp. *cremoris*) to make probiotic buttermilk or similar fermented dairy foods (Antunes *et al.*, 2007; Paraschiv *et al.*, 2011; Rodas *et al.*, 2002).

Simple, reliable, and inexpensive methods are needed to ensure that the required minimum numbers of probiotic bacteria — and those of yogurt starter organisms and mesophilic lactic/aromatic cultures as well — are present in commercial fermented milks (Ashraf and Shah, 2011; Saccaro *et al.*, 2012). However, the presence of multiple and phylogenetically closely related species in these products makes the differential or selective enumeration of probiotic organisms and starter bacteria difficult because of the similarity in growth requirements and overlapping biochemical profiles of the species. Despite the fact that culture-independent molecular tools for the quantification of probiotic organisms in commercial products have recently been developed (García-Cayuela *et al.*, 2009; Matijašić *et al.*, 2010; Reimann *et al.*, 2010; Tabasco *et al.*, 2007), food manufacturers still tend to rely on conventional plating techniques for enumeration purposes (Elahi *et al.*, 2008; Fachin *et al.*, 2008; Miranda *et al.*, 2011; Tharmaraj and Shah, 2003; Van de Castele *et al.*, 2006).

A wide range of culture media have been described for selective and differential enumeration of probiotic bacteria in mixed populations, and these have been the subject of comprehensive reviews (Ashraf and Shah, 2011; Charteris *et al.*, 1997; Karimi *et al.*, 2012; Roy, 2001; Shah, 2000; Tabasco *et al.*, 2007; Van de Castele *et al.*, 2006). It is worth pointing out that differential enumeration of LAB and bifidobacteria species is mostly based on visual observation of colonies. However, colony morphology is a relatively unstable phenotypic trait. For this reason, selective media should be preferred for enumeration purposes

(Saccaro *et al.*, 2012; Talwalkar and Kailasapathy, 2004; Van de Castele *et al.*, 2006).

In a study published in 2004, Australian authors demonstrated the unsuitability of commercially available culture media to reliably enumerate various types of probiotic bacteria in commercial fermented milks (Talwalkar and Kailasapathy, 2004). The International Organization for Standardization (ISO) and the International Dairy Federation (IDF) have jointly published international standards wherein MRS-clindamycin-ciprofloxacin (MRS-CC) agar and Transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) agar are recommended for the selective enumeration of presumptive *Lb. acidophilus* and bifidobacteria, respectively, in milk products including fermented and non-fermented milks, milk powders, and infant formulae (ISO and IDF, 2006, 2010).

The objective of this research was to test the suitability of TOS-MUP and MRS-CC agars, along with several other culture media, for selectively enumerating bifidobacteria and thermophilic and mesophilic LAB species commonly used in the dairy industry to manufacture fermented dairy foods. To our knowledge, this is the first single study evaluating the selective properties of both TOS-MUP and MRS-CC agars.

## Materials and Methods

### Bacteria cultures and propagation

*Streptococcus thermophilus* TH-4, *Lactobacillus delbrueckii* subsp. *bulgaricus* YC-X11 (isolated from FD-DVS YC-X11 Yo-Flex<sup>®</sup> yogurt culture), *Lb. delbrueckii* subsp. *bulgaricus* CH-2, *Lb. acidophilus* LA-5, and *Bifidobacterium animalis* subsp. *lactis* BB-12 were kindly supplied by Chr. Hansen (Hørsholm, Denmark). *Bifidobacterium breve* M-16 V was obtained from Morinaga Milk Industry (Tokyo, Japan). *Streptococcus thermophilus* DSM 20479 was received from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Lactobacillus acidophilus* NCAIM B.02085 and *Lb. casei* NCAIM B.01137 were provided by the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). *Lactococcus lactis* subsp. *lactis* ATCC 19435 and *Leuconostoc mesenteroides* subsp. *dextranicum* ATCC 19255 were purchased from the American Type Culture Collection (Manassas, Virginia, USA). *Lactobacillus casei* HDRI-R and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* VK-256 were obtained from the culture collection of the Hungarian Dairy Research Institute (Mosonmagyaróvár, Hungary). All these cultures were tested for purity by Gram stain and observation of cell morphology under a KF 2 ICS transmitted light microscope (Carl Zeiss Microscopy, Jena, Germany).

All strains were maintained at  $-30\text{ }^{\circ}\text{C}$  using the Microbank<sup>™</sup> low-temperature microbial preservation sys-

tem (Pro-Lab Diagnostics, Neston, United Kingdom). Organisms were retrieved from cryovials containing a special cryopreservative solution and porous beads, and were subcultured twice in enrichment media, *i.e.*, Casein-peptone Soymeal-peptone (CASO) broth or MRS broth. CASO broth, a universal culture medium free from inhibitors, contained 17.0 g of pancreatic digest of casein, 3.0 g of papaic digest of soybean meal, 5.0 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, 2.5 g of glucose, 1.0 g of skimmed milk powder, and 1000 mL of distilled water. The final pH of CASO broth was  $7.3 \pm 0.2$  at 25 °C. Commercial MRS broth (Merck, Darmstadt, Germany) was rehydrated in distilled water according to manufacturer's instructions, and hydrochloric acid (1 M HCl) was added to adjust the pH of the medium to  $6.2 \pm 0.2$  at 25 °C. Both enrichment broths were sterilized by autoclaving at 121 °C for 15 min before use.

*Streptococcus thermophilus* TH-4 and *S. thermophilus* DSM 20479 were propagated in CASO broth at 37 °C for 48 h under aerobic conditions; *Lc. lactis* subsp. *lactis* ATCC 19435, *Lc. lactis* subsp. *lactis* biovar. *diacetyllactis* VK-256, and *Ln. mesenteroides* subsp. *dextranicum* ATCC 19255 were grown in CASO broth at 30 °C for 72 h aerobically; whereas the rest of the strains tested in this study were incubated in MRS broth at 37 °C for 72 h in anaerobiosis. Anaerobic conditions were generated using anaerobic culture jars (2.5 L) and AnaeroGen AN 25 sachets (Oxoid, Basingstoke, United Kingdom). The pure culture suspensions thus prepared were used to test the performance of various culture media.

### Culture media

**Bacteriological peptone diluent:** Bacteriological peptone diluent (0.1%) was prepared by dissolving 1 g of peptone (Oxoid) in 1000 mL of distilled water. The pH value was adjusted to  $7.0 \pm 0.2$  at 25 °C, followed by autoclaving 9 mL aliquots at 121 °C for 15 min.

**CASO agar:** The Casein-peptone soymeal-peptone agar with powdered milk contained (per liter) 15.0 g of pancreatic digest of casein, 5.0 g of papaic digest of soybean meal, 5.0 g of sodium chloride, and 15.0 g of agar, which was supplemented with 1.0 g of skimmed milk powder, 1.0 g of glucose, 4.0 g of yeast extract, and 5.0 mL of Tween® 80. The ingredients were suspended in 1000 mL of distilled water, then mixed thoroughly and heated to boiling point under frequent agitation. The medium was dispensed into bottles and sterilized by autoclaving at 121 °C for 15 min. The final pH was  $7.3 \pm 0.2$  at 25 °C.

**M17 agar:** In accordance with manufacturer's instructions, 55 g of commercial M17 agar (Merck), which contained 5.0 g of lactose monohydrate, was suspended in 1000 mL of distilled water, then mixed thoroughly and brought gently to the boil under frequent agitation. The medium was dispensed into bottles and sterilized by auto-

claving at 121 °C for 15 min. Final pH was  $6.8 \pm 0.2$  at 25 °C.

**MRS pH 5.4 agar and MRS pH 6.2 agar:** Commercial MRS agar (Merck) was rehydrated in distilled water according to manufacturer's instructions, and 1 M HCl was used to adjust the pH of the medium to 5.4 or 6.2. The agar media were sterilized by autoclaving at 121 °C for 15 min.

**MRS-CC agar:** Commercial MRS agar (Merck) was rehydrated in distilled water according to manufacturer's instructions. It was distributed in portions of 200 mL into bottles of 250 mL capacity and sterilized in a Varioklav 500E autoclave (H+P Labortechnik, Oberschleißheim, Germany) set at 121 °C for 15 min. The final pH was  $6.2 \pm 0.2$  at 25 °C. Simultaneously, 2.0 mg of clindamycin hydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA) and 20.0 mg of ciprofloxacin hydrochloride (Sigma-Aldrich) were dissolved separately in two 10.0-mL aliquots of distilled water. Both solutions were then sterilized by filtering through a 0.22- $\mu$ m membrane filter (Millipore, Bedford, Massachusetts, USA). Immediately before pouring, 0.1 mL of clindamycin stock solution and 1.0 mL of ciprofloxacin stock solution were added to 200 mL of MRS agar cooled to between 44 °C and 47 °C. Thus, the complete MRS-clindamycin-ciprofloxacin (MRS-CC) agar had final clindamycin and ciprofloxacin concentrations of 0.1 mg/L and 10.0 mg/L, respectively.

**TOS-MUP agar:** The basic medium, TOS-propionate agar, was obtained from Merck. It contained (per liter) 10.0 g of peptone from casein, 1.0 g of yeast extract, 3.0 g of potassium dihydrogen phosphate, 4.8 g of dipotassium hydrogen phosphate, 3.0 g of ammonium sulphate, 0.2 g of magnesium sulphate heptahydrate, 0.5 g of L-cysteine monochloride, 15.0 g of sodium propionate, 10.0 g of galactooligosaccharide (TOS), and 15.0 g of agar. The ingredients were suspended in 950 mL of distilled water while being heated carefully with frequent agitation until completely dissolved. The basic medium was dispensed in 190-mL aliquots into 250-mL bottles and sterilized by autoclaving at 115 °C for 15 min. The final pH was  $6.3 \pm 0.2$  at 25 °C. Simultaneously, 50 mg of lithium-mupirocin (MUP) selective supplement (Merck) was dissolved in 50 mL of distilled water, and the solution was sterilized by filtration through a membrane with pore diameter of 0.22  $\mu$ m (Millipore). Immediately before pouring, at  $48 \text{ °C} \pm 1 \text{ °C}$ , 10 mL of MUP supplement solution was added to each 190 mL of TOS-propionate agar by using a syringe equipped with a sterile filter unit of 0.22- $\mu$ m pore size (Millipore). The complete Transgalactosylated oligosaccharides-mupirocin lithium salt (TOS-MUP) agar thus had a final MUP concentration of 50 mg/L.

### Enumeration of bacteria

One milliliter of each pure culture suspension was tenfold serially diluted ( $10^6$  to  $10^9$ ) in 0.1% sterile bacterio-

logical peptone diluent. Enumeration was carried out as specified in Table 1 using the pour plate technique. Anaerobic culture jars and AnaeroGen AN 25 sachets (Oxoid) were used for creating anaerobic conditions. Plates containing 25 to 250 colonies were enumerated and recorded as cfu/mL of culture suspension. The number of replicate samples was two, and the experimental program was repeated twice.

### Statistical analysis

The results were logarithmically transformed to normalize the distributions and were then subjected to ANOVA using the general linear model procedure of STATISTICA data analysis software system, version 9.0 (StatSoft, Tulsa, Oklahoma, USA). Significant differences among the  $\log_{10}$  cfu/mL means were determined by using Duncan's multiple comparison test at  $p < 0.05$  (StatSoft).

### Results and Discussion

The results of our medium evaluation study are shown in Table 2. Milk powder-supplemented CASO agar, which was used as a reference medium, supported the growth of all the 13 dairy bacteria strains tested.

*Bifidobacterium animalis* subsp. *lactis* BB-12 and *B. breve* M-16 V only grew well in CASO, TOS-MUP, and MRS pH 6.2 agars incubated at 37 °C for 72 h under anaerobic conditions. It is worth mentioning that TOS-MUP agar was superior ( $p < 0.05$ ) to both CASO and MRS pH 6.2 agars — and all the other media screened in this research, for that matter — in terms of selectivity and recovery of bifidobacteria.

Both strains of *Lb. acidophilus* (i.e., LA-5 and NCAIM B.02085) showed good growth in the majority of culture media, except in M17 agar incubated at 45 °C for 24 h under aerobic conditions and in TOS-MUP agar. Simi-

lar observations were made for *Lb. casei* NCAIM B.01137 and HDRI-R, which were present in high numbers in six of the eight media (methods) tested; however, they failed to form detectable numbers of colonies (i.e.,  $\geq 10^6$  cfu/mL) in TOS-MUP and MRS pH 5.4 agars incubated anaerobically at 37 °C for 72 h and at 45 °C for 48 h, respectively.

Yogurt starter lactobacilli, *Lb. delbrueckii* subsp. *bulgaricus* CH-2 and YC-X11, were only found to grow in CASO and MRS agars incubated under anaerobiosis at 37 °C. MRS pH 6.2 agar yielded significantly higher ( $p < 0.05$ ) *Lb. delbrueckii* subsp. *bulgaricus* counts than did MRS pH 5.4 agar, but both culture media proved to be suitable for the enumeration of *Lb. delbrueckii* subsp. *bulgaricus*. Surprisingly enough, however, this organism did not grow well in anaerobically incubated MRS pH 5.4 agar at 45 °C. Most probably, extension of the incubation period from 48 h to 72 h would have allowed sufficient growth of the strains tested, because in a previous trial by Dave and Shah (1996) *Lb. delbrueckii* subsp. *bulgaricus* could be selectively enumerated in MRS pH 5.2 agar incubated at 45 °C for  $\geq 72$  h.

Besides the reference medium, only M17 agar was capable of promoting the growth of *S. thermophilus* TH-4 and DSM 20479. It must be noted, however, that CASO and M17 agars incubated aerobically at 37 °C for 48 h yielded approximately 1 log cycle higher ( $p < 0.05$ ) *S. thermophilus* counts than aerobically incubated M17 agar at 45 °C for 24 h.

In general, recovery rates of the mesophilic LAB strains tested were rather low in the culture media involved in this study. This must have been due to the fact that, aside from CASO agar, media were incubated at temperatures higher than 30 °C. The optimum growth temperature range for mesophilic LAB is approximately 25 °C to 31 °C, with most species growing generally faster at 25 °C than at 31 °C

**Table 1** - Incubation conditions applied in the medium evaluation study.

Culture medium*	Incubation		Atmospheric conditions
	temperature (°C)	time (h)	
CASO agar	37	72	Anaerobic
CASO agar	37	48	Aerobic
CASO agar	30	72	Aerobic
TOS-MUP agar	37	72	Anaerobic
MRS-CC agar	37	72	Anaerobic
MRS pH 6.2 agar	37	72	Anaerobic
MRS pH 5.4 agar	37	72	Anaerobic
MRS pH 5.4 agar	45	48	Anaerobic
M17 agar	37	48	Aerobic
M17 agar	45	24	Aerobic

\*For composition and/or preparation of culture media, see subsection "Culture media".



**Table 2** - Evaluation of various culture media and incubation conditions for enumerating pure cultures of bifidobacteria and lactic acid bacteria strains\*.

#	Bacterial species and strain	Culture medium†, incubation temperature, incubation time, and atmospheric conditions									
		CASO agar‡	TOS-MUP agar, 37 °C, 72 h, anaerobic	MRS-CC agar, 37 °C, 72 h, anaerobic	MRS pH 6.2 agar, 37 °C, 72 h, anaerobic	MRS pH 5.4 agar, 37 °C, 72 h, anaerobic	MRS pH 5.4 agar, 45 °C, 48 h, anaerobic	M17 agar, 37 °C, 48 h, aerobic	M17 agar, 45 °C, 24 h, aerobic		
1	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BB-12	8.33 ± 0.18 <sup>b</sup>	9.30 ± 0.12 <sup>a</sup>	< 6.00	8.07 ± 0.14 <sup>b</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00
2	<i>Bifidobacterium breve</i> M-16 V	8.05 ± 0.11 <sup>c</sup>	8.76 ± 0.02 <sup>a</sup>	< 6.00	8.61 ± 0.04 <sup>b</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00
3	<i>Lactobacillus acidophilus</i> LA-5	8.20 ± 0.05 <sup>b</sup>	< 6.00	8.30 ± 0.10 <sup>ab</sup>	8.34 ± 0.02 <sup>a</sup>	8.33 ± 0.02 <sup>a</sup>	8.42 ± 0.11 <sup>a</sup>	7.84 ± 0.07 <sup>c</sup>	8.17 ± 0.09 <sup>b</sup>	7.84 ± 0.07 <sup>c</sup>	< 6.00
4	<i>Lactobacillus acidophilus</i> NCAIM B.02085	8.22 ± 0.06 <sup>b</sup>	< 6.00	8.54 ± 0.17 <sup>a</sup>	8.54 ± 0.04 <sup>a</sup>	8.54 ± 0.03 <sup>a</sup>	8.51 ± 0.14 <sup>a</sup>	8.17 ± 0.09 <sup>b</sup>	8.17 ± 0.09 <sup>b</sup>	8.17 ± 0.09 <sup>b</sup>	< 6.00
5	<i>Lactobacillus casei</i> NCAIM B.01137	7.70 ± 0.19 <sup>a</sup>	< 6.00	7.30 ± 0.24 <sup>a</sup>	7.58 ± 0.08 <sup>a</sup>	7.56 ± 0.08 <sup>a</sup>	< 6.00	7.76 ± 0.28 <sup>a</sup>	7.76 ± 0.28 <sup>a</sup>	7.76 ± 0.28 <sup>a</sup>	7.45 ± 0.17 <sup>a</sup>
6	<i>Lactobacillus casei</i> HDRI-R	7.86 ± 0.15 <sup>a</sup>	< 6.00	7.88 ± 0.18 <sup>a</sup>	7.80 ± 0.17 <sup>a</sup>	7.64 ± 0.12 <sup>a</sup>	< 6.00	7.20 ± 0.15 <sup>b</sup>	7.20 ± 0.15 <sup>b</sup>	7.20 ± 0.15 <sup>b</sup>	7.30 ± 0.11 <sup>b</sup>
7	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> YC-X11	6.30 ± 0.18 <sup>b</sup>	< 6.00	< 6.00	6.78 ± 0.05 <sup>a</sup>	6.48 ± 0.09 <sup>b</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00
8	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> CH-2	6.70 ± 0.18 <sup>b</sup>	< 6.00	< 6.00	7.11 ± 0.09 <sup>a</sup>	6.30 ± 0.33 <sup>b</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00
9	<i>Streptococcus thermophilus</i> TH-4	7.43 ± 0.12 <sup>a</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	7.57 ± 0.13 <sup>a</sup>	7.57 ± 0.13 <sup>a</sup>	7.57 ± 0.13 <sup>a</sup>	6.78 ± 0.18 <sup>b</sup>
10	<i>Streptococcus thermophilus</i> DSM 20479	8.05 ± 0.22 <sup>a</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	7.89 ± 0.17 <sup>a</sup>	7.89 ± 0.17 <sup>a</sup>	7.89 ± 0.17 <sup>a</sup>	6.90 ± 0.18 <sup>b</sup>
11	<i>Lactococcus lactis</i> subsp. <i>lactis</i> ATCC 19435	7.98 ± 0.17 <sup>a</sup>	< 6.00	< 6.00	7.81 ± 0.07 <sup>a</sup>	< 6.00	< 6.00	7.71 ± 0.19 <sup>a</sup>	7.71 ± 0.19 <sup>a</sup>	7.71 ± 0.19 <sup>a</sup>	< 6.00
12	<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar. <i>diaceplactis</i> VK-256	7.18 ± 0.12 <sup>a</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	6.30 ± 0.27 <sup>b</sup>	6.30 ± 0.27 <sup>b</sup>	6.30 ± 0.27 <sup>b</sup>	< 6.00
13	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> ATCC 19255	7.80 ± 0.18 <sup>a</sup>	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00	< 6.00

\*Values are log<sub>10</sub> cfu/mL means ± SD based on four observations (two samples, two replicates).

<sup>ab</sup>Means within a row without a common superscript differ (p < 0.05).

†For composition and/or preparation of culture media, see subsection "Culture media".

‡Incubated at 37 °C for 72 h anaerobically (strain #1 to #8), at 37 °C for 48 h aerobically (strain #9 and #10), or at 30 °C for 72 h under aerobic conditions (strain #11 to #13).

(Sohrabvandi *et al.*, 2012). Not surprisingly, lactococci grew well in M17 agar at 37 °C. In addition, *Lc. lactis* subsp. *lactis* ATCC 19435 also formed comparable numbers of colonies in MRS pH 6.2 agar at 37 °C even under anaerobic conditions. This is in agreement with the findings of Antunes *et al.* (2007) who reported that the CHN-22 mesophilic aromatic culture reached viable cell counts as high as 10<sup>8</sup> cfu/mL in MRS-based selective media incubated at 37 °C. *Leuconostoc mesenteroides* subsp. *dextranicum* ATCC 19255 could, however, only be enumerated using the reference medium.

It is clearly visible in Table 2 that TOS-MUP agar was successfully used for the selective enumeration of bifidobacteria. Both *B. animalis* subsp. *lactis* BB-12 and *B. breve* M-16 V showed significantly higher ( $p < 0.05$ ) viable cell counts in this medium than in CASO agar, and none of the 11 LAB strains tested were capable of forming detectable numbers of colonies (*i.e.*,  $\geq 10^6$  cfu/mL) in TOS-MUP agar. Recent studies also indicated that mupirocin agars such as Wilkins-Chalgren agar containing 100 mg/L of mupirocin (Rada and Koc, 2000), MRS agar fortified with cysteine hydrochloride (0.05%, w/v) and mupirocin at 50 mg/L (Simpson *et al.*, 2004), and Transoligosaccharide propionate agar supplemented with 50 mg/L of mupirocin (Ghoddusi and Hassan, 2011; ISO and IDF, 2010; Kolakowski *et al.*, 2010) are the most suitable media for isolation and selective enumeration of bifidobacteria in fermented dairy products containing other LAB. *Bifidobacterium* spp. were reported to exhibit high mupirocin resistance, with all the 40 bifidobacterial strains, belonging to a total of 30 species, tested by Serafini *et al.* (2011) giving MIC values in excess of 1,000 mg/L.

MRS-CC agar showed relatively good selectivity for *Lb. acidophilus*; however, it also supported the growth of *Lb. casei* strains. For this reason, MRS-CC agar can only be used as a selective medium for the enumeration of *Lb. acidophilus* if *Lb. casei* is not present in a product at levels comparable to or exceeding those of *Lb. acidophilus*. Similar observations were recently made by Saccaro *et al.* (2012) regarding *Lb. acidophilus* and *Lb. casei* in MRS-clindamycin agar containing the antibiotic at a concentration of 0.5 mg/L. These findings are somewhat surprising because MRS-clindamycin agar is considered to be an optimum medium for the selective enumeration of *Lb. acidophilus* grown in co-culture with yogurt starter organisms and other commercial probiotic strains (Van de Castele *et al.*, 2006). It is well known, however, that *Lb. acidophilus* is rather difficult to enumerate selectively in mixed populations because the majority of the media supporting the growth of *Lb. acidophilus* also promote *Lb. rhamnosus* and *Lb. casei* (Lima *et al.*, 2009; Tharmaraj and Shah, 2003).

Incubated at 37 °C, the nonselective MRS media provided optimum growth conditions for many of the strains. Unlike bifidobacteria and coccus-shaped LAB, all the

lactobacilli strains tested in this study were found to grow well in MRS pH 5.4 agar incubated under anaerobiosis at 37 °C for 72 h. Therefore, this method appears particularly suitable for the selective enumeration of *Lactobacillus* spp. As shown in Table 2, MRS pH 5.4 agar incubated anaerobically at 45 °C for 48 h proved to be selective for *Lb. acidophilus*. This is in agreement with a previous work performed by Tharmaraj and Shah (2003) who reported that *Lb. acidophilus* could be enumerated using MRS agar at 43 °C for 72 h under anaerobic incubation. Similarly, in another experiment aimed at comparing the growth capability of probiotic and non-probiotic cultures in a wide range of culture media, MRS pH 5.4 agar presented the best performance in determining the viable counts of *Lb. acidophilus* LA-5 (Lima *et al.*, 2009). By contrast, Dave and Shah (1996) found MRS pH 5.2 agar to be suitable for selective enumeration of *Lb. delbrueckii* subsp. *bulgaricus* when the plates were incubated at 45 °C for at least 72 h.

Under aerobic incubation at 37 °C for 48 h, M17 agar lacked selectivity, promoting the growth of the majority of LAB strains screened in this investigation. An increase of 8 °C in incubation temperature with a simultaneous decrease in incubation time, from 48 h to 24 h, resulted in an improvement in the selectivity of this medium. Besides *S. thermophilus*, only *Lb. casei* strains were observed to grow in M17 agar incubated at 45 °C for 24 h under aerobiosis. These findings are basically in accordance with those of Tabasco *et al.* (2007) who successfully used M17 agar, incubated in aerobiosis at 45 °C for 24 h, for the selective enumeration of *S. thermophilus*. Extension of the incubation period to 48 h, however, allowed the appearance of pinpoint colonies of *Lb. acidophilus* (Tabasco *et al.*, 2007).

It is strongly emphasized that this work was carried out using pure culture suspensions of starter and probiotic bacteria. When real food samples are examined, culture medium selection should be carefully considered on a case to case basis. The choice of methodology for selective enumeration of LAB and bifidobacteria is supposed to be a function of the product matrix, the target species or strains, and the composition of the bacterial background flora in the specific food product tested (Van de Castele *et al.*, 2006).

## Conclusions

TOS-MUP agar was found to be capable of selectively enumerating *Bifidobacterium* spp., and can therefore be used to determine the viable counts of bifidobacteria in food products containing mixed populations of this organism and various LAB species, including *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Lb. casei*, *Lc. lactis*, and *Ln. mesenteroides* subsp. *dextranicum*. *Lactobacillus acidophilus* could be selectively enumerated in MRS pH 5.4 agar incubated anaerobically at 45 °C for 48 h. The recently developed MRS-CC agar was also successfully employed for the same purpose unless *Lb. casei* was present at concentrations similar to or

exceeding those of *Lb. acidophilus*. MRS pH 5.4 agar incubated at 37 °C for 72 h under anaerobiosis was selective for *Lactobacillus* spp. The viable cell counts of classic yogurt bacteria, *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, could be selectively determined by using M17 agar (37 °C, 48 h, aerobiosis) and MRS agars (37 °C, 72 h, anaerobiosis), respectively. For selective enumeration of starter organisms and probiotic bacteria in ABT-type fermented milks, use of the following culture media is recommended under the incubation conditions described in this study: MRS-CC agar or MRS pH 5.4 agar for *Lb. acidophilus*, TOS-MUP agar for bifidobacteria, and M17 agar (45 °C, 24 h, aerobiosis) for *S. thermophilus*.

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