

Full Length Research Paper

# Evaluation of different selective media for enumeration of probiotic micro-organisms in combination with yogurt starter cultures in fermented milk

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The aim of this study is to assess selective plating methodologies for the enumeration and identification of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium animalis* ssp. *lactis* in fermented milks. Seven agar media (MRS with added sorbitol, clindamycin or vancomycin, acidified MRS, RCA with added aniline blue and dicloxacilin, M17 and ST) were evaluated. The results showed that RCA dicloxacilin agar is suitable for the selective enumeration of *B. animalis* ssp. *lactis* in fermented milk. Either MRS (acidified) or M17 agar could be used for enumeration of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, respectively. MRS media containing antibiotics were effective for the enumeration of the probiotic organisms (*L. rhamnosus* and *L. acidophilus*) inoculated in fermented milks.

**Key words:** Selective media, probiotic, starter cultures, fermented milk.

## INTRODUCTION

A number of health benefits have been claimed for many products containing one or more groups of probiotic organisms (Moriya et al., 2006; Saccaro et al., 2009). Acidophilus-bifidobacteria products (Mortazavian et al., 2006) or yogurts containing *Bifidobacterium bifidum* and/or *Lactobacillus acidophilus* are widely available in the world market (Hassan and Amjad, 2010; Lim et al., 1995; Nwamaka and Chike, 2010). Currently, there is an increasing commercial interest to add probiotic bacteria to fermented dairy products due to the recent discoveries in several aspects of bioscience, which support the hypothesis that, beyond nutrition, the diet may modulate various functions in the body (Oliveira et al., 2009; Saccaro et al., 2009).

Due to the fact that the yogurt starter cultures (*Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) grow fast in milk during the fermentation stage (ca. 6-8 h), whilst the probiotic micro-organisms

tend to grow slowly (ca. 18-24 h and up to 5 days depending on the used microbiota) and, as a consequence, the normal commercial practice is to blend the yogurt bacteria with the probiotic cultures in order to achieve a shorter production time. It is a well-established fact that the yogurt bacteria do not survive the gastrointestinal conditions or colonize the human gut (Shah, 2000) and, for this reason, the probiotic micro-organisms are added to the yogurt starter cultures to achieve some 'functional' health benefits to humans when they consume fermented milk products.

A variety of interactions can occur between the lactic acid bacteria (LAB) during the manufacture of dairy products (Vasiljevic and Shah, 2008). Microbial interactions, either beneficial (protoco-operation) or unfavourable (antagonism) among lactic acid bacteria may generate undesirable changes in the composition of the bacterial microbiota during the manufacture and cold storage of fermented dairy products (Bellengier et al., 1997). Therefore, for optimising the technological performance of probiotic cultures during development of 'functional' foods, possible interactions amongst probiotic cultures and LAB must be assessed (Vasiljevic and

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Shah., 2008). Champagne et al. (2005) suggested that assessment of interactive behaviour in terms of their effect on sensory properties, safety and stability in the product during processing and storage. In order to provide certain health benefits to human, the count of probiotic bacteria in the fermented milks should be  $\geq 10^6$  colony forming units (cfu)/g at the end of the shelf-life of the product (Sanders and 't Veld, 1999). It seems reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested.

An important parameter in monitoring viable organisms in assessing product quality is the ability to count probiotic bacteria selectively. In order to ensure that a minimal number of probiotic bacteria are present in the end-product, rapid and reliable methods for routine enumeration are urgently required. Furthermore, such methods are also essential to monitor possible physiological or biochemical changes in the probiotic bacterial population during the storage of commercial products (Vinderola and Reinheimer, 2000; Vinderola et al., 2002). The viability of probiotic bacteria in yogurt depends of many factors, such as the strains used, interaction between species present, culture conditions, production of hydrogen peroxide due to bacterial metabolism, final acidity of the product, and the concentrations of lactic and acetic acids. However, the main factors for loss of viability of probiotic organisms have been attributed to the decrease in the pH of the medium, and accumulation of organic acids as a result of bacterial growth during the fermentation of the milk (Lim et al., 1995; Sanders et al., 1999).

In practice, the differential enumeration of lactic acid bacteria is often difficult to achieve due to the presence of multiple (closely related) species, and the unstable phenotypic trait. Isolation media with truly selective properties are preferred for selective enumeration on a routine basis. Several studies reported selective media for enumeration of probiotic micro-organisms in mixed cultures (Beehrens, 1990; Jordano et al., 1992; Dave and Shah, 1996; Lankaputhra and Shah, 1996; Charteris et al., 1997; IDF, 1999; Lourens-Hattingh and Viljoen, 2001; Talwalkar and Kailasapathy, 2003; Champagne et al., 2005; Antunes et al., 2007; Kailasapathy et al., 2008).

Based on these findings, the aim of this study was to assess selective plating methodologies for enumeration of probiotic strains of mixed cultures of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *B. animalis* ssp. *lactis* in yogurt and fermented milks, and during the storage of the product for 21 days at  $< 10^\circ\text{C}$ .

## MATERIALS AND METHODS

### Starter cultures

Pure and freeze-dried starter cultures (*S. thermophilus* TAO40, *L.*

*delbrueckii* ssp. *bulgaricus* LB340, *L. acidophilus* LAC, *Bifidobacterium animalis* subsp. *lactis* BLO4 and *L. rhamnosus* LBA) were provided by Danisco Brasil Ltda, Cotia, Brazil. The cultures were kept in the cold store until they were required.

### Media preparation

Different agar media were used and diluents for serial dilutions were prepared as follows:

1. Bacteriological peptone diluents (0.1 g/L) were prepared by dissolving 1 g (Oxoid, Basingstoke, UK) in 1 L of distilled water, and sterilized at  $121^\circ\text{C}$  for 15 min.
2. MRS<sub>pH 5.4</sub> agar (MRS)-Commercial MRS medium (OXOID, Basingstoke, UK) was rehydrated in distilled water according to manufacturer's instructions, and hydrochloric acid (HCl) was used to adjust the pH of the medium to 5.4 (6). The agar medium (that is, including all the agar media in the present study - see subsequent sections) was sterilized at  $121^\circ\text{C}$  for 15 min.
3. MRS<sub>pH 6.2</sub> agar plus sorbitol (MRS S)-D-Sorbitol membrane-filtered sterile solution (10 g/100 ml) was prepared, and was then added to 90 ml of sterile and melted agar just before pouring the agar medium at  $40^\circ\text{C}$ .
4. MRS<sub>pH 6.2</sub> agar plus clindamycin (MRS C)-HCl was used to adjust the pH of the MRS agar to 6.2. Clindamycin hydrochloride (Sigma, St. Louis, USA) stock solution was prepared by dissolving 2.5 mg in 50 ml distilled water followed by membrane filter sterilization (Millipore, São Paulo, SP, Brazil), and 1ml of this antibiotic solution was added to 100 ml of melted and sterile MRS agar just before pouring the plates at  $40^\circ\text{C}$ .
5. MRS<sub>pH 6.2</sub> agar plus vancomycin (MRS V) - Vancomycin solution (5 ml of 100 mg vancomycin hydrochloride - Sigma, St. Louis, USA) was used to prepare the stock solution (that is, dissolving 100 mg in 5 ml of distilled water), which was filter sterilized (Millipore); 50  $\mu\text{L}$  of the stock solution was added to 100 ml of melted and sterile MRS agar just before pouring the plates (at  $40^\circ\text{C}$ ).
6. RCA agar plus aniline blue and dicloxacilin (RCA + AB & D) - Reinforced clostridia agar (RCA - Oxoid, Basingstoke, UK) was prepared according to manufacturer's instructions, and the pH of medium was adjusted with NaOH solution to 7.1 after the addition of 0.03 g/L aniline blue (Sigma). The medium was sterilized at  $121^\circ\text{C}$  for 15 min. The dicloxacilin sodium monohydrate stock solution was prepared by dissolving 100 mg of dicloxacilin in 50 ml of distilled water followed by filter sterilization (Millipore); 250  $\mu\text{L}$  solution was added to 250 ml of melted and sterile RCA agar just before pouring the plates at  $40^\circ\text{C}$ .
7. M17<sub>pH6.9</sub> agar- Commercial M17 agar (Oxoid, Basingstoke, UK) was prepared according to manufacturer's instructions. The rehydrated medium was sterilized in an autoclave at  $121^\circ\text{C}$  for 15 min, and 12.5ml the sterilized lactose solution (10 g/100 ml) was added to 250 ml melted and sterile M17 agar just before pouring the plates at  $40^\circ\text{C}$ .
8. ST agar (ST) - The ingredients of *S. thermophilus* (ST) agar (10 g of tryptone, 1 g of sucrose, 5 g of yeast extract, and 2 g  $\text{K}_2\text{HP0}_4$ ) were dissolved in 1 L of distilled water. The pH was adjusted to 6.8, and 6 ml of 0.5 g/100 ml of bromocresol purple (Sigma) and 12 g of agar were added to the rehydrated ingredients. The medium was sterilised at  $121^\circ\text{C}$  for 15 min.

### Production of yogurt and fermented milks

Pure spray-dried strains were weighed in sufficient amounts to obtain initial counts  $10^8 \log_{10}$  colony forming units (cfu)/g. A pre-culture was prepared by dissolving individually each culture in 25mL sterilized skim milk (10 g/100 ml total solids) that has been tempered at  $42^\circ\text{C}$  for 15 min. Afterwards, the rehydrated *S.*

**Table 1.** Counts (log<sub>10</sub> cfu/g) of activated yogurt starters and probiotic cultures on different selective media.

Bacterial cultures	Media (agar)						
	MRS	MRS S	MRS C	MRS V	RCA	M17	ST
<i>S. thermophilus</i> (A)	ng	ng	ng	ng	ng	8.56	8.28
<i>L. delbrueckii bulgaricus</i> (A)	7.59	7.93	8.19	ng	ng	7.26	7.26
<i>L. acidophilus</i> (An)	8.26	8.46	8.58	ng	ng	8.46	ng
<i>L. rhamnosus</i> (An)	8.62	8.68	9.31	8.64	ng	7.18	ng
<i>B. animalis</i> (An)	ng	ng	ng	ng	8.08	ng	ng

MRS - de Man, Rogosa & Sharpe; S - sorbitol; C - clindamycin; V - vancomycin; RCA - reinforced clostridium agar with aniline blue and dicloxacilin; (A) - aerobic; (An) - anaerobic. ng: no growth on any plates, and the counts are mean of four readings in each trial, that is, N=4.

*thermophilus* and *L. delbrueckii* ssp. *bulgaricus* starter cultures were added to the milk, and were used for the production of yogurt (i.e. control), and fermented milks were produced as described by (Saccaro et al., 2009) in co-culture as follows:- (a) rehydrated yogurt starter cultures were mixed with each rehydrated single strains of *L. acidophilus*, *L. rhamnosus* or *B. animalis* ssp. *lactis*, (b) rehydrated yogurt starter cultures were mixed with two rehydrated single strains of *L. acidophilus* and *L. rhamnosus*, *L. acidophilus* and *B. animalis* ssp. *lactis* or *L. rhamnosus* and *B. animalis* ssp. *lactis*, and (c) rehydrated yogurt starter cultures were mixed with all the probiotic micro-organisms. The yogurt and fermented milk products were stored at 4°C for 21 days, and all the cultures were enumerated in different selective agar media. Although the experimental was not replicated in order to minimise the effect changes in the milk composition during the lactation period, the total number of trials in this modelled experiment was eight.

#### Enumeration of micro-organisms

The counts of the yogurt starter cultures and probiotic micro-organisms were enumerated as follows:

1. Re-activated pure culture test - The activated cultures (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *B. animalis* ssp. *lactis* and *Lactobacillus rhamnosus*) were enumerated on M17, ST, MRS, MRS S, MRS C, RCA and MRS V, respectively, to evaluate the appropriate selective media for each strain (Talwalkar et al., 2003; Champagne et al., 2005; Fachin et al., 2008; Kailasapathy et al., 2008). The experiment was replicated twice.

2. Yogurt/fermented milks test -The cell counts of the yogurt starter cultures and probiotics bacteria prepared with mixed cultures were enumerated after 1, 7, 14, and 21 days storage at < 10°C. Samples (1 mL) were added to 9 ml of sterile peptone diluents (0.1g/L); appropriate dilutions were made. Enumeration was carried out using pour plate technique. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were incubated aerobically at 37°C for 48 h. The probiotic cultures (*L. acidophilus*, *B. animalis* ssp. *lactis* and *L. rhamnosus*) were enumerated after anaerobic incubation at 37°C for 72 h. Anaerobic conditions were created using AnaeroGen (Oxoid, Basingstoke, UK). Plates containing 20 to 200 colonies were enumerated, and the counts were expressed as log<sub>10</sub>cfu/g of the product. The selectivity of the growth conditions was confirmed by microscopic examination.

#### Statistical analysis

Analysis of variance for multiple comparisons (ANOVA) using

Statistica 6.0, Statsoft (Tulsa, USA), was performed in order to confirm statistical significance of differences among samples (P<0.05). Mean values were compared using the Tukey test at P<0.05.

## RESULTS AND DISCUSSION

The counts of pure freeze-dried single strains *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *B. animalis* ssp. *lactis* and *L. rhamnosus* reactivated in milk using different agar media are shown in the Table 1. It is evident that the media used demonstrated selectivity for the bacterial strains used in the present study. For example, cells of *S. thermophilus* were only evident in M17 and ST agar media, whilst *L. delbrueckii* ssp. *bulgaricus* did not grow in MRS V and RCA media. In addition, *L. rhamnosus* and *B. animalis* ssp. *lactis* grew only in MRS V and RCA agar media, respectively, and can be used as selective media when grown in co-culture with the yogurt starter cultures because the latter organisms showed no growth in these media. However, most of the probiotic organisms showed growth in the majority of the studied media (Table 1).

Enumeration of probiotic micro-organisms used in co-culture with the yogurt starter cultures in certain agar media exhibited a property of selectivity, which produced a single colony-type in fermented milks. In yogurt, the starter organisms grew well in certain agar media, but no growth was observed in other agar media (Table 1). In stored fermented milk products, cell counts of each of the yogurt starter cultures and probiotic strains varied significantly (P<0.05) when enumerated in different agar media (Table 2). Thus, the enumeration pattern using different agar media, and selectivity of these media to count these organisms in fermented milks could be summarised as follows:

#### Media for enumeration *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*

A lack of selectivity of the yogurt starter cultures on M17 and MRS 5.4 media towards the probiotic strains was

**Table 2.** Counts ( $\log_{10}$ cfu/g) of yogurt starter and probiotic cultures in fermented milk products.

Bacterial cultures	Media <sup>1</sup>		Storage time (days)			
	Agar	pH	1	7	14	21
<i>S. thermophilus</i> (A) <sup>1</sup>	M17	6.9	10.48	8.50	6.50	6.48
<i>L. delbrueckii bulgaricus</i> (A) <sup>1</sup> (A) <sup>1</sup> (A(A)	MRS	5.4	7.95	6.15	5.68	
<i>L. acidophilus</i> (An) <sup>1</sup>	MRS C	6.2	7.15 <sup>2</sup>	6.50 <sup>2</sup>	5.91 <sup>2</sup>	
<i>L. rhamnosus</i> (An)	MRS V	6.2	7.71	6.32	6.57	6.48
<i>B. animalis</i> ssp. <i>lactis</i> (An)	RCA	7.1	8.39	7.04	7.21	6.48

<sup>1</sup>For further details refer to Table 1,<sup>2</sup>Two types of colonies were evident, but easily distinguishable. *L. acidophilus* presented small and brown colonies while *L. rhamnosus* showed large and yellow colonies. Counts are mean of eight readings, that is, N=8.



**Figure 1.** The morphology of *L. acidophilus* (narrow arrows, brown colonies) and *L. rhamnosus* (wide arrows, white colonies) in fermented milks stored at 4°C. MRS<sub>pH 6.2</sub> agar plus clindamycin (MRS C) was the media used for enumeration of *L. acidophilus*.

(Table 2). However, M17 agar appeared to be selective in enumeration *S. thermophilus* in yogurt, showing a good recovery during the storage, and there was no growth evident of lactobacilli cells. The MRS<sub>pH 5.4</sub> agar was suitable for counting *L. delbrueckii* ssp. *bulgaricus* selectively, but this culture did not show good viability during the storage period (Table 2).

#### Media for enumeration *L. acidophilus*

Two types of colonies were observed when enumerating organisms in stored fermented milks using MRS clindamycin agar media, and these were *L. acidophilus* and *L. rhamnosus* (Figure 1). This observation was surprising because MRS clindamycin agar medium is

reported to be most optimal media for the enumeration of *L. acidophilus* grown in co-culture with the yogurt starter cultures and other commercial probiotic strains (Castele et al., 2005). Based on the appearance of the microbial colony, it was possible to perform a differential enumeration of *L. acidophilus* and *L. rhamnosus* cultures. According to (Thamaraj and Shah, 2003), *L. acidophilus* was found to be the most difficult probiotic to be enumerated selectively, since most of the media that support the growth of *L. acidophilus* also provide the growth of *L. rhamnosus* and *Lactobacillus casei*. However, in the present study, *L. rhamnosus* formed well developed colonies (1.5 mm in diameter) while *L. acidophilus* formed smaller colonies (0.1 to 1.0 mm) (Figure 1). Furthermore, the addition of clindamycin was the main factor that allowed optimal enumeration and differentiation of *L. acidophilus* in fermented milks during the storage of the product for 21 days. Appreciable cell counts of *L. acidophilus* were present in fermented milks up to 14 days storage, but none could be recovered at 21 days (Table 2). It could be argued, however, that the survival of such strain in fermented milk is limited to 14 days to achieve counts  $>10^6 \log_{10}$  cfu/g, and further studies are required to confirm such observation.

#### Media for enumeration *L. rhamnosus*

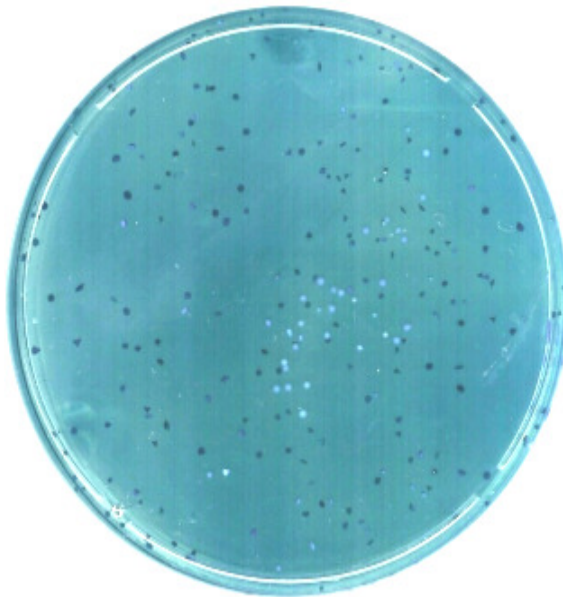
The MRS vancomycin agar (pH 6.2) could be used for selective enumeration of *L. rhamnosus* when the plates were incubated anaerobically at 37°C for 72 h. These conditions did not allow the recovery of the yogurt starter cultures (Figure 2 and Table 1). (Thamaraj and Shah, 2003) reported that the lower incubation temperatures ( $\leq 37^\circ\text{C}$ ) formed well developed smooth white discs-like colonies that were 2 mm or more in diameter in MRS vancomycin agar at 37°C under anaerobic incubation (Figure 2).

#### Media for enumeration *B. animalis* ssp. *Lactis*

RCA dicloxacilin (pH 7.1) agar gave an excellent



**Figure 2.** The morphology of *L. rhamnosus* (wide arrows, yellow colonies) in fermented milks stored at 4°C. MRS<sub>pH 6.2</sub> agar plus vancomycin (MRS V) was the media used for enumeration of *L. rhamnosus*.



**Figure 3.** The morphology of *B. animalis ssp. lactis* (wide arrows, white and blue colonies) in fermented milks stored at 4°C. RCA agar plus aniline blue and dicloxacilin (RCA + AB & D) was the media used for enumeration of *B. animalis ssp. Lactis*.

differentiation and recovery of *B. animalis ssp. lactis* in comparison to the yogurt starter cultures and other probiotic organisms (Figure 3 and Table 1). Dave and Shah (1996) reported that some bifidobacteria and

*L. delbrueckii ssp. bulgaricus* grew on RCA agar at pH 5.3; however, the colonies formed by *L. delbrueckii ssp. bulgaricus* could be easily differentiated from those of bifidobacteria. Adjusting the pH of the agar medium to 7.1 and the addition of dicloxacilin were efficient strategies to allow conclusive identification and selective enumeration of *B. animalis ssp. lactis* in fermented milks.

Bacterial stress could lead to the inability of some cells to develop colonies in agar media or even develop colonies that have different morphological characteristics. Together with poor selectivity, this can prove a major difficulty in the suitability of the various selective and differential media to accurately enumerate each type of probiotic bacteria in the product (Shah and Jelen, 1990).

The counts observed during 21 days of probiotic and yogurt starter cultures in fermented milks, *S. thermophilus*, *B. animalis ssp. lactis* and *L. rhamnosus* were presented in appreciable counts in the product as mixed cultures to allow the manifestation of probiotic effects. However, the counts of *L. acidophilus* and *L. delbrueckii ssp. bulgaricus* declined during the storage period, and no counts were observed at the dilution tested after 21 days (Table 2).

According to (Shah, 2000), the decrease in the counts was highest for *L. bulgaricus* and the numbers declined to  $<10^5 \log_{10}$  cfu/g after 14 to 20 days of storage in mixed culture with *S. thermophilus*, *L. acidophilus* and *Bifidobacterium sp.* in fermented milks. The multiplication of bifidobacteria can be stimulated by the proteolytic activity of *L. delbrueckii ssp. bulgaricus* resulted in availability of free amino acids, which have been reported to be essential as a growth factors for these organisms. Counts of *B. animalis ssp. Lactis* dropped to  $<10^7 \log_{10}$  cfu/g at day 21 in mixed culture in yogurt (Table 2). The same author also reported that the pH level in the product was the most crucial factor for the survival of *L. acidophilus*. If the pH in fermented milks dropped to below 4.4 it resulted in a 3 to 4  $\log_{10}$  cycle decrease in the counts of *L. acidophilus*. Generally, yogurt bacteria grow faster than probiotic bacteria during fermentation, and produce acids, which could reduce the viability of some probiotic bacteria.

Currently, procedures for enumerating probiotic micro-organisms in fermented milk products rely solely on plate counts. The International Dairy Federation - IDF (1999) emphasizes that standard media need to be developed for enumeration probiotic bacteria in fermented milks. Developing standard methodologies for enumeration probiotic bacteria, which are industrially viable, would greatly assist dairy manufacturers and researchers in evaluating the exact status of probiotic micro-organisms in commercial products.

## Conclusions

Seven agar media were evaluated for their suitability to recover selectively and to enumerate *S. thermophilus*, *L.*

*delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *B. animalis* ssp. *lactis* in fermented milks. RCA dicloxacilin (pH 7.1) agar was appropriate for the selective enumeration of *B. animalis* ssp. *lactis* in fermented milk. Either MRS agar (pH 5.2) or M17 agar could be used for the enumeration of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, respectively. The addition of antibiotics to the base MRS agar medium (pH 6.2) provided an effective enumeration of *L. rhamnosus* and *L. acidophilus* inoculated in fermented milks.

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