Selective enumeration of dairy based strains of probiotic and lactic acid bacteria

[1*]Ashraf, R. and [2]Smith, S.C.

[1]*Advanced Food Systems Research Unit, College of Health and Biomedicine, Victoria University, Werribee Campus, P.O. Box 14428, Melbourne, Vic 8001, Australia*
[2]*School of Exercise and Nutrition Sciences, Faculty of Health, Gut Health SRC Molecular and Medical Research, Deakin University, Burwood, Victoria 3125, Australia*

Abstract

Reliable methods for selective enumeration of probiotic and lactic acid bacteria (LAB) are required for improving the functional food quality of probiotics. Various methods were evaluated for selective enumeration of seventeen LAB and probiotic strains. Tested sugars failed to select any species however, improved recovery of total LAB count. The strains were viable and physiologically active within a range of oxygen tension levels, temperature and acidic conditions. Prior methods showed varied results such as De Man Rogosa Sharpe containing bile (MRSB), MRS containing nalidixic acid, paromomycin, neomycin sulphate and lithium chloride (MRS-NPNL), M17 and *L. casei* (LC) agar failed to select *Lactobacillus acidophilus, Bifidobacterium*, starter LAB and *L. casei* strains respectively. However, LC agar appears appropriate for *L. paracasei* and MRSB for yoghurt starter bacteria in the absence of *L. reuteri* and *L. rhamnosus*. The study suggests selective potential of culture media largely depends on target species.

Introduction

Probiotic products have opened up new horizons for functional foods and because of their well-documented health-benefits they are gaining increasing popularity in dairy-based food products globally. In the past decade, the global market has experienced an estimated increase of more than 500 probiotic products, which includes *Lactobacillus acidophilus* and *Bifidobacterium* spp. as predominant probiotic bacteria. The currently accepted definition of probiotics is 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014). The suggested therapeutic level of $\geq 10^6$ cfu/g probiotic bacteria in a product has often been cited (Vasiljevic and Shah, 2008; Raeisi *et al.*, 2013). However, low viability of probiotic organisms in these products has often been found (Klein *et al.*, 1998; Shah, 2000; Temmerman *et al.*, 2003). The viability and survival of probiotic bacteria are the important parameters for assessing the product quality, which necessitates having customary methods for selective enumeration of these probiotic organisms. The major constraint in this regard, is to identify and selectively enumerate these bacteria from within a mixture of multiple and closely related species sharing similar cultural characteristics. The contemporary use of culture-independent methods and as well as poly-phasic approaches to identify and enumerate these bacteria has been an alternative strategy (Dumonceaux *et al.*, 2006; Fu *et al.*, 2006; Lahtinen *et al.*, 2006; Masco *et al.*, 2007; Tsai *et al.*, 2008; García-Cayuela *et al.*, 2009; Colombo *et al.*, 2014) but does not necessarily limit the value of culture-dependant techniques to selectively enumerate probiotic and LAB from a mixture of closely related species. Several media for enumeration of probiotic bacteria have previously been proposed (Roy *et al.*, 1997; Shah, 2000; Bonaparte *et al.*, 2001; Roy, 2001; Tabasco *et al.*, 2007; Lima *et al.*, 2009). However, these media and culture conditions have never been tested with the large array of probiotic bacteria being considered for functional foods and may not always be suitable or applicable to other probiotic strains because there is extensive variability between species and even strains on their responses to plating methods (Champagne *et al.*, 2011; Lahtinen *et al.*, 2011). Therefore, a more thorough review of standardised plating media for varied probiotic species and strains needs to be undertaken before continuing studies with probiotics are accomplished and this is the basis of our study.

In our previous studies seventeen lactic acid bacteria (LAB) and probiotic strains were tested for

*Corresponding author.
Email: *Rabia.Ashraf@live.vu.edu.au*
Tel: (+61-3)-9919-8059; Fax: (+61-3)-9919-8284

their immuno-modulatory activities and functions (Ashraf *et al*., 2014ab). The ability to cultivate these bacteria, especially probiotic strains, is paramount to our understanding of their physiological functionality. Moreover, the knowledge of possible interactions amongst probiotic strains and LAB is essential from the perspective of selection and preparation of probiotic cultures to improve functional food quality. Therefore it seems reasonable to determine the viability of these bacteria through specific and reliable methodology. A number of methods proposed for selective enumeration of *Lactobacillus casei, Streptococcus thermophilus, L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus, Bifidobacterium* spp. and *Lactococcus lactis* in yoghurt and cheese have been extensively reviewed earlier (Ashraf and Shah, 2011) which established several uncertainties in relation to the reliability of these methods for enumeration (Onggo and Fleet, 1993; Dave and Shah, 1996; Champagne *et al*., 1997; Camaschella *et al*., 1998; Ravula and Shah, 1998; Nebra and Blanch, 1999; Vinderola and Reinheimer, 1999; Roy, 2001; Tharmaraj and Shah, 2003; Talwalkar and Kailasapathy, 2004; Darukaradhya *et al*., 2006; Van de Casteele *et al*., 2006; Oberg *et al*., 2011). The methods either relied on colonial differences to identify and enumerate the bacteria in products or were applicable for only certain strains of the same species. In addition, selective enumeration methods are not available to specifically quantify all probiotic strains or newly added strains. Also, some probiotic organisms such as *L. reuteri, L. plantarum, L. rhamnosus* and *L. casei* have not been studied extensively for selective methods for quantitation. Based on these findings, this study was aimed at evaluating suitability of various existing media for the selective enumeration of different probiotic and LAB strains commonly used in yoghurt and cheese preparation and assessing plating methods for selective enumeration of probiotic and LAB strains including *L. delbrueckii* subsp. *bulgaricus, S. thermophilus, Lc. lactis, L. casei, Bifidobacterium* spp., *L. acidophilus, L. rhamnosus* and *L. paracasei* from mixed cultures that could form the basis for probiotic foods. The selective factors influencing the viability of seventeen LAB and probiotic strains were evaluated in the present study in order to optimize plating methodology.

Materials and Methods

Bacterial strains

Seventeen LAB and probiotic strains previously described (Ashraf *et al*., 2014a) including

Lactobacillus paracasei 292, *L. salivarius* 5248, *L. reuteri, Lactococcus lactis, L. rhamnosus* G5435, *L. acidophilus* 2401, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *Streptococcus thermophilus* 1342, *L. casei* 290, *Bifidobacterium breve* BB99, *B. animalis* subsp. *lactis* BB12, *B. longum* 1941, *Lc. lactis* R704, *L. plantarum* 276, *L. rhamnosus* 5434 and *S. thermophilus* M5 were used in the current study. The parent stock cultures were kept at -80°C in phosphate buffered saline (PBS; Oxoid, Melbourne Australia) containing 40% glycerol whereas lyophilized cultures were retained at -20°C freezer. Prior to experiment, the cultures were activated in de Man Rogosa and Sharpe (MRS) broth (Oxoid) at 37°C for 18 h and samples were removed for gram stain to check for purity and bacterial morphology. Bacteria were further grown on MRS agar (1.5% w/v agar) as reference medium to observe the colonial characteristics.

Inoculum and media preparation

The cultures were activated successively three times in sterile 12% (w/v) reconstituted skim milk (RSM) supplemented with 2% (w/v) glucose and 1.2% (w/v) yeast extract using 1% (v/v for frozen cultures and w/v for freeze-dried cultures) inoculum at 37°C for 18h. MRS agar was used as a reference medium and as a control for enumeration of LAB and probiotic strains. For preparing mixtures, freshly prepared (18h activated) commercially available starter and probiotic (yoghurt and cheese) cultures of *S. thermophilus* M5 (T) and *L. delbrueckii* subsp. *bulgaricus* 11842 (D), *B. lactis* BB12 (B), *L. acidophilus* 2401 (A), *L. rhamnosus* 5434 (R), *L. casei* 290 (C), *Lc. lactis* subsp. *lactis* R704 (L) and *L. paracasei* 292 (P) were equally mixed (1:1) in different combinations (ABT, ABY, ABRY, ABC, LAP and LAR). The initial numbers of T, D, B, A, R, C, L and P were 9.6, 9.3, 8.8, 9.5, 9.8, 9.3 and 9.1 $\log_{10}$ cfu/ml respectively.

The pH modified Reinforced clostridial agar (RCA 6.1 and 6.8) and MRS (5.2, 5.8 and 6.1), MRS supplemented with sorbitol and Basal agar (BA) added with 2% w/v sugars (galactose, sorbitol, fructose, raffinose, sucrose or mannitol) were used in the study for testing the viability pattern of seventeen LAB and probiotic strains and for evaluating the applicability of these media for enumeration purpose. Various existing media, proposed for the selective enumeration of different LAB and probiotic organisms were evaluated in the study. In reference to this approach, MRS 5.2 was used to target *L. delbrueckii* subsp. *bulgaricus* (Dave and Shah, 1996; Van de Casteele *et al*., 2006), M17 was used to target

S. thermophilus and *Lc. lactis* (IDF, 1981; Ravula and Shah, 1998; Mc Brearty *et al.*, 2001; Ong and Shah, 2009), LC and MRS-bile (MRSB) were tested to allow selective growth of *L. casei* (Ravula and Shah, 1998; Vinderola and Reinheimer, 1999; Bergamini *et al.*, 2005; Lima *et al.*, 2009) and MRS-NPNL was evaluated to support *Bifidobacterium* spp. (Dave and Shah, 1996; Roy, 2001; Tharmaraj and Shah, 2003; Darukaradhya *et al.*, 2006; Moriya *et al.*, 2006; Ong and Shah, 2009). For selective enumeration of *L. acidophilus*, MRSB, LC, MRS-sorbitol and BA-sorbitol were tested (Dave and Shah, 1996; Vinderola and Reinheimer, 1999; Tharmaraj and Shah, 2003; Bergamini *et al.*, 2005; Lima *et al.*, 2009; Ong and Shah, 2009) and for *L. rhamnosus* and *L. paracasei*, LC and MRS 5.2 agar were evaluated (Ravula and Shah, 1998; Van de Casteele *et al.*, 2006). These media were prepared as follows:-

Rehydrated MRS broth was prepared according to the manufacturer instructions. The pH of the broth was adjusted to 5.2, 5.8 and 6.1 using 1.0 M HCl to obtain the pH-modified agar (MRS 5.2, MRS 5.8 and MRS 6.1). RCA (Oxoid) was prepared according to the manufacturer instructions and pH was adjusted to 6.1 and 6.8 using 1.0 M HCl to prepare pH modified RCA (RCA 6.1 and 6.8). Basal agar (BA; composition: tryptone 10.0 g (Oxoid), Lablemco powder 10.0 g (Oxoid), yeast extract 5.0 g (Oxoid), Tween 80 1.0 g (Sigma-Aldrich Pty Ltd. NSW Australia), dipotassium hydrogen orthophosphate 2.6 g (K_2HPO_4; Unilab, Ajax chemicals, Sydney, Australia), sodium acetate trihydrate 5.0 g ($CH_3COONa. 3H_2O$; Sigma-Aldrich), ammonium citrate tri-basic 2.0 g (Sigma-Aldrich), magnesium sulphate heptahydrate 0.2 g ($MgSO_4.7H_2O$; Sigma-Aldrich), manganese sulphate tetrahydrate 0.05 g ($MnSO_4.4H_2O$; BDH AnalaR, Australia), bacteriological agar 15 g (Oxoid) and milli-Q water 1000 ml)) as a minimal nutrient agar was also prepared and autoclaved at 121°C for 15 min. Filter sterilized solutions of galactose, sorbitol, fructose, raffinose, sucrose, or mannitol (Sigma-Aldrich) were added to basal agar to the final concentration of 2% w/v. Similarly, MRS-sorbitol agar with 2% w/v D-sorbitol was tested comparatively. Inoculated agar plates in duplicates were incubated aerobically and anaerobically at 37°C for 72 h. For selective enumeration of *L. delbrueckii* subsp. *bulgaricus* 11842, MRS 5.2 plates were incubated anaerobically at 45°C for 72 h.

MRSB agar was prepared by adding 2% w/v filter-sterilized pure bile salts (Sigma-Aldrich) solution to sterilized MRS agar. MRS-NPNL agar was prepared by adding filter-sterilized stock solutions of NPNL ingredients (neomycin sulphate 100 mg/L; paromomycin 200 mg/L; nalidixic acid 15 mg/L; LiCl 3 g/L; Sigma-Aldrich) to autoclaved MRS base just before pouring the media. Nalidixic acid was solubilized by preparing a basic stock solution using 0.5 N sodium hydroxide solution. Filter-sterilized L-cysteine-HCl (0.05% w/v) was added to lower the oxidation-reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria. Inoculated plates in duplicates were incubated anaerobically at 37°C for 72 h.

L. casei (LC) agar (composition: bacteriological peptone 10.0 g (Oxoid); yeast extract 1.0 g (Oxoid); Lab Lemco 4.0 g (Oxoid); potassium phosphate monobasic 2.0 g (KH_2PO_4; Sigma-Aldrich); sodium acetate trihydrate 3.0 g (CH_3COONa; Sigma-Aldrich); ammonium citrate tri-basic 1.0 g (Sigma-Aldrich); magnesium sulphate heptahydrate 0.2 g ($MgSO_4.7H_2O$; Sigma-Aldrich); manganese sulphate tetrahydrate 0.05 g ($MnSO_4.4H_2O$; BDH AnalaR, Australia); acid casein hydrolysate 1.0 g; tween 80 1.0 g (Sigma-Aldrich), bromocresol green 6 ml (Sigma-Aldrich); bacteriological agar 15 g (Oxoid); milli-Q water 1000 ml; pH 5.1 ± 0.2) was prepared using the method described by Ravula and Shah (1998) and sterilised at 121°C for 15 min. Filter sterilized solutions of ribose (Sigma-Aldrich) was added to LC agar to the final concentration of 1% w/v. Incubations were carried out anaerobically at 25°C for 72 h.

M17 (Oxoid) agar was prepared according to manufacturer's instruction and sterilized by autoclaving at 121°C for 15 min. Incubations were made under aerobic conditions at 30°C or 37°C for 24 h according to set of experiments. Bacteriological peptone solution was used as a diluent and it was prepared by dissolving 0.15% (w/v) bacteriological peptone (Oxoid) in milli-Q water. The pH of diluent was adjusted to 7.0 ± 0.2, and it was dispensed in McCartney bottles in 9 ml aliquots followed by autoclaving at 121°C for 15 min. Bacteriological agar (Oxoid) was used at 1.5% w/v and all media were sterilized by autoclaving at 121°C for 15 min.

Enumeration of bacteria

Enumeration of bacterial cells was carried out by preparing 10-fold serial dilutions of freshly prepared culture (18 h activated) in sterile bacteriological peptone-water diluents using the pour plate technique as methods described earlier (Ashraf *et al.*, 2014a). Particular dilutions (at least 4) were plated in duplicate with respective media, plates were set to solidify and incubations were carried out according to individual method. After appropriate incubation time, plates containing 25 to 250 colonies were enumerated and

recorded as colony forming units (cfu) per ml of culture sample. These are described as viable count (Log$_{10}$ cfu/ml ± SD) and survival percentage in Table 1-3 and Figure 1. LAB mixtures (ABT, ABY, ABRY, ABC, LAP and LAR) were also serially diluted and plated in various selective media using pour plate technique followed by incubations and viable counts (log$_{10}$ cfu/ml) were obtained (Table 4).

Statistical analysis

All experiments and analyses were repeated twice and the results were presented as logarithmic values for averages of at least two replicates with their standard deviation. The recovery rate of bacterial strains on the selective medium was expressed as the per cent of their number on control medium. The Statistical Analysis System (SAS) was used to perform data analysis. Results were analysed using the General Linear Model (GLM) and significance was considered at $p < 0.05$ for all analyses.

Results and Discussion

Bacterial cultures, media, incubation condition (presence or absence of oxygen) and their interactions all had a significant effect ($p < 0.01$) on the bacterial counts using analysis of variance (ANOVA) table. Viable counts (Log$_{10}$ cfu/ml) and colony size (mm) of the seventeen different probiotic and LAB strains were obtained in MRS agar (pH 6.1, 5.8 and 5.2) and RCA (6.8 and 6.1) after different sets of aerobic and anaerobic incubation and presented in Tables 1 and 2. Likewise viable counts (Log$_{10}$ cfu/ml) for individual bacteria cultures and strains in mixture were obtained in different media presumptive for selective enumeration of starter lactic acid bacteria (SLAB), non-starter lactic acid bacteria (NSLAB) and probiotic organisms (Table 4). The recovery rates of the strains evaluated in BA supplemented with different sugars (sorbitol, galactose, fructose, raffinose, sucrose or mannitol) after different sets of aerobic and anaerobic incubation, using MRS agar as control is presented in Figure 1. The results are presented and discussed successively as follows.

RCA and MRS agar (pH modified)

The results presented in Table 1 and 2 show effective growth (log$_{10}$ cfu/ml) of all tested strains in MRS and RCA media at different pH values, under aerobic and anaerobic conditions. From initial screening of media, it was observed that MRS 5.2 was able to hinder the growth of *Lactobacillus* spp. including *L. rhamnosus* G5434, *L. acidophilus* 388, *L. plantarum* 276, *L. paracasei* 292 and *L. rhamnosus*
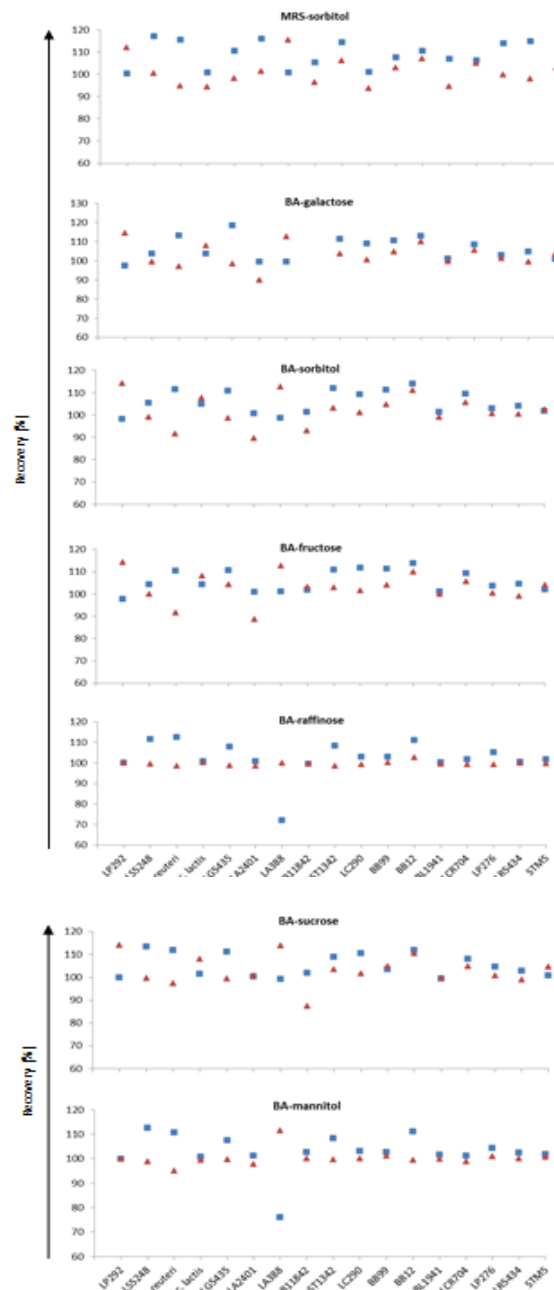

Figure 1. Recovery rate (%) of LAB and probiotic organisms on various sugar added media. Recovery rate (%) of LAB and probiotic strains (*L. paracasei* 292, *L. salivarius* 5248, *L. reuteri, Lc. lactis, L. rhamnosus* G5435, *L. acidophilus* 2401, *L. acidophilus* 388, *L. delbrueckii* subsp. *bulgaricus* 11842, *S. thermophilus* 1342, *L. casei* 290, *B. breve* BB99, *B. animalis* subsp. *lactis* BB12, *B. longum* 1941, *Lc. lactis* R704, *L. plantarum* 276, *L. rhamnosus* 5434 and *S. thermophilus* M5) on Basal agar (BA) supplemented with different sugars is expressed as their per cent count on tested medium in comparison to the count obtain on MRS medium (100%) as control, incubations carried out 37ºC for 72 h either at ■anaerobic or▲ aerobic conditions.

5434 when incubations were carried out aerobically at 37ºC for 72 h. On the other hand, *Bifidobacterium* sp. (BB99, BL1941 or BB12), *S. thermophilus* 1342, *L. casei* 290, *L. reuteri, L. acidophilus* 2401, *Lc. lactis*

Table 1. Viable count (Log_{10} cfu/ml ± SD) and colony size (mm) of lactic acid bacteria and probiotic strains cultivated on pH modified MRS agar (5.2, 5.8 and 6.1) and RCA (6.1 and 6.8) after anaerobic incubation at 37°C for 72h

Cultures	MRS 5.2		MRS 5.8		MRS 6.1		RCA 6.1		RCA 6.8	
	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)
LP292	9.19± 0.08	2.0-3.0	9.27± 0.01	2.0-4.0	8.61± 0.02	2.0-4.0	8.72± 0.04	2.0	9.03± 0.06	2.0
LS5248	8.03± 0.03	5.0-6.0	8.02± 0.04	3.0	8.17± 0.06	4.0-5.0	8.74± 0.00	<1.0	8.02± 0.02	3.0
L. reuteri	8.16± 0.01	4.0-5.0	8.08± 0.04	3.0-4.0	7.77± 0.07	3.0-5.0	8.14± 0.10	1.0	7.94± 0.01	2.0-3.0
Lc. lactis	9.06± 0.06	1.0	9.05± 0.03	<1.0	8.54± 0.00	<1.0	8.65± 0.10	<1.0	8.93± 0.03	<1.0
LG5434	9.23± 0.11	1.0-1.5	9.13± 0.12	1.5-2.0	8.33± 0.11	2.0	7.99± 0.01	<1.0	8.81± 0.05	<1.0
LA2401	8.66± 0.00	<1.0	8.53± 0.01	<1.0	8.38± 0.10	<1.0	8.29± 0.06	<1.0	8.61± 0.07	<1.0
LA388	9.08± 0.01	1.0-1.5	9.13± 0.03	<1.0	7.84± 0.03	<1.0	7.71± 0.07	<1.0	8.46± 0.07	1.5-2.0
LB11842	8.95± 0.05	<1.0	8.92± 0.00	< 1.0	8.35± 0.04	1.0	8.56± 0.00	<1.0	8.26± 0.06	< 1.0
ST1342	9.63± 0.01	1.0-2.0	9.61± 0.00	1.0	8.77± 0.10	3.0-5.0	9.22± 0.09	1.0-2.0	9.10± 0.07	<1.0
LC290	9.61± 0.13	1.0-2.0	9.57± 0.05	2.0-3.0	8.68± 0.05	3.0-4.0	9.08± 0.01	1.0	8.92± 0.03	2.0
Bb99	9.28± 0.03	1.0	9.21± 0.01	3.0	8.84± 0.06	3.0-5.0	9.18± 0.06	3.0	9.14± 0.01	3.0
BB12	9.79± 0.03	2.0-3.0	9.71± 0.03	2.0-4.0	8.56± 0.13	4.0-5.0	9.08± 0.01	1.0-2.0	9.29± 0.09	4.0
BL1941	9.00± 0.04	3.0	9.03± 0.00	3.0-4.0	8.70± 0.10	3.0	8.96± 0.07	1.0-2.0	8.63± 0.01	3.0
LCR704	<5	-	6.01± 0.03	<1.0	6.98± 0.24	<1.0	9.05± 0.02	1.0	8.36± 0.10	1.0
LP276	8.96± 0.08	<1.0	8.88± 0.02	<1.0	8.49± 0.10	<1.0	8.96± 0.04	<1.0	9.03± 0.06	2.0
LR5434	8.98± 0.01	3.0	9.03± 0.02	1.0-2.0	8.35± 0.01	1.0-2.0	8.54± 0.06	<1.0	8.96± 0.01	1-2.0
STM5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

MRS: deMan Rogosa Sharpe, RCA: Reinforced clostridial agar; values shows pH of the medium
ND- Not determined

R704, *Lc. lactis* and *L. salivarius* 5248 successfully grow under these incubation conditions. Colonies of *L. delbrueckii* subsp. *bulgaricus* 11842 and *Lc. lactis* were minute in size <1.0 mm and were easily differentiated from other bacteria grown under these condition (Table 2). On MRS 5.2, when anaerobic incubations were carried out at 37ºC for 72 h, only *Lc. lactis* failed to grow. However when anaerobic incubations were carried out at 45ºC rather than 37 ºC, MRS 5.2 agar was able to exclude the growth of *L. rhamnosus* G5434, *S. thermophilus* (ST1342, STM5), *B. lactis* BB12 and *Lc. lactis* R704 (Table 5). In general, MRS 5.2 incubated at 45ºC for 72h, was not found differential for *L. delbrueckii* subsp. *bulgaricus* in the presence of *Bifidobacterium* spp. and could not eliminate *L. casei* and *Bifidobacterium* spp., contradicting the findings of others (Dave and Shah, 1996; Tharmaraj and Shah, 2003). In line with the findings of Van de Casteele *et al.* (2006), our results suggest that selective recovery of the starter culture *L. delbrueckii* subsp. *bulgaricus* could be achieved using MRS 5.2 providing that the counts of the starter cultures are expected to be higher than probiotic culture. If the starter and probiotic cultures are in the same Log_{10} concentration range, the subtraction method can be used to enumerate SLAB by differentially subtracting the number of counts obtained on selective medium for the probiotic culture from the total count obtain on medium supporting the growth of both cultures.

The obtained results were evaluated according to the recovery rate of these bacteria using MRS as control media (results not shown). The overall recovery was improved under anaerobic condition for thirteen tested strains (*L. salivarius* 5248, *L. reuteri, Lc. lactis, L. rhamnosus* LG5434, *L. acidophilus* 2401, *L. delbrueckii* subsp. *bulgaricus*

11842, *S. thermophilus* 1342, *L. casei* 290, *L. paracasei* 276 and *L. rhamnosus* LR5434) including *Bifidobacterium* strains (BB99, BB12, BL1941). The results demonstrate a higher recovery rate of tested strains in all media particularly when incubations were made anaerobically with the exception of *L. acidophilus* LA388 and *L. paracasei* LP292 where % recovery was greater under aerobic incubations. Moreover, % recovery of tested strains was improved on RCA when pH was lowered to 6.1 in formulated media.

Our results demonstrated that colony sizes of bacteria were affected by incubation conditions (oxygen stress) and pH of the medium (Cooper *et al.*, 1968; Hochberg and Folkman, 1972; Lacasta *et al.*, 1999). For example, a colony size of *Bifidobacterium* spp. increased when grown anaerobically and decreased with pH reduction. Lowering the pH of MRS medium to 5.2, and for RCA to 6.1 affected the colony size of *Bifidobacterium* spp. Similarly, *L. rhamnosus* LG5434, *Lc. lactis, L. acidophilus* (LA2401, LA388), *S. thermophilus* 1342 and *L. delbrueckii* subsp. *bulgaricus* 11842 formed extremely minute colonies (<1.0 mm) on RCA 6.8 when incubations were carried out anaerobically. Although colonial morphology (such as size of colonies) is not a stable phenotypic trait to identify and quantify LAB strains it can be used as an additional attribute for rapid confirmation of the bacteria.

BA with sugars

Overall recovery of tested LAB and probiotic strains was above 90% (with only few exceptions) for majority of BA supplemented with different sugars that shows their versatile metabolic activity (Fig 1). In contrast with other studies (Dave and

Table 2. Viable count (Log_{10} cfu/ml ± SD) and colony size of lactic acid bacteria and probiotic strains cultivated on pH modified MRS agar (5.2, 5.8 and 6.1) and RCA (6.1 and 6.8) under aerobic incubation at 37°C for 72h

Cultures	MRS 5.2		MRS 5.8		MRS 6.1		RCA 6.1		RCA 6.8	
	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)
LP292	<5.0		9.31± 0.09	2.0	8.32± 0.00	2.0-3.0	8.11± 0.05	1.0-2.0	9.30± 0.09	<1.0
LS5248	9.16± 0.02	3.0	9.21± 0.03	2.0-5.0	9.65± 0.04	1.0-2.0	9.56± 0.15	<1.0	7.35± 0.08	1.0
L. reuteri	8.45± 0.08	2.0-3.0	8.48± 0.03	2-2.5	9.54± 0.09	2.0-3.0	9.41± 0.00	1.0-2.0	8.34± 0.10	1.0
Lc. lactis	8.76± 0.04	<1.0	8.77± 0.04	<1.0	9.10± 0.01	1.5-2.0	9.14± 0.03	1.0-2.0	9.19± 0.01	1.0
LG5434	<5.0	-	9.09± 0.01	1.0-2.0	9.39± 0.10	1.5-3.0	9.37± 0.01	<1.0	9.09± 0.01	1.0
LA2401	9.24± 0.08	2.0	9.28± 0.04	2.0-4.0	9.57± 0.05	2.0-4.0	9.39± 0.15	1.0-1.5	9.28± 0.10	2.0
LA388	<5.0	-	8.95± 0.03	2.0-3.0	8.28± 0.06	2.0-3.0	8.29± 0.18	1.5-2.0	9.08± 0.01	1.0-1.5
LB11842	9.12± 0.04	<1.0	8.76± 0.07	<1.0	9.39± 0.06	1.0-2.5	9.44± 0.09	<1.0	8.99± 0.05	1.0
ST1342	9.72± 0.01	2.0	9.65± 0.10	1.5-2.0	9.39± 0.06	1.0-2.5	9.37± 0.07	<1.0	9.64± 0.10	2.0
LC290	9.66± 0.01	2.0-2.5	9.69± 0.11	2.0-.03	9.33± 0.11	1.5-3.0	9.25± 0.03	1.0	9.68± 0.11	1.0
Bb99	9.29± 0.09	2.0	9.31± 0.03	2.0-3.0	9.38± 0.09	1.5-2.0	9.43± 0.08	1.0-1.5	9.56± 0.04	1.0
BB12	9.42± 0.04	2.0-3.0	9.36± 0.01	1.0-2.0	8.81± 0.00	2.0-3.0	9.62± 0.08	2.0	9.80± 0.01	1.0
BL1941	8.71± 0.12	2.0	8.98± 0.04	2.0-3.0	9.87± 0.02	1.5-2.5	9.85± 0.04	1.0-1.5	8.69± 0.16	2.0
LCR704	<5.0	-	<5.0	-	8.23± 0.11	2.0-3.0	9.24± 0.16	1.0-1.5	9.74± 0.02	1.5
LP276	<5.0	-	<5.0	-	9.69± 0.03	1.5-3.0	9.66± 0.07	<1.0	<5.0	-
LR5434	<5.0	-	7.35± 0.07	1.0-2.0	9.87± 0.01	1.0-2.0	9.88± 0.02	<1.0	8.69± 0.04	2.0
STM5	ND	ND	ND	ND	9.38± 0.03	1.0-2.5	9.48± 0.13	<1.0	ND	ND

MRS: deMan Rogosa Sharpe, RCA: Reinforced clostridial agar; values shows pH of the medium
ND- Not determined

Shah, 1996; Lankaputhra and Shah, 1996; Tabasco *et al.*, 2007; Miranda *et al.*, 2014), sugars did not act as a discriminating tool for selecting colonies; however, this attribute could be helpful to improve the overall recovery of LAB strains or total LAB count.

The utilization of different sugars by *Bifidobacterium* spp. is reflected by the fact that bifidobacteria are well adapted to the fluctuations in carbon source availability in the gut and exhibit high metabolic versatility within human intestinal microbiota allowing for utilization of different carbon sources (Pokusaeva *et al.*, 2011; Ruiz *et al.*, 2011). For some reason *Bifidobacterium* spp. were able to grow under aerobic conditions. This aero-tolerance could have been due to a possible mutation due to oxygen or acid stress (Ruiz *et al.*, 2011) or these sugars might have provided a shielding effect and thus protected the cell surface from detrimental impact of oxidation, or else it might be due the presence of certain NADH oxidases and peroxidases capable of detoxifying the cells (Shimamura *et al.*, 1992; Shin and Park, 1997). Although *Bifidobacterium* spp. are considered strict anaerobe but there is growing evidence that some species such as *B. animalis* are aero-tolerant and are able to form colonies on agar plates exposed to air (Von Ah *et al.*, 2007; Ruiz *et al.*, 2012). *Bifidobacterium* species including *B. animalis* subsp. lactis BB-12 were found aerotolerant in the study by Simpson *et al.* (2005), and *B. bifidum, B. breve* and *B. longum* were found highly aerotolerant in the recent study by Andriantsoanirina *et al.* (2013). Other study (Talwalkar *et al.*, 2001) has shown the growth of *B. lactis, B. pseudolongum* and *B. longum* at 21% oxygen. The aero-tolerance in *Bifidobacterium* spp. is desired trait for strains used in food industry

because it may warrant high bacterial viability in the end product and indeed, is the interesting finding of this study.

L. delbrueckii subsp. *bulgaricus* 11842 formed minute (<1.0 mm size) colonies with all the sugars and was largely differentiated from others (data not shown). Interestingly, *S. thermophilus* 1342 gave excellent growth on BA supplemented with galactose as sole carbon source in the media. A similar pattern was found for *S. thermophilus* M5, which also showed appreciable galactose utilisation. The later strain was included in the enumeration experiments in order to confirm and compare the metabolic activity of the strain in regards to galactose utilisation; for this reason counts for *S. thermophilus* M5 were not presented for initially screened media. Competence for galactose fermentation presents a valuable feature to *S. thermophilus* strains in various dairy fermentation industries where gal-positive phenotype is crucial to have increased yield of exopolysaccharides (EPS). Additionally, gal-positive strains have valuable application in dairy products where galactose accumulation in the milk or curd can cause product defects, such as growth of undesirable hetero-fermentative LAB and cheese browning during baking (O'Leary and Woychik, 1976; Mukherjee and Hutkins, 1994; De Vin *et al.*, 2005). The metabolic activity for galactose brings about the presence of metabolic enzymes required to utilize this sugar. This provides an additional significant attribute to the tested strains including *S. thermophilus* (STM5, ST1342), *B. lactis* BB12, *L. casei* 290 and *L. rhamnosus* G5434, which have shown increased FoxP3 expression in our previous study (Ashraf *et al.*, 2014a) and offers their functional application in

Table 3. Viable count (Log$_{10}$ cfu/g ± SD) of lactic acid bacteria and probiotic strains cultivated on different existing media selective for the enumeration of *L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. casei, L. rhamnosus, L. paracasei, Bifidobacterium* sp., *S. thermophilus* and *Lc. lactis.*

Culture	MRSB 37ºC 72h anaerobic		MRS 5.2 45ºC 72h anaerobic		LC 25ºC 72h anaerobic		MRS-NPNL+ L-cysteine 37ºC 72h anaerobic		M17* 30ºC 24h aerobic		M17* 37ºC 24h aerobic	
	count	size (mm)	count	size (mm)	count	size (mm)	count	size (mm)	mean	size (mm)	mean	size (mm)
LP292	<5.00	-	9.69±0.02	1.0	<5.00	-	<3.00	-	<5.00	-	<5.00	-
LS5248	<5.00	-	9.71±0.02	1.0	<5.00	-	<3.00	-	<5.00	-	<5.00	-
L. reuteri	8.07±0.05	1.0	9.00±0.00	1.0	7.85±0.00	<1.0	6.48±0.00	<1.0	<5.00	-	<5.00	-
Lc. lactis	<5.00	-	9.70±0.05	1.0	<5.00	-	<3.00	-	<5.00	-	<5.00	-
LG5435	9.10±0.01	<1.0	<5.00	-	8.81±0.04	<1.0	6.60±0.00	<1.0	<5.00	-	<5.00	-
LA2401	<5.00	-	9.63±0.00	1.0	<5.00	-	<3.00	-	<5.00	-	<5.00	-
LA388	<5.00	-	9.04±0.06	1.0-1.5	<5.00	-	<3.00	--	<5.00	-	<5.00	-
LB11842	8.63±0.06	<1.0	8.54±0.18	1.0	8.74±0.08	<1.0	5.85±0.00	<1.0	<5.00	-	ND	-
ST1342	9.25±0.01	<1.0	<5.00	-	8.52±0.07	<1.0	7.22±0.06	<1.0	<5.00	-	9.22±0.01	<1.0
LC290	8.97±0.02	<1.0	8.11±0.00	1.0-1.5	8.94±0.03	<1.0	7.07±0.52	<1.0	<5.00	-	<5.00	-
BB99	<5.00	-	9.56±0.12	1.0	9.64±0.08	1.0	6.41±0.01	1.0	<5.00	-	<5.00	-
BB12	<5.00	-	<5.00	-	9.20±0.00	1.0	8.38±0.03	1.0	<5.00	-	<5.00	-
BL1941	<5.00	-	9.54±0.02	1.0	<5.00	-	7.67±0.05	1.0	<5.00	-	<5.00	-
LCR704	<5.00	-	<5.00	-	<5.00	-	<3.00	-	9.37±0.01	<1.0	9.30±0.00	<1.0
LP276	<5.00	-	9.52±0.03	1.5	<5.00	-	<3.00	-	<5.00	-	<5.00	-
LR5434	9.54±0.13	<1.0	9.42±0.00	1.0	<5.00	-	<3.00	-	<5.00	-	<5.00	-
STM5	9.11±0.00	<1.0	<5.00	-	9.04±0.03	<1.0	7.66±0.03	<1.0	8.77±0.01	<1.0	9.31±0.01	<1.0

ND- Not determined

* caused co-recovery of other strains

the prevention and control of autoimmune disorders but also they may be of benefit for treating and managing sugar malabsorption such as lactose- and fructose- intolerances. These particular traits provide improved value to food industries and suggest avenues for future investigations.

MRSB agar

The results demonstrate *L. paracasei* 292, *L. salivarius* 5248, *Lc. lactis, L. acidophilus* (LA2401 and LA388), *Bifidobacterium* spp. (BB12, BB99 and BL1941), *Lc. lactis* R704 and *L. plantarum* 276 failed to grow in MRSB incubated anaerobically at 37ºC for 72 h whereas *S. thermophilus* strains (ST1342, STM5), *L. delbrueckii* subsp. *bulgaricus* 11842, *L. casei* LC290, *L reuteri*, and *L. rhamnosus* strains (LG5434 and LR5434) displayed appreciable growth. Although *L. acidophilus* species were not recovered on MRSB at 37ºC anaerobically, at the same time MRSB incubated aerobically at 42ºC reasonably supported growth of strains in ABT, ABY, ABRY, ABC, LAP or LAR mixture (Table 4). This could either represent true recovery of *L. acidophilus* or co-recovery with other strains. The colonial morphology was found unique, of single colony type and did not offer differences to account for multiple counts. This could have resulted due to a change in the physiology of probiotics when they were added in combination with other probiotics or starter cultures (Ruiz *et al.*, 2011). MRSB at 37ºC or 42°C under aerobic incubation was proposed by Lima *et al.* (2009) for enumerating *L. acidophilus* and Mortazavian *et al.* (2007) reported MRSB agar for differential enumeration of probiotic bacteria in the presence of *L. acidophilus, L. casei* and yoghurt bacteria under both aerobic and anaerobic conditions. Talwalkar and Kailasapathy (2004) categorized MRSB as unselective in final identification and enumeration of probiotic bacteria. Also Dave and Shah (1996) found low recovery of *L. acidophilus* and *Bifidobacterium* on MRSB. In contrast, our findings indicated unsuccessful recovery of *L. acidophilus* and *Bifidobacterium* strains but the method could be used for the selective enumeration of yoghurt starters (*L. delbrueckii* subsp. *bulgaricus* or *S. thermophilus*) in the absence of *L. reuteri* and *L. rhamnosus*. Furthermore, the subtraction method could be used to quantify *L. acidophilus* and *Bifidobacterium* by subtracting counts obtain on MRSB incubated anaerobically at 37ºC for 72 h, from total LAB counts obtained on MRS agar.

LC agar

Selective recovery of the tested strains was not uniform on LC agar and resulted in strain-to-strain variations. LC agar, incubated anaerobically at 25ºC for 72 h, inhibited the growth of *L. paracasei* 292, *L. salivarius* 5248, *L. acidophilus* (LA2401 and LA388), *B. longum* 1941, *lc. lactis* R704, *L. plantarum* 276 and *L. rhamnosus* 5434. On the other hand, *L. delbrueckii* subsp. *bulgaricus* 11842, *L. reuteri* and *S. thermophilus* (ST1342 and STM5) were recovered on this media. Moreover, *Bifidobacterium* spp. (BB99 and BB12), *L. rhamnosus* G5434 and *L. casei* 290 grew very well on the LC agar. *Bifidobacterium* spp. formed colonies bigger (1 mm) than the rest of the tested strains that formed very minute colonies. Similar to the findings of Shah (2000) and, Ravula

Table 4. Viable count ($\log_{10}$ cfu/ml) of yoghurt and cheese cultures in different bacterial combinations in selective media

product	type	Target culture	code	Selective media	Incubation conditions	Mixture	Count ($\log_{10}$ cfu/ml)
Yoghurt	Starter	*S. thermophilus* M5	T	M17	37ºC, Aerobic, 24 h	ABT	10.11 ± 0.08
						ABY	9.95 ±0.01
						ABRY	10.17±0.01
		L. delbrueckii subsp. *bulgaricus* 11842	D	MRS 5.2	45 ºC, Anaerobic, 72 h	ABD	10.30±0.02
						ABY	10.20±0.02
						ABRY	10.30±0.04
	Probiotic	*Bifidobacterium* sp. BB12	B	MRS NPNL	37 ºC, Anaerobic, 72 h	ABT	6.70±0.15
						ABY	6.60±0.08
						ABRY	6.45±0.22
						ABC	6.90±0.10
		L. acidophilus 2401	A	MRS B	42 ºC, Aerobic, 72 h	ABT	10.23±0.02
						ABY	10.14±0.01
						ABRY	10.28±0.00
						ABC	10.40±0.01
		L. rhamnosus 5434	R	LC	25 ºC, Anaerobic, 72 h	ABRY	10.38±0.01
		L. casei 290	C	LC	25 ºC, Anaerobic, 72 h	ABC	10.21±0.08
Cheese	SLAB	*Lc. lactis* subsp. *Lactis* R704	L	M17	30 ºC, Aerobic, 24 h	LAP	10.25±0.01
						LAR	10.42±0.01
	NSLAB Probiotic	*L. acidophilus* 2401	A	MRS B	42 ºC, Aerobic, 72 h	LAP	10.19±0.02
						LAR	10.44±0.04
		L. paracasei 292	P	LC	25 ºC, Anaerobic, 72 h	LAP	10.25±0.02

and Shah (1998), our results indicated that the tested *L. acidophilus* strains failed to grow on LC media. L. paracasei was recovered on LC agar from a mixture of LAP combination, thus LC agar can be used for selective enumeration of NSLAB *L. paracasei*. LC media was not found inhibitory to the growth of *S. thermophilus, L. delbrueckii* subsp. *bulgaricus* and bifidobacteria. Our results with these strains contradict prior studies (Ravula and Shah, 1998; Talwalkar and Kailasapathy, 2004) and thus the medium should not be recommended for selective enumeration of *L. casei* from yoghurts and fermented milk drinks. However, results demonstrated that selective counts of *Bifidobacterium* spp. (BB12 and BB99) could be generated by subtracting MRSB counts from those obtained on LC agar.

MRS-NPNL agar

MRS-NPNL, recommended for selective enumeration of Bifidobacterium, also allowed the growth of *L. reuteri, L. rhamnosus* LG5434, *L. delbrueckii* subsp. *bulgaricus* 11842, S. thermophilus (ST1342, STM5) and *L. casei* 290. Though recovery of *B. lactis* BB12 was highest, the overall growth of bifidobacteria was poorly affected in this medium (Table 3 and 4). *Bifidobacterium* spp. formed colonies of 1mm size that could be differentiated from the rest of the strains, which formed very minute (<1.0 mm) colonies. Moreover, MRS-NPNL failed to recover bifidobacteria when L-cysteine HCl was not incorporated into the medium (results not shown). This proved L-cysteine HCl as an important element in reducing oxygen tension for the recovery of

Bifidobacterium strains in the presence of antibiotics. Talwalker and Kailasapathy (2004) reported MRS-NPNL as one of the most differentiating media for enumerating bifidobacteria from yoghurts in the presence of *L. acidophilus* and *L. casei*. In contrast, our findings showed insufficient selectivity of MRS-NPNL towards SLAB (*L. delbrueckii* subsp. *bulgaricus* 11842, and *S. thermophilus* M5 and 1342), *L. casei, L. reuteri* and *L. rhamnosus* G5434. Non-selective behaviour, inconsistent counts and poor recovery of bidfidobacteria suggest MRS-NPNL as unsuitable for selective enumeration of Bifidobacterium spp. Besides time-consuming preparation of NPNL solution, concomitant use of antibiotics must be avoided and requires substitution of MRS-NPNL with other method for selective quantitation of bifidobacteria in products.

M17 agar

M17 recovered *S. thermophilus* and *Lactococcus* when incubations were conducted at 30ºC or 37ºC for 24 h but the colonies were extremely minute. Interestingly, M17 incubated at 37ºC under aerobic condition could not hinder the growth of all tested LAB cultures when counts were obtained after 36 h (data not shown). Similar results were obtained in the study by Oberg *et al.* (2011), where variety of NSLAB strains were capable of growing and forming colonies on M17 media. Our results indicated that the colonies formed by all tested LAB strains at earlier stage were extremely small and failed to differentiate. However, after 72 h incubations at 37ºC, colonies formed by *Bifidobacterium* spp. (BB99, BL1941 and BB12),

L. casei 290, *L. plantarum* 276 and *L. rhamnosus* R5434 were bigger (>1.0 mm) than the rest of the tested strains (data not shown). *S. thermophilus* and *Lc. lactis* strains as yoghurt and cheese SLAB were enumerated from mixture of different formulations and gave log higher recovery on M17 agar (Table 4) than found in pure culture, suggest possible co-recovery of other strains. Since LAB strains can be recovered, M17 is not recommended to obtain selective counts of streptococci and lactococci in the presence of other LAB.

Conclusion

The present study demonstrated that altering the selectivity of the media using pH and sugar addition can improve overall recovery of probiotic and LAB strains, and can be used to obtain total LAB counts. The colony size of strains was affected by pH of media, sugar addition and incubation conditions (oxygen stress) which suggest that differential evaluation of colony size could be misleading in some cases. On the other hand, carbohydrate catabolic diversity and compatibility of tested LAB strains to a range of oxygen tension levels, temperature and acidic conditions define their overall survival in the gastrointestinal tract and preservation of their functional probiotic properties. An interesting finding of the study is aero-tolerance demonstrated by the species including *Bifidobacterium* spp. which refer to their wide adaptability to industrial stresses and offer value to these strains for use in functional food industry. In addition, some probiotics may offer added value to foods especially dairy-based food products, with their ability to reduce the symptoms of food intolerances through metabolism of lactose and fructose.

The prior methods for selective enumeration of *L. acidophilus*, *Bifidobacterium* spp., *L. casei* and SLAB (*S. thermophilus*, *Lactococcus* sp. and *L. delbrueckii* subsp. *bulgaricus*) showed varied and inconsistent results. MRSB, LC, MRS-NPNL proved to be non-selective for *L. acidophilus* strains, *L. casei* and *Bifidobacterium* strains respectively. M17 agar used for selective enumeration of SLAB (*S. thermophilus* and *Lc. lactis*) has the capacity to recover other LAB, hence cannot be used for selective enumeration of strains. However, MRSB could be used to target yoghurt starter (*L. delbrueckii* subsp. *bulgaricus* or *S. thermophilus*) and LC agar can be used to target *L. paracasei*. Selective recovery of *L. delbrueckii* subsp. *bulgaricus* could be achieved using MRS 5.2 incubated at 45°C anaerobic for 72 h, providing that the counts of starter LAB are expected

to be higher than those of probiotics. On the basis of these findings, it is concluded and confirmed that the choice of media and method for selective enumeration of probiotic and LAB strains largely depends on the target specie and strain.

Despite its exploratory nature, the present study provides insight about metabolic features of the LAB and probiotic strains. Taken together, the findings will help in the development of better selective and differential media for enumeration of LAB and probiotics strains and it would be interesting to explore modern methods too for this purpose.

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