Selective Enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, Bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and Propionibacteria

N. Tharmaraj and N. P. Shah

School of Molecular Sciences

Victoria University, Werribee Campus

PO Box 14428 Melbourne City Mail Centre, Victoria 8001 Australia

ABSTRACT

Nineteen bacteriological media were evaluated to assess their suitability to selectively enumerate *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, bifidobacteria, and propionibacteria. Bacteriological media evaluated included *Streptococcus thermophilus* agar, pH modified MRS agar, MRS-vancomycin agar, MRS-bile agar, MRS-NaCl agar, MRS-lithium chloride agar, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paramomycin sulfate) agar, reinforced clostridial agar, sugar-based (such as maltose, galactose, sorbitol, mannitol, esculin) media, sodium lactate agar, arabinose agar, raffinose agar, xylose agar, and *L. casei* agar. Incubations were carried out under aerobic and anaerobic conditions at 27, 30, 37, 43, and 45°C for 24, 72 h, and 7 to 9 d. *S. thermophilus* agar and aerobic incubation at 37°C for 24 h were suitable for *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* could be enumerated using MRS agar (pH 4.58 or pH 5.20) and under anaerobic incubation at 45°C for 72 h. MRS-vancomycin agar and anaerobic incubation at 43°C for 72 h were suitable to enumerate *L. rhamnosus*. MRS-vancomycin agar and anaerobic incubation at 37°C for 72 h were selective for *L. casei*. To estimate the counts of *L. casei* by subtraction method, counts of *L. rhamnosus* on MRS-vancomycin agar at 43°C for 72 h under anaerobic incubation could be subtracted from total counts of *L. casei* and *L. rhamnosus* enumerated on MRS-vancomycin agar at 37°C for 72 h under anaerobic incubation. *L. acidophilus* could be enumerated using MRS-agar at 43°C for 72 h or Basal agar-maltose agar at 43°C for 72 h or BA-sorbitol agar at 37°C for 72 h, under anaerobic incubation. Bifidobacteria could be enumerated on MRS-NNLP agar under anaero-

bic incubation at 37°C for 72 h. Propionibacteria could be enumerated on sodium lactate agar under anaerobic incubation at 30°C for 7 to 9 d. A subtraction method was most suitable for counting propionibacteria in the presence of other lactic acid bacteria from a product. For this method, counts of lactic bacteria at d 3 on sodium lactate agar under anaerobic incubation at 30°C were subtracted from counts at d 7 of lactic bacteria and propionibacteria.

(**Key words:** *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, propionibacteria).

Abbreviation key: BA = basal agar, NNLP = nalidixic acid, neomycin sulfate, lithium chloride and paramomycin sulfate, **ST agar** = *Streptococcus thermophilus* agar, **RCA** = reinforced clostridial agar, **RSM** = reconstituted skim milk.

INTRODUCTION

A number of health benefits have been claimed for probiotic bacteria and more than 90 probiotic products containing one or more groups of probiotic organisms are available worldwide. Probiotic food can be defined as “food containing live microorganisms which actively enhance the health of consumers by improving the balance of microflora in the gut” (Fuller, 1992).

A number of probiotic organisms including *L. acidophilus*, *Bifidobacterium* spp., *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Propionibacterium* are incorporated in dairy foods. These organisms grow slowly in milk during product manufacture. Therefore the usual practice is to incorporate yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. Yogurt bacteria do not survive in the gastric passage or colonize in the gut (Shah and Jelen, 1990) and are unlikely to provide any therapeutic benefits. However, yogurt bacteria grow rapidly and thus are added to speed up the fermentation process.

To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Shah, 2000). It seems reasonable to assume that the beneficial

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Corresponding author: N. P. Shah; e-mail: Nagendra.Shah@vu.edu.au.

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 differentially. Differential enumeration of probiotic bacteria is difficult owing to the presence of several types of similar microbes in a product. In order to assess viability and survival of probiotic bacteria, it is important to have a working method for selective enumeration of these bacteria.

Several media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. have been previously proposed (Hunger, 1986; Hull and Roberts, 1984; Laroia and Martin, 1991; Dave and Shah, 1996; Lankaputhra and Shah, 1996; Wijsman et al., 1989; Shah, 1997, 2000). Similarly, several media have been proposed for selective enumeration of yogurt cultures (Onggo and Fleet, 1993; Samona and Robinson, 1984). There are only few reports that have described selective enumeration of *Lactobacillus casei* in the presence of other probiotic bacteria and yogurt bacteria (Champagne et al., 1997; Ravula and Shah, 1998). Selective enumeration of *L. casei* from probiotic products based on a 15°C incubation temperature and 14 day incubation was studied by Champagne et al. (1997). Ravula and Shah (1998) developed a medium, known as LC agar, for selective enumeration of *L. casei*. Selective enumeration of *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and propionibacteria has not been studied extensively. The aim of this study was to develop and evaluate media for selective enumeration of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, bifidobacteria, and propionibacteria.

MATERIALS AND METHODS

Bacteria Cultures and Propagation

L. delbrueckii ssp. *bulgaricus* (LB 100B), *S. thermophilus* (ST 2362), *L. casei* (DS 930), *L. acidophilus* (DS 910), *Bifidobacterium lactis* (DS920), and *Propionibacterium freudenreichii* ssp. *globosum* (type standard 10360) were provided by DSM Gist brocades (DSM Gist brocade Australia Pty. Ltd., Werribee, Australia). *L. paracasei* ssp. *paracasei* (LC01), *L. acidophilus* (LA 5), *Bifidobacterium lactis* (Bb 12), and *Propionibacterium freudenreichii* ssp. *shermanii* (PS1) were obtained from Chr. Hansen (Chr. Hansen Pty. Ltd., Bayswater, Australia). *L. rhamnosus* (LC 705), *L. acidophilus* (74-2), and *Bifidobacterium* spp. (BB 420) were received from Danisco Cultor (Danisco Cultor, Dingley, Australia). *S. thermophilus* (TA040), *L. paracasei* ssp. *paracasei* (LBC81), *L. rhamnosus* (LBA), *L. acidophilus* (LAC 4), and *Bifidobacterium* spp. (BL) were from Rhodia (Rhodia Australia Pty. Ltd., Notting Hill, Australia).

All the strains were tested for purity using Gram stain. All cultures except propionibacteria were propagated weekly in sterile 12% reconstituted skim milk (RSM) supplemented with 2% glucose and 1.2% yeast extract. Cultures were grown using 1% inoculum at 37°C for 18 h. Propionibacteria were grown in sodium lactate broth (composition: 10 g of pancreatic digest of casein, 10 g of sodium lactate, 10 g of yeast extract, 0.5 g of Tween 80, and 1 L of distilled water) and incubated at 30°C for 2 d using 1% inoculum. Bifidobacteria were propagated using 1% inoculum in sterile RSM supplemented with 0.05% L-cysteine-hydrochloride in order to provide anaerobic condition and to enhance their growth. Cultures were maintained in the same media at 4°C. Before enumeration the cultures were transferred successively three times for activation.

Media Preparation

Bacteriological peptone and water diluent. Bacteriological peptone and water diluent (0.15%) were prepared by dissolving 1.5 g of bacteriological peptone (Oxoid (Australia) Pty. Ltd., West Heidelberg, Australia) in 1 L of distilled water. The pH was adjusted to 7.0 ± 0.2 , followed by autoclaving 9 ml aliquots at 121°C for 15 min.

Streptococcus thermophilus (ST) agar. The ST agar was prepared according to the method described by Dave and Shah (1996).

MRS agar, pH-modified (pH 5.20, 4.58) MRS agar, MRS-vancomycine agar, MRS-bile (0.2% and 0.5%) agar, MRS-NaCl agar, and MRS lithium chloride agar. Rehydrated MRS broth (Oxoid) was prepared according to the manufacturer instructions. The pH of the broth was adjusted to 5.20 and 4.58 using 1.0 M HCl to obtain the pH-modified agar. Two and five grams of pure bile salts (Amyl Media, Dandenong Australia)/L were added to obtain 0.2% and 0.5% MRS-bile agar. Forty grams of NaCl/L was added for MRS-NaCl agar (4% final concentration) and 5 g/L lithium chloride (LiCl) was added for MRS-LiCl agar (0.5% final concentration). To prepare MRS-vancomycine (MRS-V) agar, 2 ml of 0.05 g vancomycine (Sigma Chemical Co., Castle Hill, Australia)/100 ml solution was added to 1 L of MRS broth to obtain 1 mg/L final concentration. Agar powder was added to each broth at the rate of 1.2% and the media were autoclaved at 121°C for 15 min. Inoculated plates in duplicates were incubated anaerobically at 37°C and 43°C for 72 h.

MRS-NNLP agar. The MRS-nalidixic acid, neomycine sulfate, lithium chloride and paromomycine sulfate agar (NNLP; Sigma Chemical Co.) was prepared according to the method described by Laroia and Martin (1991). MRS agar was the basal medium. Filter-sterilized NNLP was added to the autoclaved MRS base just

Table 1. Viable counts ($\log_{10}$ cfu/g) and colony size of bacterial cultures in different sugar-based media (anaerobic incubation, 37°C, 72 h).

Cultures	MRS		BA ¹ -maltose		BA-galactose		BA-sorbitol		BA-mannitol		BA-esculin	
	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)	Counts	Size (mm)
LB-LB 100B	8.28	0.1-0.5	<3.00	—	—	—	<3.00	—	<3.00	—	<3.00	—
ST-TA 040	7.14	0.5 mm	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
ST-DS 2362	8.09	0.5 mm	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
LC-DS 930	9.30	2.0	9.48	2.0	9.30	2.0	9.23	2.0	9.42	2.0	9.21	2.0
LC-LCO 1	9.28	2.0	8.30	2.0	9.15	2.0	9.23	2.0	9.23	2.0	9.21	1.0-1.5
LC-LBC 81	9.07	2.0	9.34	2.0	9.18	2.0	9.28	2.0	9.22	2.0	9.22	1.0-1.5
LR-LC 705	9.20	2.0	9.30	2.0	9.09	2.0	9.18	2.0	9.35	2.0	9.20	1.0-1.5
LR-LBA	9.36	2.0	9.36	2.0	9.04	2.0	9.36	2.0	9.40	2.0	9.15	1.0-1.5
LA-LA 5	7.00	0.1-0.5	8.00	0.5-1	7.70	0.5-1	8.09	0.1-0.5	7.43	0.1-0.5	7.50	0.1-0.5
LA-DS 910	8.41	0.5-1	8.59	1.0-1.5	8.30	1.0-1.5	8.70	0.1-0.5	9.18	0.1-0.5	7.83	0.1-0.5
LA-LAC 4	7.15	0.1-0.5	7.13	0.5-1.0	7.19	.5-1	7.08	0.1-0.5	7.10	0.1-0.5	7.84	0.1-0.5
LA-74-2	7.11	0.1-0.5	8.81	0.5-1.0	7.26	.5-1	8.08	0.1-0.5	7.20	0.1-0.5	7.24	0.1-0.5
BB-Bb 12	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	9.26	1.0-1.5
BB-DS 920	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	8.95	1.0-1.5
BB-420	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	8.88	1.0-1.5
BB-BL	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—
PS-PS 1	10.56	0.1-0.5	<3.00	—	8.11	0.5-1.0	<3.00	—	<3.00	—	<3.00	—
PS-10360	10.84	0.1-0.5	<3.00	—	8.90	0.5-1.0	<3.00	—	<3.00	—	<3.00	—

¹BA = Basal agar, LB = *L. delbrueckii* ssp. *bulgaricus*, ST = *S. thermophilus*, LC = *L. casei*, LA = *L. acidophilus*, LR = *L. rhamnosus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

before pouring. Filter-sterilized L-cysteine-HCl (0.05% final concentration) was also added at the same time 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.

Reinforced clostridial agar. Reinforced clostridial agar (RCA; Oxoid) was made according to the manufacturer instructions and sterilized by autoclaving at 121°C for 15 min.

Basal agar, BA-maltose agar, BA-galactose agar, BA-sorbitol agar, BA-mannitol agar, and BA-esculin agar. Basal agar was prepared (composition: 10 g of trypton, 10 g of Lablemco powder, 5 g of yeast extract, 1 g of Tween 80, 2.6 g of K_2HPO_4 , 5 g of sodium acetate, 2 g of tri-ammonium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot 4H_2O$, 12 g of bacteriological agar, and 1 L of distilled water) and autoclaved at 121°C for 15 min. Ten milliliters of membrane filtered sterile 20% solutions of maltose, galactose, sorbitol, mannitol or esculin were added to 90 ml of basal agar (2% final concentration) just before pouring the agar medium. Inoculated plates in duplicates were incubated aerobically and anaerobically at 37°C and 43°C for 72 h.

Sodium lactate agar (NaLa agar), arabinose agar, xylose agar, and raffinose agar. The base for these agar media was prepared (composition: 10 g of pancreatic digest of casein, 10 g of yeast extract, 2 g of sodium pyruvate, 2 g of glycine, 1.5 g of sodium chloride, 0.5 g of Tween 80, 0.25 g of di-potassium hydrogen phosphate, 12 g of bacteriological agar and 1 L of distilled water). The pH was adjusted to 7 ± 0.2 using 1 M HCl

and 10 M NaOH. To make NaLa agar, 10 g of sodium lactate was added before autoclaving. The medium was then autoclaved at 121°C for 15 min. For other media, 10 ml of 10% membrane filtered arabinose, raffinose or xylose was added to 90 ml of autoclaved media (1% final concentration) before pouring the plates. Inoculated plates in duplicates were incubated anaerobically at 30°C for 7 to 9 d.

LC agar. LC agar was made using the method described by Ravula and Shah (1998). The incubation was carried out under anaerobic condition at 27°C for 72 h.

Enumeration of Bacteria

Cultures were activated by three successive transfers in nutrient medium before enumeration. One gram of each culture was 10-fold serially diluted (10^3 to 10^7) in 0.15% sterile bacteriological peptone and water diluents. Enumeration was carried out using the pour plate technique. Anaerobic jars and gas generating kits (Anaerobic System, BR 38; Oxoid Ltd., Hampshire, England) were used for creating anaerobic condition. Plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (cfu) per gram of the product or culture.

All experiments and analyses were repeated at least twice. The results presented are averages of at least two replicates.

RESULTS AND DISCUSSION

Viable counts ($\log_{10}$ cfu/g) and colony sizes (in mm diameter) of 7 species of bacterial cultures containing 18

strains of bacteria including 1 strain of *L. delbrueckii* ssp. *bulgaricus*, 2 strains of *S. thermophilus*, 3 strains of *L. casei*, 2 strains of *L. rhamnosus*, 4 strains of *L. acidophilus*, 4 strains of *Bifidobacterium* spp., and 2 strains of propionibacteria in various sugar based media are presented in Table 1. *L. delbrueckii* ssp. *bulgaricus* did not grow in any sugar-based media except in MRS agar. MRS agar was particularly suitable for growing lactobacilli. *S. thermophilus* did not grow in any sugar based medium and formed small colonies in MRS agar.

RCA agar supported the growth of all tested organisms. Bifidobacteria grew in this medium even without the addition of L-cysteine.hydrochloride (data not shown). Therefore RCA agar was not suitable for selective enumeration.

ST agar was found to be suitable for *S. thermophilus* (data not shown). *S. thermophilus* formed tiny (0.1–0.5 mm) colonies in ST agar at 37°C under aerobic incubation after 24 h. The incubation time was insufficient for growth of other cultures even if ST agar did not inhibit the growth of other organisms. Therefore, ST agar at 37°C for 24 h and aerobic condition were selective for *S. thermophilus*. This is in agreement with a previous report (Dave and Shah, 1996).

Other organisms such as, *L. casei*, *L. rhamnosus* and *L. acidophilus* grew in all sugar-based media. Bifidobacteria did not grow in any media, except in BA-esculin agar. *Propionibacterium freudenreichii* ssp. *shermanii* grew only in MRS agar and BA-galactose agar. Thus based on sugar utilization patterns, probiotic organisms could not be selectively enumerated.

Table 2 shows the counts of bacterial cultures in media containing different inhibitory substances including vancomycin, NNLP, hydrochloric acid, NaCl, LiCl, and bile at 37°C and 43°C incubations. All the organisms except *Bifidobacterium* spp. grew in MRS agar. When the pH of MRS agar was reduced to 5.20 and the incubation temperature increased to 43°C, only *L. delbrueckii* ssp. *bulgaricus* (which formed 1.0 mm, white rough irregular colonies), *L. rhamnosus* (which formed 2 mm, shiny smooth white colonies) and *L. acidophilus* (which formed 0.1 to 0.5 mm, brown, rough irregular colonies) showed good growth. When the pH of MRS agar was reduced to 4.58 using 1 M HCl, only *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus* showed good growth similar to that formed in MRS agar at pH 5.20 and the growth of *L. acidophilus* was inhibited except that of DS 910. Therefore, MRS agar at pH 5.20, under anaerobic incubation at 43°C could be selective for *L. delbrueckii* ssp. *bulgaricus* if *L. rhamnosus* and *L. acidophilus* DS 910 were not present in a product. The colony morphology of *L. delbrueckii* ssp. *bulgaricus* and *L. rhamnosus* was very different and these two organisms could be easily differentiated if *L. rhamnosus* was present in the product.

Table 2. Viable counts (log₁₀ cfu/g) of bacterial cultures under anaerobic incubation at 37°C and 43°C (LC agar at 27°C) for 72 h in media containing different inhibitory substances.

Cultures	MRS 37°C inc.	MRS- vancomycin 37°C inc.	MRS- vancomycin 43°C inc.	MRS- NNLP 37°C inc.	MRS- pH 5.20 43°C inc.	MRS- pH 4.58 43°C inc.	MRS- NaCl (4%) 37°C inc.	MRS-LiCl (0.5%) 37°C inc.	LC agar 27°C inc.	MRS-bile (0.2%) 43°C inc.	MRS-bile (0.5%) 43°C inc.
LB-LB 100B	9.28	<3.00	<3.00	<3.00	9.37	9.38	<3.00	<3.00	<3.00	<3.00	<3.00
ST-TA 040	7.14	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
ST-DS 2362	8.09	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
LC-DS 930	9.46	9.38	<3.00	<3.00	<3.00	<3.00	9.11	9.14	9.08	<3.00	<3.00
LC-LCO 1	9.37	9.23	<3.00	<3.00	<3.00	<3.00	9.05	9.21	9.09	<3.00	<3.00
LC-LBC 81	9.28	9.22	<3.00	<3.00	<3.00	<3.00	9.26	9.08	9.26	<3.00	<3.00
LR-LC 705	9.20	9.28	9.18	<3.00	9.09	9.08	9.04	9.00	9.45	5.48	<3.00
LR-LBA	9.36	9.37	9.40	<3.00	9.40	9.30	6.36	<3.00	9.00	9.40	7.85
LA-LA 5	7.09	<3.00	<3.00	<3.00	6.48	<3.00	<3.00	<3.00	<3.00	6.04	6.04
LA-DS 910	8.31	<3.00	<3.00	<3.00	8.26	6.89	<3.00	<3.00	<3.00	8.08	<3.00
LA-LAC 4	7.15	<3.00	<3.00	<3.00	6.04	<3.00	<3.00	<3.00	<3.00	6.26	5.04
LA-74-2	7.19	<3.00	<3.00	<3.00	6.04	<3.00	<3.00	<3.00	<3.00	6.10	5.04
BB-Bb 12	<3.00	<3.00	<3.00	7.40	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	8.78
BB-DS 920	<3.00	<3.00	<3.00	9.18	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
BB-420	<3.00	<3.00	<3.00	7.35	<3.00	<3.00	<3.00	<3.00	<3.00	8.18	<3.00
PS-PS 1	10.54	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
PS-10360	10.84	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00

LB = *L. delbrueckii* ssp. *bulgaricus*, LC = *L. casei*, LR = *L. rhamnosus*, LA = *L. acidophilus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

Table 3. Viable counts ($\log_{10}$ cfu/g) of bacterial cultures in different sugar based media under aerobic incubation at 43°C for 72 h.

Cultures	MRS	BA ¹ -sorbitol	BA-mannitol	BA-maltose	BA-esculin
LB-LB 180	7.95	<3.00	<3.00	<3.00	<3.00
ST-DS 2362	8.00	<3.00	<3.00	<3.00	<3.00
ST-TA 040	7.69	<3.00	<3.00	<3.00	<3.00
LC-LCO 1	<3.00	<3.00	<3.00	<3.00	<3.00
LC-LBC 81	<3.00	<3.00	<3.00	<3.00	<3.00
LR-LC 705	9.30	9.28	9.24	9.31	9.13
LR-LBA	9.36	9.32	9.30	9.36	9.46
LA-DS 930	<3.00	<3.00	<3.00	<3.00	<3.00
LA-LA 5	6.48	<3.00	<3.00	<3.00	<3.00
LA-DS 910	7.60	<3.00	<3.00	7.08	7.27
LA-74-2	8.93	<3.00	4.00	8.27	8.45
BB-Bb 12	<3.00	<3.00	<3.00	<3.00	<3.00
BB-DS 920	<3.00	<3.00	<3.00	<3.00	<3.00
BB-420	<3.00	<3.00	<3.00	<3.00	<3.00
BB-BL	<3.00	<3.00	<3.00	<3.00	<3.00

¹Basal agar, LB = *L. delbrueckii* ssp. *bulgaricus*, ST = *S. thermophilus*, LA = *L. acidophilus*, LC = *L. casei*, LR = *L. rhamnosus*, BB = *Bifidobacterium*.

Therefore, pH modified MRS (pH 4.58) agar and anaerobic incubation at 43°C could be used to selectively enumerate *L. delbrueckii* ssp. *bulgaricus* from a product.

L. casei grew in MRS-NaCl (4%), MRS-LiCl (0.5%) at 37°C under anaerobic incubation and LC agar. *L. casei* did not grow in NNLP agar and at 43°C. Lower incubation temperatures ($\leq 37^\circ\text{C}$) supported the growth of *L. casei*. *L. casei* and *L. rhamnosus* were resistant to 1 mg vancomycin/L. *L. rhamnosus* formed well developed smooth white discs like colonies that were 2 mm or more in diameter in MRS-vancomycin (MRS-V) agar at 37°C under anaerobic incubation. *L. rhamnosus* grew at both incubation temperatures of 37°C and 43°C and in all sugar based media under aerobic and anaerobic conditions, except in MRS-NNLP agar and showed varying growth pattern (between strains) in MRS-bile agar, and MRS-LiCl agar. The organisms grew well in MRS-V agar at both incubation temperatures of 37°C and 43°C as well as in LC agar at 27°C. MRS-V agar at 43°C and anaerobic incubation supported the growth of only *L. rhamnosus*. No other cultures tested including *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. casei*, *L. acidophilus*, *Bifidobacterium lactis* and *Propionibacterium* grew in this medium.

Basal agar (BA)-sorbitol agar and BA-mannitol agar under aerobic incubation at 43°C (Table 3) and BA-sorbitol agar and BA-mannitol agar at 43°C and anaerobic incubation (data not shown) also supported the growth of only *L. rhamnosus*. Therefore, MRS-V agar at 43°C under anaerobic incubation, BA-sorbitol agar, or BA-mannitol agar at 43°C, either under aerobic or anaerobic incubations, were selective for *L. rhamnosus*.

MRS-V agar at 37°C or LC agar at 27°C under anaerobic incubation (Table 2) could be selective for *L. casei* when *L. rhamnosus* was not present in a product. When

L. rhamnosus was present, total counts of *L. casei* and *L. rhamnosus* could be obtained using MRS-V agar at 37°C and anaerobic incubation for 72 h. The count of *L. rhamnosus* on MRS-V agar at 43°C and anaerobic incubation for 72 h could be subtracted from the total counts of *L. casei* and *L. rhamnosus* to obtain the counts of *L. casei*.

MRS-NNLP agar (which contains 0.05% L-cysteine in the formula) at 37°C and anaerobic incubation supported the growth of only bifidobacteria (Table 2). When L-cysteine was not present in the media, bifidobacteria either did not grow or formed pinpoint colonies (data not shown). Therefore, MRS-NNLP agar with 0.05% L-cysteine and anaerobic incubation at 37°C were selective for bifidobacteria and the absence of L-cysteine was able to control the growth of bifidobacteria from other media.

Table 4 shows the colony counts and colony sizes of various bacterial cultures in different agar media. Colonies of ≥ 0.5 mm in diameter were only counted as developed colonies for the enumeration purpose. NaLa agar, arabinose agar, raffinose agar, and xylose agar supported the growth of *L. casei*, *L. acidophilus*, and *L. rhamnosus* as well as of propionibacteria. In these media, *L. casei* and *L. rhamnosus* formed white shiny smooth colonies of 1 mm diameter.

Propionibacteria formed colonies of 0.5 mm diameter in all the media. However, in NaLa agar, propionibacteria formed colonies that were dull brown with lighter margin of 1.0 to 2.5 mm in diameter. The colonies were very different to those formed by *L. casei* and *L. rhamnosus*. *L. acidophilus* formed pinpoint colonies. To eliminate the possibility of *L. acidophilus* being counted, colonies of ≥ 0.5 mm diameter were only counted. Proper colonies of propionibacteria formed only after 72 h of incubation and after 7 d colony size grew to 2 mm in

Table 4. Viable counts ($\log_{10}$ cfu/g) and colony size of various cultures in different bacteriological media under anaerobic incubation at 30°C (27°C for LC agar) for 7 to 9 d.

Cultures	MRS		NaLa agar		Arabinose agar		Raffinose agar		Xylose agar		LC agar		NaLa agar, day 7– NaLa agar, day 3 ¹	
	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)	Count	Colony size (mm)
LC-DS 930	9.46	2.00	9.45	0.5–1.0	9.18	1.0	9.26	1.0	8.30	1.0	9.08	1.5–2.0	<3.00	<3.00
LC-LC0 1	9.37	2.00	8.78	0.5–1.0	9.18	1.0	9.32	1.0	9.30	1.0	9.10	1.5–2.0	<3.00	<3.00
LR-LC 705	9.20	2.00	8.86	0.5–1.0	9.23	1.0	9.08	1.0	9.10	1.0	9.35	1.5–2.0	<3.00	<3.00
LR-LBA	9.14	2.00	8.23	0.5–1.0	8.26	1.0	9.30	1.0	8.43	1.0	9.45	1.5–2.0	<3.00	<3.00
LA-La 5	7.09	0.1–0.5	6.78	0.1	7.70	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00	<3.00
LA-DS 910	8.31	0.5–1.0	7.40	0.1	9.08	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00	<3.00
LA-74-2	7.19	0.1–0.5	6.80	0.1	7.38	0.1	<3.00	—	<3.00	—	<3.00	—	<3.00	<3.00
BB-Bb 12	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	<3.00
BB-DS 920	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	—	<3.00	<3.00
PS-PS 1	10.54	0.1–0.5	9.76	1.5–2.0	9.58	1.0	9.75	0.5	9.60	0.5	<3.00	—	9.76	9.76
PS-10360	10.84	0.1–0.5	9.90	1.0–2.5	8.66	1.0	9.40	0.5	8.41	0.5	<3.00	—	9.90	9.90

¹Subtraction method (counts in sodium lactate (NaLa) agar at d 7 – counts in NaLa agar at d 3).LB = *L. delbrueckii* ssp. *bulgaricus*, LA = *L. acidophilus*, LC = *L. casei*, LR = *L. rhamnosus*, BB = *Bifidobacterium*, PS = *Propionibacterium freudenreichii* ssp. *shermanii*.

diameter. The colony sizes of *L. casei* and *L. rhamnosus* did not change. In NaLa agar, the recovery was the highest and colony morphology and colony size were different than those formed in arabinose agar, and xylose agar. Thus, NaLa agar could be used to selectively enumerate propionibacteria. The propionibacteria could also be counted by subtracting the counts of *L. casei* and *L. rhamnosus* at day 3 from the total counts of *L. casei*, *L. rhamnosus* and propionibacteria obtained on d 7 using NaLa agar, raffinose agar or xylose agar (Table 4).

L. acidophilus was found to be the most difficult to enumerate selectively, since most of the media that supported the growth of *L. acidophilus* also supported the growth of *L. casei* and *L. rhamnosus*. When the incubation temperature was increased to 43°C, *L. casei* was eliminated. However, *L. rhamnosus* still formed well developed (1.5 mm in diameter) colonies and *L. acidophilus* formed smaller colonies (0.1 to 1.0 mm) depending on the sugar used (Table 1). When galactose was used, bifidobacteria formed pinpoint colonies in the absence of L-cysteine, and the colonies could be confused with *L. acidophilus*. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* formed colonies in MRS agar when incubated anaerobically at 43°C. Therefore, MRS agar and anaerobic incubation at 43°C could be used to enumerate *L. acidophilus* when *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were not present in the product (data not shown).

When incubated anaerobically at 43°C in BA-mannitol agar, BA-sorbitol agar, BA-esculin agar and BA-maltose agar, *L. rhamnosus* formed large (2.0 to 2.5 mm diameter) smooth shiny disc like colonies (data not shown), while *L. acidophilus* formed smaller (0.1 to 1.0 mm diameter) rough dull colonies. BA-maltose agar supported the growth of *L. acidophilus* more than the other BA-based agar media at this incubation temperature, but one strain of *L. acidophilus* DS 910 formed large colonies that could be confused with *L. rhamnosus*. In MRS agar, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. rhamnosus* and *L. acidophilus* formed colonies (Table 3) while in BA-maltose agar, only *L. acidophilus* and *L. rhamnosus* formed colonies. *L. rhamnosus* formed large (2.0 to 2.5 mm diameter) smooth, shiny, and disc like colonies, while strains of *L. acidophilus* formed smaller rough brownish colonies of 0.1 to 1.0 mm diameter that could be easily distinguished. Therefore, MRS agar under aerobic or anaerobic incubation at 43°C could be used to count *L. acidophilus*, except DS 910, when *L. delbrueckii* ssp. *bulgaricus* was not present in the product. If *L. delbrueckii* ssp. *bulgaricus* is present, BA-maltose agar and anaerobic incubation at 43°C could be used and only small rough brownish colonies should be counted as *L. acidophilus*.

Table 5. Media recommended for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and propionibacteria and viable counts of in a mixture of bacteria.

Agar	Bacteria	Incubation conditions	Colony morphology	Counts in a mixture of bacteria (cfu/ml)
<i>S. thermophilus</i> agar	<i>S. thermophilus</i>	Aerobic, 37°C, 24 h	0.1–0.5 mm, round yellowish	3.9×10^4
MRS ¹ agar (pH 4.58)	<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	Anaerobic, 45°C, 72 h	1.0 mm, white, cottony, rough, irregular	7.0×10^7
MRS-sorbitol agar	<i>L. acidophilus</i>	Anaerobic, 37°C, 72 h	Rough, dull, small (0.1–0.5), brownish	10.0×10^7
MRS-NNLP ² agar	Bifidobacteria	Anaerobic, 37°C, 72 h	1 mm, white, smooth, shiny	7.0×10^7
MRS-vancomycin agar ³	<i>L. casei</i>	Anaerobic, 37°C, 72 h	1.0 mm, white shiny, smooth	5.3×10^7
MRS-vancomycin agar	<i>L. rhamnosus</i>	Anaerobic, 43°C, 72 h	1.0–2.0 mm, white shiny, smooth	7.6×10^7
Sodium lactate agar	Propionibacteria ⁴	Anaerobic, 30°C, 7 to 9 d	1.0–2.5 mm, dull brown, lighter margin	4.9×10^7

¹deMan, Rogosa, and Sharpe agar.

²Nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate.

³If *L. rhamnosus* was not present; however, if *L. rhamnosus* was present, then subtraction methods could be used (i.e., subtracting *L. rhamnosus* counts on MRS-vancomycin agar under anaerobic incubation at 43°C for 72 h from total counts of *L. casei* and *L. rhamnosus* obtained in MRS-vancomycin agar under anaerobic incubation at 37°C for 72 h).

⁴Subtraction method could also be used to determine the counts of propionibacteria (i.e., counts of *L. casei* and *L. rhamnosus* (anaerobic incubation, 30°C, 72 h) could be subtracted from counts of *L. casei*, *L. rhamnosus*, and propionibacteria (anaerobic incubation, 30°C, 7 d).

Among the media tested for *L. acidophilus*, BA-sorbitol agar gave the highest recovery (Table 1). In this medium, *L. casei* and *L. rhamnosus* formed shiny, large, smooth and white colonies, while all strains of *L. acidophilus* tested formed rough dull, small, and brownish colonies. Therefore, only the small dull rough brownish colonies should be enumerated as the counts of *L. acidophilus*.

Table 5 summarizes the media that could be used for selective enumeration of the seven groups of bacteria and their incubation conditions and colony morphology. To verify the efficacy of the method selected in this study, mixtures of *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. acidophilus*, bifidobacteria, *L. casei*, *L. rhamnosus*, and propionibacteria cells were added at approximately 10^7 cfu/ml in the ratio of 0.1, 0.5, 4, 2, 1, 1, and 1, respectively, and the organisms were plated in the media under incubations outlined in Table 5. The identity of each organism was verified by biochemical tests (Kandler and Weiss, 1986). The results are presented in Table 5. As shown in the table, the media were discriminatory for the various groups of bacteria. Thus it appears that the methods could be used for selective enumeration of the seven groups of bacteria used in this study.

Enumeration of Bacteria in Commercial Products

Because the evaluation of media for selective enumeration of yogurt and probiotic bacteria was carried out using pure cultures, it was desirable to validate the efficacy of the method selected using commercial products. Five brands of commercial yogurts and one brand of Swiss cheese were purchased from a local super market, and their bacterial populations analyzed using the different selective bacteriological media. Enumeration of *S. thermophilus* was carried out using ST agar and aerobic

incubation at 37°C for 24 h. *L. delbrueckii* ssp. *bulgaricus* was enumerated using MRS-agar (pH 4.58) and anaerobic incubation at 45°C for 72 h. For *L. rhamnosus*, MRS-V agar and anaerobic incubation at 43°C were used. *L. casei* was enumerated using subtraction method, in which viable counts of *L. rhamnosus* on MRS-V agar at 43°C under anaerobic incubation were subtracted from the total counts of *L. casei* and *L. rhamnosus* on MRS-V at 37°C under anaerobic incubation. Bifidobacteria were enumerated on MRS-NNLP agar. Enumeration of *L. acidophilus* was carried out using BA-sorbitol agar at 37°C and anaerobic incubation for 72 h, and BA-maltose agar and anaerobic incubation at 43°C for 72 h. Only the small rough brownish colonies (0.1 to 0.5 mm) were counted as *L. acidophilus*. Propionibacteria were enumerated by subtracting the counts at d 3 of lactic acid bacteria on NaLa agar and anaerobic incubation at 30°C from the total counts of lactic acid bacteria and propionibacteria at day 7.

Table 6 shows the organisms claimed to be present in commercial products and the actual recovery of the organisms. *S. thermophilus* was present in all of yogurts tested. *L. delbrueckii* ssp. *bulgaricus* was present only in product 5 (skinny yogurt). Many commercial products are manufactured using *L. acidophilus*, bifidobacteria and *S. thermophilus* cultures, which do not contain *L. delbrueckii* ssp. *bulgaricus*. *L. casei* was claimed to be present in both products 4 and 5, however, only product 4 (natural yogurt) showed reasonable population of this organism. The stage of shelf life and the pH of yogurt might have affected the viability of the probiotic organism.

Product 2 (natural yogurt) and product 3 (flavored yogurt) had high counts of all organisms claimed including *S. thermophilus*, *L. rhamnosus*, *L. acidophilus*, and

Table 6. Recovery of organisms in commercial products.

Products	Organisms claimed to be present	Organisms present in commercial products						
		<i>L. bulgaricus</i> ¹	<i>S. thermophilus</i> ²	<i>L. acidophilus</i> ³	<i>L. casei</i> ⁴	<i>L. rhamnosus</i> ⁵	<i>B. lactis</i> ⁶	<i>Propionibacteria</i> ⁷
Product 1 (yogurt)	Yogurt culture	<3.00	8.69	<3.00	<3.00	<3.00	<3.00	<3.00
Product 2 (natural yogurt)	Yogurt culture, <i>L. acidophilus</i> , <i>Bifidobacterium</i> , and <i>L. rhamnosus</i> GG	<3.00	9.17	5.23	<3.00	7.36	7.15	<3.00
Product 3 (flavoured yogurt)	Yogurt culture, <i>L. acidophilus</i> , <i>Bifidobacterium</i> , and <i>L. rhamnosus</i> GG	<3.00	9.01	6.53	<3.00	7.72	7.40	<3.00
Product 4 (natural yogurt)	Yogurt culture, <i>L. acidophilus</i> , <i>Bifidobacterium</i> and <i>L. casei</i>	7.68	8.83	7.83	5.53	<3.00	6.54	<3.00
Product 5 (skinny yogurt)	Yogurt culture, <i>L. acidophilus</i> , <i>Bifidobacterium</i> and <i>L. casei</i>	4.92	8.62	5.51	4.01	<3.00	6.36	<3.00
Product 6 (Swiss cheese)	Cheese culture	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	3.59

¹Enumerated using MRS agar (pH 5.58).²Enumerated using *S. thermophilus* agar T agar.³Enumerated using BA-sorbitol agar.⁴Enumerated using subtraction method (counts in MRS-vancomycin agar at 37°C – counts in MRS-vancomycin agar at 43°C).⁵Enumerated using subtraction method (counts in MRS-vancomycin agar at 43°C).⁶Enumerated using MRS-NNLP agar.⁷Enumerated using subtraction method (counts in NaLa agar at d 7 – counts in NaLa agar at day 3).

bifidobacteria. Products 2 and 3 contained *L. rhamnosus*. Bifidobacteria were found in products 4 and 5 and products 2 and 3 in high concentrations (10^6 to 10^7). *L. acidophilus* also was found in appreciable concentration in all yogurt claimed to contain this organism. Propionibacteria were only found in Swiss cheese (product 6) and this was the only product that claimed to contain propionibacteria. The identity of the organisms was confirmed using Gram stain. Thus it appears that the enumeration methods developed and selected in this study were suitable for enumeration of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, bifidobacteria and propionibacteria.

CONCLUSIONS

In this study 19 bacteriological media were evaluated under different incubation conditions for their suitability to recover and enumerate 7 species containing 18 strains of bacteria including *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, bifidobacteria, and propionibacteria. The evaluation was based on sugar fermentation patterns, use of inhibitory substances (such as acid, bile, salt and antibiotics), different incubation temperatures (27, 30, 37, 43, and 45°C), incubation conditions (such as aerobic and anaerobic) and the duration of incubation (24, 72 h, or 7 to 9 d). ST agar under aerobic incubation at 37°C for 24 h was suitable for *S. thermophilus*. *L. delbrueckii* ssp. *bulgaricus* could be enumerated using MRS agar (pH 4.58) and anaerobic incubation at 45°C for 72 h. MRS-vancomycin agar and anaerobic incubation for 72 h at 43°C were suitable for enumeration of *L. rhamnosus*. MRS-vancomycin agar and anaerobic incubation at 37°C for 72 h or LC agar at 27°C for 72 h and anaerobic incubation were selective for enumeration of *L. casei*, when *L. rhamnosus* was not present in the mixture. *L. casei* could also be enumerated by subtraction method if *L. rhamnosus* was present in the product. The counts of *L. rhamnosus* on MRS-vancomycin agar under anaerobic incubation at 43°C for 72 h could be subtracted from total counts of *L. casei* and *L. rhamnosus* on MRS-vancomycin agar at 37°C for 72 h under anaerobic incubation to obtain *L. casei* count. Bifidobacteria could be enumerated on MRS-NNLP agar. The most suitable method for counting propionibacteria was by subtracting the counts at d 3 of all bacteria except propionibacteria on NaLa agar under anaerobic incubation at 30°C from the total counts at d 7 of all bacteria including propionibacteria under same incubation conditions. Counting large (1.0 to 2.5 mm diameter), smooth brownish colonies with lighter margin on sodium lactate agar after 7 to 9 d at 30°C under anaerobic incubation could also be used to count propionibacteria. *L. acidophilus* could be enumerated on BA-

sorbitol agar at 37°C for 72 h under anaerobic incubation or MRS-agar at 43°C for 72 h under anaerobic incubation or BA-maltose agar at 43°C under anaerobic incubation.

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