Evaluation of Media for Selective Enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and Bifidobacteria

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**ABSTRACT**

Fifteen media were evaluated to determine their suitability for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria using 5 to 6 strains of each of the four groups of organisms. *Streptococcus thermophilus* agar was found to be suitable for selective enumeration of *S. thermophilus* under aerobic incubation at 37°C for 24 h. The MRS agar at pH 5.2 or reinforced clostridial agar at pH 5.3 could be used for the selective enumeration of *L. delbrueckii* ssp. *bulgaricus* when the incubation was carried out at 45°C for ≥72 h. However, the recovery of this organism was lower on MRS agar at pH 5.2 or reinforced clostridial agar at pH 5.3 than that obtained on MRS agar. The recovery of *L. acidophilus* and bifidobacteria on MRS agar and MRS-maltose agar was similar; MRS-maltose agar could be used to enumerate total counts of *L. acidophilus* and bifidobacteria. For selective enumeration of *L. acidophilus*, MRS-salicin agar or MRS-sorbitol agar could be used. For selective enumeration of bifidobacteria, MRS NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) agar was suitable; however, determination of bifidobacteria by differential counts between *L. acidophilus* enumerated on MRS-salicin agar or MRS-sorbitol agar and the total counts of *L. acidophilus* and bifidobacteria obtained from MRS-maltose agar resulted in higher recovery of some strains of bifidobacteria. Other media that were evaluated in this study were not suitable for selective enumeration.

**Key words:** enumeration, *Streptococcus thermophilus*, *Lactobacillus* spp., bifidobacteria

**INTRODUCTION**

A number of health benefits have been claimed for *Lactobacillus acidophilus* and bifidobacteria (7, 12, 16, 18, 19, 20, 22, 34, 36). Because of the potential health benefits, these organisms are increasingly incorporated into dairy products. More than 90 products containing acidophilus and bifidus are available in the market worldwide (19, 20, 24).

*Lactobacillus acidophilus* and bifidobacteria grow slowly in milk during product manufacture. Therefore, the usual production practice is to incorporate yogurt cultures (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. It seems reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested. In order to realize therapeutic properties, a minimum of $10^5$ viable cells of *L. acidophilus* and bifidobacteria/g should be present in a product (26). An important parameter in monitoring viable organisms during assessment of product quality is the ability to count *L. acidophilus* and bifidobacteria differentially.

Several media have been developed for differential enumeration of yogurt culture organisms, including lactic acid bacteria agar (5), Lee's agar (17), and reinforced clostridial agar (RCA) at pH 5.5 (10). Jordono et al. (11) examined different M17 media for the recovery of *S. thermophilus*. Hamann and Marth (6) evaluated four differential and two general purpose media for enumerating yogurt culture organisms.

Several media have been suggested for the enumeration of *L. acidophilus*, including bile medium (4), Rogosa agar, oxygen-reducing membrane fraction (2), MRS medium containing maltose, salicin, raffinose, or melibiose in place of dextrose (8), cellobiose esculin agar (9), and agar medium based on X-Glu (14). Similarly, several selective media have been developed for enumeration of bifidobacteria (1, 2, 3, 15, 21, 23, 29, 27, 28, 29, 31, 32, 33, 35). However, these media may not be suitable for selective enumeration of *L. acidophilus* and Bifidobac-
terium spp. in the presence of yogurt culture organisms (S. thermophilus and L. delbrueckii ssp. bulgaricus). Further, differences exist among the strains of the same species in low pH tolerance, bile salts, NaCl, and sugar fermentation characteristics (13). There is growing concern that some media that contain bile or antibiotics might also restrict the growth of L. acidophilus or bifidobacteria and that the counts obtained are not representative of the actual number of viable cells that are present in the product. This study examined a range of media that could possibly be used in selective enumeration of yogurt organisms and L. acidophilus and bifidobacteria.

**MATERIALS AND METHODS**

**Cultures**

Pure cultures of 6 strains of S. thermophilus (2000, 2002, 2008, 2010, 2013, and 2014), 5 strains of L. delbrueckii ssp. bulgaricus (2501, 2505, 2515, 2517, and 2519), 5 strains of L. acidophilus (2400, 2401, 2404, 2408, and 2415), 2 strains of Bifidobacterium bifidum (1900 and 1901), 2 strains of Bifidobacterium longum (1941 and 20097), and 1 strain each of Bifidobacterium adolescentis (1920), Bifidobacterium pseudolongum (20099), Bifidobacterium breve (1930), and Bifidobacterium infantis (1912) were obtained from the Dairy Research Laboratory (Commonwealth Scientific and Industrial Research Organisation, Highett, Victoria, Australia). The cultures were grown at 37°C for 16 to 22 h in sterile reconstituted 12% nonfat dry milk supplemented with 1% glucose and 0.3% yeast extract. The cultures were maintained in the same medium at 4 to 5°C and were transferred weekly. Before enumeration, the cultures were transferred successively three times for activation.

**Media Preparation**

**Diluent of peptone and water.** Diluent of peptone and water (0.15%) was prepared by dissolving 1.5 g of peptone water medium (Oxoid, West Heidelberg, Australia) in 1 L of distilled water, adjusting the pH to 7.0 ± 0.2, followed by autoclaving 9-ml portions at 121°C for 15 min.

**Streptococcus thermophilus agar.** The ingredients of S. thermophilus agar (ST agar; 10.0 g of tryptone, 10.0 g of sucrose, 5.0 g of yeast extract, and 2.0 g K2HPO4) were dissolved in 1 L of distilled water. The pH was adjusted to 6.8 ± 0.1, and 6 ml of 0.5% bromocresol purple and 12 g of agar were added to the medium. The medium was sterilized at 121°C for 15 min.

**MRS agar, pH-modified MRS agar (pH 5.2 and 5.8), MRS-bile agar, MRS-oxgall agar, and MRS-NaCl agar.** Rehydrated MRS broth (Oxoid) was prepared according to manufacturer instructions. To obtain pH-modified MRS agars, 1.0 M HCl was used to adjust the pH of the medium to 5.2 or 5.8. To prepare MRS-bile agar, 2.0 g of pure bile salts (Amyl Media, Dandenong, Australia)/L of MRS broth were added; for MRS-oxgall agar, 10.0 g of oxgall powder (Oxoid)/L of MRS broth were added. Twenty grams of NaCl/L (2% final concentration) of MRS broth were dissolved to obtain MRS-NaCl agar. After the broth was prepared, agar powder was added at the rate of 1.2%, and the media were autoclaved at 121°C for 15 min.

**MRS-maltose agar, MRS-salicylic acid, and MRS-sorbitol agar.** Rehydrated MRS-maltose agar (Amyl Media) was prepared according to the manufacturer. To prepare MRS-salicylic acid and MRS-sorbitol agars, MRS basal medium was prepared without dextrose, and 10 ml of membrane-filtered sterile solutions of 10% salicin or D-sorbitol were added per 90 ml of basal medium (1% final concentration) just before pouring the agar medium.

**MRS-NNLP agar.** The MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate; Sigma Chemical Co., Castle Hill, Australia) agar was prepared according to the method described by Laroia and Martin (15). The basal medium was MRS agar. Filter-sterilized NNLP was added to the autoclaved MRS base just 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.

**RCA agar (pH 6.8 and 5.3).** Rehydrated RCA agar (Oxoid) was prepared according to manufacturer instructions, and the pH of the medium was adjusted with 1.0 M HCl to 6.8 or 5.3. The medium was brought to boiling to dissolve all the ingredients properly and then autoclaved at 121°C for 15 min.

**Rogosa acetate agar.** Rogosa acetate agar (Oxoid) was prepared according to instructions of the manufacturer. All contents were properly dissolved in distilled water, and the medium was brought to boiling after the pH was adjusted to 5.4 with glacial acetic acid. The medium was then filled in sterile bottles and stored at 7°C or less until used.

**Cellbiose esculin agar.** This medium was prepared according to the formula given by Hunger (9).
TABLE 1. Viable counts of *Streptococcus thermophilus* strains on different bacteriological agars.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>ST2</th>
<th>MRS (pH 5.8)</th>
<th>MRS (pH 5.2)</th>
<th>RCA (pH 5.3)</th>
<th>RCA (pH 6.8)</th>
</tr>
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<tr>
<td></td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
</tr>
<tr>
<td>2000</td>
<td>8.72</td>
<td>8.28</td>
<td>5.93</td>
<td>8.08</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>8.26</td>
<td>8.20</td>
<td>7.11</td>
<td>8.18</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>8.99</td>
<td>8.96</td>
<td>7.30</td>
<td>8.93</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>9.08</td>
<td>9.10</td>
<td>7.04</td>
<td>8.93</td>
<td></td>
</tr>
<tr>
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<td>8.85</td>
<td>8.69</td>
<td>&lt;3.00</td>
<td>7.95</td>
<td></td>
</tr>
<tr>
<td>2014</td>
<td>8.79</td>
<td>8.64</td>
<td>7.00</td>
<td></td>
<td>8.15</td>
</tr>
</tbody>
</table>

1All strains were <10³ cfu/g for the following media: MRS (5.2 pH), MRS-bile, MRS-oxgall, MRS-NaCl, MRS-maltose, MRS-salicin, MRS-sorbitol, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate), RCA (reinforced clostridial agar) (5.3 pH), Rogosa acetate, and cellubiose esculin.

2*Streptococcus thermophilus* agar.

3Reinforced clostridial agar.

4Tiny colonies.

Enumeration of Bacteria

The activated cultures obtained after three successive transfers were used for the purpose of enumeration. One gram of each culture was 10-fold serially diluted (10³ to 10⁷) in 0.15% sterile peptone water. Enumeration was carried out using the pour plate technique, except when cellubiose esculin agar was used. For this agar, the spread-plate technique was used. Duplicate plates were incubated anaerobically (except for ST agar plates) at 37°C for 72 h using a gas mixture of 10% CO₂, 5% H₂, and 85% N₂ (CIG gases) in Oxoid jars (Oxoid). The ST agar plates were incubated aerobically (except for bifidobacteria plates) at 37°C for 24 h. Plates containing 25 to 250 colonies were enumerated and recorded as colony-forming units per gram of culture.

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

RESULTS AND DISCUSSION

Viable counts of *S. thermophilus* in various bacteriological media are presented in Table 1. The ST agar gave higher recovery of *S. thermophilus* than did MRS agar. Lower recovery of *S. thermophilus* in MRS medium could possibly have been due to the presence of sodium acetate in this medium. The cultures of *S. thermophilus* formed well-developed, yellow colonies on ST agar plates within 24 h of incubation; *L. delbrueckii* ssp. *bulgaricus* either did not grow (strains 2515 and 2519) or formed tiny, white, cottony colonies (strains 2501, 2505, and 2517) (Table 2) that could easily be distinguished from *S. thermophilus*. Shankar and Davies (30) reported that the growth of *L. delbrueckii* ssp. *bulgaricus* was suppressed on pour plates, when the pH of the M17 medium was adjusted to 6.8. The pH of ST agar is 6.8, which might have been crucial in the growth suppression of lactobacilli when the plates were incubated for 24 h. Cultures of *L. acidophilus* failed to grow on ST agar plates within 24 h of incubation under aerobic conditions (Table 3); however, this organism formed yellow colonies after 72 h of incubation. Bifidobacteria, which are anaerobic, did not grow on ST agar when incubated aerobically (data not shown). Thus, ST agar could be used for the enumeration of *S. thermophilus* from a product containing all 4 organisms, provided that the plates were incubated aerobically at 37°C for 24 h. Recovery of *S. thermophilus* in MRS agar at pH 5.8 was 30 to 90% less than that on ST agar or RCA agar (pH 5.8) (Table 1). Growth of *S. thermophilus* on other bacteriological media did not occur (Table 1). *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and bifidobacteria grew on RCA agar (pH 6.8) (Tables 2 to 4), and, thus, RCA agar could not be used for the differential enumeration of *S. thermophilus*.

TABLE 2. Viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains on different bacteriological agars.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>ST2</th>
<th>MRS (pH 5.8)</th>
<th>MRS (pH 5.2)</th>
<th>RCA3 (pH 6.8)</th>
<th>RCA (pH 5.3)</th>
<th>Cellubiose esculin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
<td>(log₁₀ cfu/g)</td>
</tr>
<tr>
<td>2501</td>
<td>8.63</td>
<td>8.60</td>
<td>8.46</td>
<td>7.34</td>
<td>8.66</td>
<td>7.60</td>
</tr>
<tr>
<td>2505</td>
<td>9.08</td>
<td>9.08</td>
<td>8.15</td>
<td>8.00</td>
<td>9.28</td>
<td>8.18</td>
</tr>
<tr>
<td>2515</td>
<td>&lt;3.00</td>
<td>7.23</td>
<td>6.40</td>
<td>6.04</td>
<td>7.28</td>
<td>4.00</td>
</tr>
<tr>
<td>2517</td>
<td>8.64</td>
<td>8.72</td>
<td>7.64</td>
<td>7.30</td>
<td>8.73</td>
<td>7.76</td>
</tr>
<tr>
<td>2519</td>
<td>&lt;3.00</td>
<td>8.20</td>
<td>8.08</td>
<td>7.88</td>
<td>8.20</td>
<td>7.23</td>
</tr>
</tbody>
</table>

1All strains were <10³ cfu/g for the following media: MRS-bile, MRS-oxgall, MRS-NaCl, MRS-maltose, MRS-salicin, MRS-sorbitol, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate), and Rogosa acetate.

2*Streptococcus thermophilus* agar.

3Reinforced clostridial agar.

4Tiny white cottony colonies.

5No typical appearance of cottony colonies.
Table 2 shows the viable counts of *L. delbrueckii* ssp. *bulgaricus* on various bacteriological media. Recovery of *L. delbrueckii* ssp. *bulgaricus* was highest on RCA agar (pH 6.8), followed by recovery on MRS agar. The growth of *L. delbrueckii* ssp. *bulgaricus* was partially inhibited when the pH of the MRS agar was lowered to 5.8, and the growth was further inhibited at pH 5.2. A similar pattern occurred with RCA medium. Growth of 1 strain of *L. delbrueckii* ssp. *bulgaricus* (strain 2515) did not occur on RCA agar adjusted to pH 5.3. These results differed from those observed by Johns et al. (10), who found complete recovery of *L. delbrueckii* ssp. *bulgaricus*. Lactobacillus *delbrueckii* ssp. *bulgaricus* did not grow on MRS-bile, MRS-oxgall, MRS-NaCl, MRS-maltose, MRS-saliciin, MRS-sorbital, MRS-NNLP, or Rogosa acetate agars. At pH 5.3, RCA agar allowed the growth of 2 strains of *L. acidophilus* (strains 2400 and 2415) (Table 3) and 4 strains of bifidobacteria (Table 4); therefore, this medium may not be suitable for purposes of differential enumeration.

In a separate experiment, we found that MRS agar at pH 5.2 or RCA agar at 5.3 could be used for differential enumeration of *L. delbrueckii* ssp. *bulgaricus* when the plates were incubated anaerobically at 45°C for 72 h (data not included). Some of the bifidobacteria also grew on these media; however, the colonies formed by *L. delbrueckii* ssp. *bulgaricus* could easily be differentiated from those of bifidobacteria. The recovery of *L. delbrueckii* ssp. *bulgaricus* was one to two log cycles lower than that obtained on MRS agar at 37°C.

*Lactobacillus delbrueckii* ssp. *bulgaricus* either did not grow (strain 2517) or formed tiny white cottony colonies (strains 2505, 2515, and 2519), except for strain 2501 on cellobiose esculin agar. The results are comparable with those reported by Hunger (9). Cellobiose esculin agar supported the growth of *L. acidophilus* (except for strain 2415), and, thus, this medium may not be suitable for the selective enumeration of *L. delbrueckii* ssp. *bulgaricus*.

A subtraction method (subtracting counts of *S. thermophilus* enumerated on ST agar and *L. acidophilus* and bifidobacteria enumerated on MRS-maltose agar from the total counts of all four groups of organisms enumerated on MRS agar) could be used; however, the number of organisms present in a product should be in equal proportion, or the subtraction method might not be suitable because the plates containing 25 to 250 colonies are considered to be ideal for enumeration purposes. Onggo and Fleet (23) observed no growth of *S. thermophilus* in MRS agar. Thus, if a strain of *S. thermophilus* used in a product did not form colonies on MRS agar plates, as was observed by Onggo and Fleet (23), counts of *L. delbrueckii* ssp. *bulgaricus* could be obtained by subtracting counts of *L. acidophilus* and bifidobacteria enumerated on MRS-maltose agar from the total counts obtained using MRS agar.

Viable counts of *L. acidophilus* enumerated on various bacteriological media are shown in Table 3. *Lactobacillus acidophilus* culture did not grow on ST agar (incubated at 37°C for 24 h) or on MRS-NNLP agar. Of the several media evaluated, MRS, MRS-maltose, MRS-saliciin, and MRS-sorbital agars gave almost the same recovery for all of the *L. acidophilus* cultures. The growth of this organism was slightly inhibited on MRS agar at pH 5.8 and was further inhibited at pH 5.2. The recovery of *L. acidophilus* was one to two log cycles lower in MRS medium containing bile or oxgall. The MRS-NaCl agar showed almost 40 to 60% inhibition.

Growth of bifidobacteria was supported by MRS-maltose agar (Table 4); aerobic incubation to suppress the growth of this organism resulted in 40 to 70% less recovery of some of the *L. acidophilus* cul-

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**TABLE 3. Viable counts of *Lactobacillus acidophilus* strains on different bacteriological agars.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MRS (pH 5.8)</th>
<th>MRS (pH 5.2)</th>
<th>MRS-Bile</th>
<th>MRS-Oxgall</th>
<th>MRS-NaCl</th>
<th>MRS-Maltose</th>
<th>MRS-Saliciin</th>
<th>RCA (pH 6.8)</th>
<th>RCA (pH 5.3)</th>
<th>Rogosa acetate</th>
<th>Cellobiose esculin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2400</td>
<td>8.64</td>
<td>8.63</td>
<td>8.60</td>
<td>7.43</td>
<td>7.38</td>
<td>8.48</td>
<td>8.63</td>
<td>8.65</td>
<td>8.65</td>
<td>6.38</td>
<td>5.88</td>
</tr>
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<td>6.90</td>
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<td>6.75</td>
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<td>7.48</td>
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<td>&lt;3.00</td>
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<td>&lt;3.00</td>
<td>&lt;3.00</td>
</tr>
<tr>
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<td>8.53</td>
<td>8.49</td>
<td>7.20</td>
<td>7.52</td>
<td>7.72</td>
<td>7.51</td>
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<td>8.62</td>
<td>8.61</td>
<td>&lt;3.00</td>
<td>&lt;3.00</td>
</tr>
<tr>
<td>2415</td>
<td>8.70</td>
<td>8.64</td>
<td>6.18</td>
<td>7.00</td>
<td>7.08</td>
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<td>8.65</td>
<td>8.72</td>
<td>8.64</td>
<td>7.59</td>
<td>8.48</td>
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</table>

1. All strains were <10³ cfu/g for MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) and ST (*Streptococcus thermophilus* agar); the latter showed no growth of *L. acidophilus* within 24 h of aerobic incubation at 37°C.
2. Reinforced clostridial agar.
3. No typical appearance; i.e., dark-centered, 1.0- to 1.5-mm colonies of *L. acidophilus* with greenish-brown zone.

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L. acidophilus. The colonies formed by bifidobacteria that grew on MRS-salicin and MRS-sorbitol agar plates were easily distinguishable from those of L. acidophilus. Thus, MRS-salinic agar or MRS-sorbitol agar could be used for differential enumeration of L. acidophilus because S. thermophilus and L. delbrueckii ssp. bulgaricus did not grow on these media (Tables 1 to 2). These results are similar to the findings of Hull and Roberts (8), who observed almost the same recovery of L. acidophilus when dextrose in MRS agar was replaced with maltose and salicin. However, salicin was uneconomical for the routine testing, and, hence, the use of sorbitol to enumerate L. acidophilus selectively was investigated in this study. Use of MRS-sorbitol medium for differential enumeration of L. acidophilus has not been reported earlier.

The MRS-salicin and MRS-sorbitol agars supported the growth of L. acidophilus (Table 3), but bifidobacteria either did not grow or grew poorly (Table 4) on these complex media based on sugar. The colonies formed by bifidobacteria that grew on MRS-salicin and MRS-sorbitol agar plates were easily distinguishable from those of L. acidophilus. Thus, MRS-salinic agar or MRS-sorbitol agar could be used for differential enumeration of L. acidophilus because S. thermophilus and L. delbrueckii ssp. bulgaricus did not grow on these media (Tables 1 to 2). These results are similar to the findings of Hull and Roberts (8), who observed almost the same recovery of L. acidophilus when dextrose in MRS agar was replaced with maltose and salicin. However, salicin was uneconomical for the routine testing, and, hence, the use of sorbitol to enumerate L. acidophilus selectively was investigated in this study. Use of MRS-sorbitol medium for differential enumeration of L. acidophilus has not been reported earlier.

Similarly, 2 strains of L. acidophilus (2401 and 2404) did not grow on Rogosa acetate agar, and the recovery of the other 3 strains was adversely affected on this medium.

Cellobiose esculin agar suppressed the growth of S. thermophilus (Table 1) and L. delbrueckii ssp. bulgaricus (Table 2). All of the strains of L. acidophilus (except strain 2415) (Table 3) formed colonies, but bifidobacteria (except for B. pseudolongum 20099) formed tiny colonies (Table 4), which could be easily differentiated. Thus, cellobiose esculin agar could be used for differential enumeration of L. acidophilus (except strain 2515) from a product containing all 4 organisms, provided B. pseudolongum (strain 20099) was not incorporated into the product (Table 4). However, the recovery of L. acidophilus on cellobiose esculin agar was less than that on MRS, MRS-maltose, MRS-salicin, or MRS-sorbitol agars.

Viable counts of bifidobacteria on different bacteriological media are presented in Table 4. As shown, ST agar supported the growth of B. longum (20097) and B. pseudolongum (20099) when grown anaerobically, but the other strains formed tiny colonies or failed to grow. The MRS agar provided the best recovery of bifidobacteria, but the other three groups of organisms grew as well on this medium (Tables 1 to 3). At pH 5.8, MRS agar did not inhibit bifidobacteria cultures. However, recovery of bifidobacteria was adversely affected on MRS agar at pH 5.2. Recovery of bifidobacteria on MRS medium containing bile, oxgall, or NaCl varied, and the counts remained lower than those obtained on MRS agar. Lactobacillus acidophilus also grew on MRS-bile, MRS-oxgall, and MRS-NaCl agars, and these media therefore may not be suitable for the purposes of selective enumeration.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ST1</th>
<th>MRS (pH 5.8)</th>
<th>MRS (pH 6.8)</th>
<th>MRS-bile</th>
<th>MRS-oxgall</th>
<th>MRS-NaCl</th>
<th>MRS-maltose</th>
<th>MRS-salicin</th>
<th>MRS-sorbitol</th>
<th>MRS-NLNP1 (pH 6.8)</th>
<th>RCA (pH 5.3)</th>
<th>RCA (pH 5.8)</th>
<th>Cellobiose esculin</th>
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<tr>
<td>1920</td>
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<td>8.15</td>
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<td>7.15</td>
<td>8.15</td>
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<td>&lt;3.00</td>
<td>&lt;3.00</td>
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<td>9.89</td>
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1Streptococcus thermophilus agar.
2Nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate.
3Reinforced clostridial agar.
4Tiny colonies.
5Pinpoint colonies.
6No poor growth of bifidobacteria on cellobiose esculin agar.
The MRS-maltose agar gave almost the same recovery of bifidobacteria as that obtained on MRS agar, but bifidobacteria either did not grow or formed pinpoint colonies on MRS-salicyn agar plates (except for B. pseudolongum 20099, which formed normal colonies) and on MRS-sorbitol agar.

Several media containing one or more antimicrobial substances have been suggested for the enumeration of bifidobacteria. The MRS-NNLP agar is widely used for the recovery and enumeration of bifidobacteria by researchers and quality control laboratories. This medium was originally developed by Teraguchi et al. (33). We found poor recovery of B. adolescentis (1920) and B. pseudolongum (20099) strains in MRS-NNLP medium (Table 4). Four types of antibiotics are used as selective agents in MRS-NNLP medium, and one or more of these might have affected the growth of B. adolescentis and B. pseudolongum. Therefore, the counts obtained on MRS-NNLP agar may not be representative of the viable cells that are present in the product, which suggests a need to countercheck the efficacy of this medium with pure cultures before the medium is adopted for enumeration purposes.

To obtain a true picture of bifidobacterial strains tested in this study, L. acidophilus count obtained using MRS-salicyn agar or MRS-sorbitol agar should be subtracted from a total count of L. acidophilus and bifidobacteria obtained from MRS-maltose agar; other strains or species not tested might use salicin and sorbitol. Therefore, we recommend the counterchecking of pure strains of bifidobacteria in these media before they are adopted for enumeration purposes in the presence of yogurt cultures and L. acidophilus. Similarly, enumeration of bifidobacteria using MRS-NNLP agar should be carried out simultaneously, because a difference of more than one to two log cycles in the counts of bifidobacteria in a product containing L. acidophilus do not give an accurate count of bifidobacteria by the subtraction method.

Recovery of all the bifidobacteria obtained on RCA agar was almost identical to that on MRS agar at pH 6.8, but the recovery was slightly lower on RCA agar at pH 5.3. Bifidobacteria formed well-developed colonies on RCA agar plates at 5.3 (except for B. bifidum 1900 and 1901). Recovery of bifidobacteria on Rogosa acetate agar was two to three log cycles lower. Lactobacillus acidophilus counts on this medium were also lower (Table 3), which could have been due to the presence of high concentration of sodium acetate, low pH of the medium, or use of glacial acetic acid to adjust the pH.

Bifidobacteria formed tiny colonies on cellobiose esculin agar (except for B. adolescentis 1920, B. longum 1941, and B. pseudolongum 20099). Hunger (9) also observed poor growth of bifidobacteria on cellobiose esculin agar. Conversely, in our study, 3 of 6 strains of bifidobacteria exhibited good growth. Lactobacillus acidophilus strain 2415 failed to show typical colony development. Therefore, cellobiose esculin agar might not be an ideal medium for the enumeration purposes.

**Commercial products.** Because the evaluation of media for the selective enumeration of yogurt bacteria, L. acidophilus, and bifidobacteria was carried out using pure cultures, it was desirable to enumerate these organisms selectively from products containing mixtures of these organisms. In a separate experiment, 6 commercial products were analyzed using the different selective bacteriological media. The samples that were analyzed included probiotic capsules (Blackmores Ltd, Balgowlah, Victoria, Australia) claimed to contain L. acidophilus and bifidobacteria, a commercial yogurt sample (Yoplait, Echuca, Victoria, Australia), yogurt prepared in our laboratory using a commercial starter culture (Chr. Hansen, Bayswater, Victoria, Australia) containing all four groups of organisms, and three frozen Bulla brand Fruit’ N Yoghurt sticks (Regal Cream Products Pty. Ltd., North Melbourne, Victoria, Australia) claimed to contain L. acidophilus and Bifidobacterium spp. The results presented in Table 5 as the log_10 viable counts per capsule (for Blackmores’ capsules) and per gram for the rest of the samples. Presumptive enumeration of S. thermophilus was carried out on ST and M17 agars (aerobic incubation at 37°C for 24 h); L. delbrueckii spp. bulgaricus was enumerated on MRS (pH 5.2) agar (anaerobic incubation at 45°C for 72 h); L. acidophilus was enumerated on MRS-salicyn agar, MRS-sorbitol agar, cellobiose esculin agar, and MRS-maltose agar (using aerobic incubation at 37°C for 72 h); and bifidobacteria were enumerated on MRS-NNLP agar and also by the subtraction method (i.e., subtracting counts of L. acidophilus obtained on MRS-salicyn or MRS-sorbitol agar from the total counts of L. acidophilus and bifidobacteria obtained on MRS-maltose agar).

As shown in Table 5, ST agar appeared to be selective in enumerating S. thermophilus; the counts obtained on ST agar were higher than or similar to those on M17 agar. The ST agar was more economical than the M17 media, and the presence of sucrose in ST agar could make ST agar more practical for the recovery of S. thermophilus in the presence of other organisms. The MRS agar (pH 5.2) was suitable for counting L. delbrueckii spp. bulgaricus differentially.
Both MRS-salicin agar and MRS-sorbitol agar appeared to be selective in enumerating \textit{L. acidophilus}. Cellobiose esculin agar and MRS-maltose agar (using aerobic incubation) also enumerated \textit{L. acidophilus} selectively; however, counts were 40 to 70% fewer than those obtained on MRS-salicin agar or MRS-sorbitol agar. The MRS-NNLP agar appeared to be selective for bifidobacteria; however, counts of bifidobacteria obtained by the subtraction method were higher than those enumerated using MRS-NNLP agar.

Further, we found growth of \textit{B. brevii} (strain 1930) and \textit{B. infantis} (strain 1912) on MRS-sorbitol and MRS-salicin agars (data not included). The colonies formed by these two organisms were not able to be differentiated from those of \textit{L. acidophilus}. Therefore, in products containing salicin and sorbitol-positive bifidobacteria, aerobic incubation of MRS-maltose agar would be useful for selective enumeration of \textit{L. acidophilus} in the presence of other groups of organisms.

CONCLUSIONS

In this study, 15 media were evaluated for their suitability to recover selectively and to count \textit{S. thermophilus}, \textit{L. delbrueckii} ssp. \textit{bulgaricus}, \textit{L. acidophilus}, and bifidobacteria. Provided the enumeration was carried out aerobically at 37°C for 24 h, ST agar was suitable for the selective enumeration of \textit{S. thermophilus} from a mixed culture containing \textit{S. thermophilus}, \textit{L. delbrueckii} ssp. \textit{bulgaricus}, \textit{L. acidophilus}, and bifidobacteria. Either MRS agar (pH 5.2) or RCA agar (pH 5.3) could be used for the enumeration of \textit{L. delbrueckii} ssp. \textit{bulgaricus} when the plates were incubated at 45°C for \geq 72 h. These conditions did not allow complete recovery of the four types of microorganisms. Either MRS agar (pH 5.2) or RCA agar (pH 5.3) could enumerate \textit{L. delbrueckii} ssp. \textit{bulgaricus} when the plates were incubated at 45°C for \geq 72 h. The conditions, however, did not allow complete recovery of \textit{L. delbrueckii} ssp. \textit{bulgaricus}, and colonies of \textit{L. delbrueckii} ssp. \textit{bulgaricus} needed to be differentiated from those of bifidobacteria on the basis of colony characteristics. In a product containing yogurt organisms, \textit{L. acidophilus}, and bifidobacteria, MRS-maltose agar could be used to estimate total counts of \textit{L. acidophilus} and bifidobacteria; MRS-salicin agar or MRS-sorbitol agar could be used to enumerate \textit{L. acidophilus} differentially, provided the product did not contain bifidobacteria that were positive for salicin or sorbitol. In such products, aerobic incubation of MRS-maltose agar plates would inhibit the anaerobic bifidobacteria and could selectively enumerate \textit{L. acidophilus}. A 40 to 70% inhibition of \textit{L. acidophilus} was observed under aerobic incubation. Bifidobacteria could be counted either by selective enumeration on MRS-NNLP agar or by subtraction of the \textit{L. acidophilus} counts obtained on MRS-salicin agar or on MRS-sorbitol agar from the total counts obtained from MRS-maltose agar, provided that the population of bifidobacteria was not one log cycle less than that of \textit{L. acidophilus}. The subtraction method could be useful because some strains of bifidobacteria were inhibited 40 to 60% on MRS-NNLP agar. The MRS

### TABLE 5. Recovery of yogurt and probiotic organisms in commercial products.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Agar</th>
<th>Sample&lt;sup&gt;1&lt;/sup&gt;</th>
<th>BDC</th>
<th>Yop</th>
<th>ChH</th>
<th>BCS</th>
<th>BS</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Streptococcus thermophilus}</td>
<td>ST&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>&lt;2.00</td>
<td>9.24</td>
<td>8.83</td>
<td>7.86</td>
<td>8.90</td>
<td>8.26</td>
</tr>
<tr>
<td>\textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus}</td>
<td>MRS (pH 5.2)</td>
<td></td>
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<td>&lt;3.00</td>
<td>7.18</td>
<td>7.13</td>
<td>7.18</td>
<td>7.45</td>
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<td>MRS-Salicin</td>
<td></td>
<td>8.40</td>
<td>7.48</td>
<td>7.26</td>
<td>4.72&lt;sup&gt;3&lt;/sup&gt;</td>
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<td></td>
<td>8.38</td>
<td>7.33</td>
<td>7.21</td>
<td>4.69&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.84&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.15&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>\textit{Bifidobacteria}</td>
<td>MRS-NNLP&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>7.59</td>
<td>&lt;4.00</td>
<td>&lt;4.00</td>
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</table>

<sup>1</sup>BDC = Blackmores' capsule (Blackmores Ltd., Balgowlah, Victoria, Australia); Yop = commercial yogurt sample (Yoplait, Euchuca, Victoria, Australia); ChH = Yogurt from culture (Chr. Hansen, Bayswater, Victoria, Australia); BCS = Bulla's (Regal Cream Products Pty. Ltd., North Melbourne, Victoria, Australia) chocolate-coated strawberry stick; BS = Bulla's strawberry stick, and BB = Bulla's banana stick.

<sup>2</sup>Two types of colonies; however, colonies of \textit{L. acidophilus} were easily distinguishable.

<sup>3</sup>Nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate.

medium that contained bile salts, oxgall, NaCl, and Rogosa acetate agar showed gross inhibition of L. acidophilus and bifidobacteria and might not be suitable for the enumeration purposes. Cellobiose esculin agar gave variable results with the cultures used in this study. Moreover, use of salicin, cellobiose, and esculin in enumeration media was uneconomical for routine testing of L. acidophilus compared with use of D-sorbitol.

ACKNOWLEDGMENTS

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REFERENCES