ABSTRACT

One of the key focuses of today’s dairy industry worldwide is the continued development of new products, especially probiotic-based products. Buttermilk is originally a by-product of butter making fermented by Mesophilic Aromatic Cultures (MAC). It can also be made by fermentation of pasteurized whole milk or skimmed milk. This product is not marketed in Brazil. The objectives of this work were: (1) to develop a selective medium for *Bifidobacterium animalis* subsp. *lactis* enumeration and (2) to determine the viability of this microorganism during the shelf life of the buttermilk. Skim milk added with 10% sucrose or 0.03% sucralose was pasteurized and inoculated with a composite starter culture consisting of 1% MAC (containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*) and 2% *Bifidobacterium animalis* subsp. *lactis*. To attain selective counts of *Bif. animalis* subsp. *lactis* the MRS agar supplemented with 0.5% L-cysteine hydrochloride at 10%, 1% lithium chloride at 10%, 0.01% aniline blue and 0.5% dicloxacillin at 0.1% was modified by increasing the antibiotic concentration, addition of NaCl, adjusting pH to 4.8 or increasing the incubation temperature (from 37 to 45ºC). Raising the incubation temperature to 45ºC was found to be efficient in inhibiting the MAC cultures, even in media not added with dicloxacillin. *Bif. animalis* subsp. *lactis* exhibited high viability in the product. The buttermilk product prepared with sucrose and sweetener contained in excess of 10⁸ cfu.ml⁻¹ bifidobacteria throughout the shelf life of the product (28 days).

Key words: Mesophilic cultures, buttermilk, selectivity, viability

INTRODUCTION

Buttermilk is originally obtained as a by-product of the butter making process. Sweet cream buttermilk is usually treated with butter starter cultures after separation of the butterfat to yield so-called fermented buttermilk (8). However, Nordic cultured buttermilk is made by microbial fermentation of pasteurized whole milk or skimmed milk by mesophilic lactic acid bacteria, such as *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris* (23). Buttermilk manufactured in Ireland is mostly made from skimmed milk (3).

Due to its delicate flavor, as well as to its high nutritional value, buttermilk is of great interest to the dairy industry (8). Although the nutritional and functional value of skim milk components is well known, only recently buttermilk has received attention as a potential source of functional ingredients (4). This is due to the presence of the phospholipids that play an important role in many metabolic processes. Phospholipid-enriched fractions are marketed today as important ingredients in a variety of dairy products (4,18).

The functional food market has experienced spiraling growth over the past few years and new fermented milks, containing probiotic microorganisms, have been developed by the dairy industry. The idea of using buttermilk as a vehicle for probiotics is relatively new. Only one paper was found in literature describing the addition of probiotic strains to buttermilk (17). According to Rodas et al. (17), when 1% of a probiotic strain...
(Lactobacillus reuteri) was added prior to buttermilk fermentation, its viability remained greater than 10^6 cfu.ml^-1 for 10 days storage. However, after 16 days, the Lb. reuteri population had statistically significantly decreased. Also, the amount of inoculum used (0.5 to 1.0%) produced different counts. Proportionally higher counts of Lb. reuteri were observed in the samples inoculated with 1% of the probiotic culture.

Most probiotics belong to the Lactobacillus and Bifidobacterium species (13). In humans, bifidobacteria are considered to be beneficial, since by producing acetic and lactic acids, they lower the pH of the colon and inhibit the proliferation of pathogens (10). There has been increasing evidence that probiotic cultures may have the ability to modulate the composition of the intestinal microflora and deliver a series of health benefits (12). Saavedra et al. (20) demonstrated that the consumption of an infant formula containing bifidobacteria decreased the rate of diarrhea in pediatric patients.

In the present study, Bifidobacterium animalis subsp. lactis Bb12 was selected as probiotic strain since it has been widely used in infant formulas and baby foods, dietary supplements and cultured milk products for more than 10 years in many countries all over the world (14, 16). According to Haschke et al. (7), Bif. animalis subsp. lactis Bb12 has an excellent ability to survive passage through the gastrointestinal tract, in addition to extraordinary adherence to enterocytes. Bb12 is also a technologically suitable strain for the purpose of this study since it does not have any adverse effects on either the flavor, appearance or mouthfeel of the foods in which it is used and survives in high enough concentrations until the probiotic product is consumed (14).

Moubareck et al. (15) analyzed the antibiotic susceptibility of various Bifidobacterium strains and found that they were sensitive to penicillin G, amoxicillin, piperracillin, ticarcillin, imipenem and common anti-Gram-positive antibiotics. Bifidobacterium strains isolated from dairy products are resistant to dicloxacillin (22).

Few media are truly selective for bifidobacteria, and hence the aims of this work were (i) to evaluate a range of possible medium formulations and determine which could be employed to selectively enumerate Bif. animalis subsp. lactis Bb12 in the presence of other lactic cultures (Mesophilic Aromatic Culture) and (ii) to determine the viability of the probiotic and mesophilic cultures in the buttermilk throughout the shelf-life of the product (28 days).

**MATERIAL AND METHODS**

**Microbial cultures**

The lyophilized cultures of Bif. animalis subsp. lactis (Bb12) and Mesophilic Aromatic Cultures (MAC CHN-22), composed of multiple mixed strains including Lactococcus lactis subsp. cremoris, Lac. lactis subsp. lactis, Lac. lactis subsp. lactis biovar. diacetylactis and Leuconostoc mesenteroides subsp. cremoris, used in this study were kindly provided by Christian Hansen (Brazil). The probiotic and mesophilic cultures were suspended in 1L and 2L sterile whole milk, respectively, and stored at -20ºC.

**Preparation of Buttermilk Product**

Skimmed UHT milk was added with 10% (m/v) sucrose or 0.03% (m/v) sucrrose (Taste and Lyle Sucrose, kindly provided by Tovani Benzaquen / Brazil), pasteurized (65ºC for 30 min), inoculated with 1% and 2% mesophilic and probiotic culture (susended as described above), respectively, and incubated at 21 ± 1ºC for 15-20 h until pH 5.0 ± 0.1 was reached.

**Culture media preparation**

Specific culturing conditions aiming at inhibiting MAC were defined in accordance with the procedures and methods described in Bergey’s Manual of Determinative Bacteriology (9): incubation temperature above 37ºC or pH 4.8 for Leu. mesenteroides inhibition and temperature of 45ºC or 4% NaCl for Lactococcus spp inhibition.

MRS Agar (Oxoid) supplemented with 0.5% L-cysteine hydrochloride at 10%, 1% lithium chloride at 10%, 0.01% aniline blue and 0.5% dicloxacillin at 0.1% modified as described by Fávaro-Trindade and Grosso (5) was used with some alterations to achieve selective counts of Bif. animalis subsp. lactis Bb12 in the presence of MAC CHN-22. The basic culture media (MRS agar, 0.5% L-cysteine HCL at 10%, 1% lithium chloride at 10% and 0.02% aniline blue) were tested with gradient addition of antibiotic, however, dicloxacillin was not added when inhibition was achieved by addition of NaCl, raising the pH or incubation temperature adjustments (see Table 1).

The culture medium modified by Nickels and Leesment, prepared as described by Vogensen et al. (25), was used for selective enumeration of mesophilic cultures.

**Enumeration procedure**

The MAC and Bb12 cells present in the inocula were enumerated using the media described above. Serial dilutions were prepared using 0.1% peptone water. Plates containing 0.5 to 1 mL diluted mesophilic and probiotic culture samples were prepared using the pour plate technique. The experiment was performed in duplicate. The results were expressed as log_{10} cfu.ml^-1.

When MRS basic media were used (see Table 1), all the plates were incubated under anaerobiosis (Anaerogen, Oxoid) for 72h at 37 or 45ºC. The plates containing Nickels and Leesment media were incubated under aerobic conditions at 25ºC for 3 days, and then added with 0.5 mL Xgal solution and subsequently incubated for one more day. Blue colonies are indicative of Leu. cremoris, whereas white colonies indicate
**B. animalis** in fermented milk

**RESULTS**

First of all, the MAC inocula stored at -20ºC were thawed at room temperature and serially diluted to determine the number of each mesophilic culture present in the mix inoculated onto the Nickels and Leesment media (25). The same was done to determine the number of viable *Bif. animalis* subsp. *lactis* cells plated on MRS agar (Oxoid) supplemented with 0.5% L-cysteine hydrochloride at 10%, 1% lithium chloride at 10%, 0.01% aniline blue and 0.5% dicloxacillin at 0.1% (5). The viable cell counts (log10 cfu.ml-1) of MAC were:

- Leu. mesenteroides subsp. cremoris: 8.08
- Lac. lactis subsp. lactis biovar. diacetylactis: 7.63
- Lac. lactis subsp. lactis and subsp. cremoris: 7.93

The inocula of *Bif. animalis* subsp. *lactis* were found to contain 9.69 log10 cfu.ml-1.

Fávaro-Trindade and Grosso (5) used selective media consisting of MRS agar supplemented with 0.5% L-cysteine hydrochloride at 10%, 1% lithium chloride at 10%, 0.01% aniline blue and 0.5% dicloxacillin at 0.1% for the enumeration of *bifidobacteria* in samples of acidified milk and yoghurt (containing *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus acidophilus*, *Bif. animalis* subsp. *lactis* Bb12). However, these media - previously tested by our research team - are not selective for *bifidobacterium* counts in buttermilk, which is fermented by *Lactococcus* spp and *Leu. mesenteroides*. Therefore, the supplemented MRS agar (5) was modified as described in Table 1. The counts of MAC and *Bif. animalis* subsp. *lactis* are shown in Table 2.

The MAC and Bb12 cultures exhibited similar patterns of dicloxacillin resistance, that is, no significant differences (P > 0.05) between counts were found among the samples added with up to 4% dicloxacillin. However, the addition of 5% of antibiotic solution completely inhibited all microbial growth.

### Table 1. Variations of MRS agar media (added with 0.5% L-cysteine HCL at 10%, 1% lithium chloride at 10% and 0.02% aniline blue) and incubation conditions to achieve selectivity for *Bif. animalis* subsp. *lactis* Bb12.

<table>
<thead>
<tr>
<th>Media</th>
<th>Dicloxacillin (0.1% mL/100 mL media)</th>
<th>NaCl addition (%)</th>
<th>pH adjustment</th>
<th>Incubation temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS diclox 0.5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS diclox 1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS diclox 3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS diclox 4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS diclox 5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS NaCl 4.5</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>37</td>
</tr>
<tr>
<td>MRS pH 4.8</td>
<td>-</td>
<td>-</td>
<td>4.8</td>
<td>37</td>
</tr>
<tr>
<td>MRS 45ºC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
</tbody>
</table>

### Table 2. Viable counts of selected microorganisms on different media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Enumeration (log10 cfu.ml-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC*</td>
<td>Bif. animalis subsp. lactis Bb12</td>
</tr>
<tr>
<td>MRS diclox 0.5</td>
<td>8.10a</td>
</tr>
<tr>
<td>MRS diclox 1</td>
<td>7.98a</td>
</tr>
<tr>
<td>MRS diclox 3</td>
<td>8.17a</td>
</tr>
<tr>
<td>MRS diclox 4</td>
<td>7.98a</td>
</tr>
<tr>
<td>MRS diclox 5</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>MRS NaCl 4.5</td>
<td>6.24b</td>
</tr>
<tr>
<td>MRS pH 4.8</td>
<td>5.92c</td>
</tr>
<tr>
<td>MRS 45ºC</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

* Mesophilic Aromatic Culture CHN-22 (*Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leu. mesenteroides* subsp. *cremoris*). Means in the columns with a common superscript do not differ significantly (P > 0.05).

**Statistical analysis**

The mean values of microbial counts were statistically analyzed and compared using ANOVA and Tukey’s test (P < 0.05) (SAS software - version 8.2).

The appearance of the mesophilic colonies varied in size and color throughout the medium. Mesophilic cultures plated on MRS and added with dicloxacillin formed blue and grey colonies with an average diameter of 1 mm; on MRS added with NaCl they formed grey and blue 0.5 mm-diameter colonies and on MRS acidified to pH 4.8 they formed blue 0.5 mm-diameter colonies.

Selective counts of *Bif. animalis* subsp. *lactis* in buttermilk product samples were performed using MRS agar (enriched with L-cysteine HCL, lithium chloride and aniline blue) and incubation at 45ºC. The mesophilic counts of buttermilk products were determined in samples plated onto Nickels and Leesment media. Both results are shown in Table 3.
DISCUSSION

According to Lankaputhra et al. (11) there is some concern that some media containing antibiotic or bile may also restrict the growth of bifidobacteria and consequently counts obtained with such media are not necessarily representative of viable cells that are in the product. Bif. animalis subsp. lactis showed considerable antibiotic resistance when plated onto media containing up to 4% dicloxacillin at 0.1%. However, the highest counts of Bif. animalis subsp. lactis were obtained when dicloxacillin was not incorporated into the culture medium and incubation was performed at 45ºC.

Addition of NaCl to supplemented MRS agar, as well as the drop in pH of the media, suppressed significantly, but not completely, MAC growth. NaCl was added with the aim of inhibiting Lactococcus ssp (9). One log cycle reduction in MAC counts was observed. However, NaCl did also completely inhibit the growth of bifidobacteria; as a conclusion, this medium variation is useless. Lowering the pH suppressed the growth of mesophilic cultures to a certain extent and did not negatively affect Bif. animalis subsp. lactis counts. As described by Holt et al. (9), pH 4.5 should suppress Leu. mesenteroides cells, but, as a 2 log cycle reduction was observed, some Lactococcus subsp. cells are also believed to have been inhibited.

The growth of Bif. animalis subsp. lactis Bb12 on supplemented MRS agar (5) is characterized by lenticular, brilliant blue colonies with an average diameter of 1 mm. At the bottom of the Petri dish the colonies were larger and rounded, or irregular in shape. When Bb12 incubation was performed at 45ºC, larger colonies were observed, with an average diameter of 2 mm. While growing on the plates, the culture acidifies the medium that became bluer.

The thermophilic incubation temperature was efficient for selective growth of Bif. animalis subsp. lactis Bb12 in the presence of mesophilic cultures.

Several culture media have been developed for differential enumeration of Bifidobacterium (11,19,21,24). The modified media presented in this paper are easy to prepare and antibiotic-free, which represents an ecological and economical advantage since dicloxacillin is the most expensive medium ingredient and - like any other antibiotic substance - poses a problem to be discarded.

The Bifidobacterium genus was found to have lower viability during storage, especially in acidic foods, such as yoghurt and cultured milk. Antunes et al. (1) developed probiotic yoghurt containing Bif. longum and obtained counts lower than 5 log10 cfu.ml⁻¹. Barreto et al. (2) evaluated the viability of bifidobacteria in yoghurts and cultured milk products sold on the Brazilian market and obtained the same result. Similarly, Gueimonde et al. (6) observed bifidobacterial counts lower than 10⁵ in some probiotic products sold in Spain.

Despite the fastidious characteristics of the genus Bifidobacterium, it is technologically feasible to use Bif. animalis subsp. lactis (Bb12) in buttermilk due to its compatibility with mesophilic cultures and excellent viability, as shown by the results of this work.

ACKNOWLEDGEMENTS

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RESUMO

Enumeração seletiva e viabilidade de Bifidobacterium animalis subsp. lactis em um novo produto lácteo fermentado

Atualmente, um dos principais focos da indústria de lácteos em todo o mundo é o desenvolvimento de novos produtos, especialmente probióticos. Buttermilk é originalmente um sub-produito do processamento da manteiga fermentado por Culturas Aromáticas Mesofílicas (MAC). Pode também ser feito pela fermentação de leite integral ou desnatado. Este produto não
é comercializado no Brasil. Os objetivos deste trabalho foram o desenvolvimento de meio de cultura seletivo para Bifidobacterium animalis subsp. lactis e a determinação da viabilidade deste microrganismo durante a vida de prateleira do buttermilk produzido. Leite desnatado foi adicionado de 10% da sacarose ou 0,03% de sacarose, pasteurizado e inoculado com 1% de MAC composto por Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. lactis biovar. diacetylactis e Leuconostoc mesenteroides subsp. cremoris e por 2% de Bifidobacterium animalis subsp. lactis. Para obter contagens seletivas de Bif. animalis subsp. lactis, o meio MRS agar suplementado com 0,5% L-cisteína HCl a 10%, 1% cloreto de lítio a 10%, 0,01% azul de anilina e 0,5% dicloxacilina a 0,1% foi modificado pelo aumento da concentração de antibiótico, adição de NaCl, ajuste de pH para 4,8 ou aumento da temperatura de incubação (de 37 para 45ºC). A temperatura de incubação de 45ºC foi eficiente para inibir as culturas MAC mesmo sem adição de antibiótico ao meio. Bif. animalis subsp. lactis apresentou alta viabilidade no produto. O buttermilk preparado com sacarose e edulcorante, apresentou mais de 10^8 ufc.ml⁻¹ de Bif. animalis subsp. lactis durante a vida-de-prateleira (28 dias).

**Palavras-chave:** Buttermilk, culturas mesofílicas, seletividade, viabilidade

**REFERENCES**