Brazilian Journal of Microbiology (2007) 38:173-177
ISSN 1517-8283

SELECTIVE ENUMERATION AND VIABILITY OF BIFIDOBACTERIUM ANIMALIS SUBSP. LACTIS IN A NEW FERMENTED MILK PRODUCT

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Submitted: March 27, 2006; Returned to authors for corrections: May 22, 2006; Approved: October 13, 2006

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 10^8 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 *Leu. 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

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(*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 microbiota and deliver a series of heath 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, piperacillin, ticarcillin, imepenem 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) sucralose (Taste and Lyle Sucrose, kindly provided by Tovani Benzaquen / Brazil), pasteurized (65ºC for 30 min), inoculated with 1% and 2% mesophilic and probiotic culture (suspended 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* ssp 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

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.

Media	Dicloxacillin 0.1% (mL/ 100 mL media)	NaCl addition (%)	pH adjustment	Incubation temperature (ºC)
MRS diclox 0.5	0.5	-	-	37
MRS diclox 1	1	-	-	37
MRS diclox 3	3	-	-	37
MRS diclox 4	4	-	-	37
MRS diclox 5	5	-	-	37
MRS NaCl 4.5	-	4.5	-	37
MRS pH 4.8	-	-	4.8	37
MRS 45ºC	-	-	-	45

Lac. diacetylactis and white colonies without a clear zone indicate *Lac. lactis* or *Lac. cremoris* (25). This medium does not allow differentiation between *Lac. lactis* and *Lac. cremoris,* which were counted together.

The growth and viability of MAC and Bb12 cultures in the buttermilk product were evaluated on the 1st and 28th day of storage of the product.

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).

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% dicloxacilin at 0.1% (5). The viable cell counts ($\log_{10}$ 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 $\log_{10}$ 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*, *Streptoccoccus salivarius* subsp. *thermophilus*, *Lactobacillus acidophilus* and *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* ssp 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 2. Viable counts of selected microorganisms on different media.

Medium	Enumeration ($\log_{10}$ cfu.ml^{-1})	
	MAC*	*Bif. animalis* subsp. *lactis* Bb12
MRS diclox 0.5	8.10^{a}	9.69^{b}
MRS diclox 1	7.98^{a}	10.02^{a,b}
MRS diclox 3	8.17^{a}	9.82^{a,b}
MRS diclox 4	7.98^{a}	9.73^{b}
MRS diclox 5	< 1	< 1
MRS NaCl 4.5	6.24^{b}	< 1
MRS pH 4.8	5.92^{c}	9.77^{a,b}
MRS 45ºC	< 1	10.21^{a}

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

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.

Table 3. Probiotic and mesophilic cultures in sweet and dietetic buttermilk after 1 and 28 days storage.

Cultures	Buttermilk with sucrose		Buttermilk with sucralose	
	1 day	28 days	1 day	28 days
	$\log_{10}$ cfu.ml^{-1}			
Probiotic				
Bifidobacterium animalis subsp. *lactis* Bb12	8.32	8.23	8.07	8.20
Mesophilic aromatic				
Leu. mesenteroides subsp. *cremoris*	7.44	8.00	8.34	7.57
Lac. lactis subsp. *lactis* biovar. *diacetylactis*	8.56	6.92	8.46	<6.00
Lac. lactis subsp. *lactis* and subsp. *cremoris*	8.73	7.11	8.56	6.74

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 1mm. 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 $\log_{10}$ 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 10^5 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

The authors wish to acknowledge the financial support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

RESUMO

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

Atualmente, um dos principais focos da indústria de laticínios em todo o mundo é o desenvolvimento de novos produtos, especialmente probióticos. *Buttermilk* é originalmente um sub-produto 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 sucralose, 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 ágar 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

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