

EVALUATION OF CULTURE MEDIA FOR COUNTS OF *BIFIDOBACTERIUM ANIMALIS* IN THE PRESENCE OF YOGHURT BACTERIA

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ABSTRACT

Much attention has been paid to the use of *Bifidobacterium* sp. in yoghurts due to their excellent therapeutic and nutritional properties. However, in order to present health properties, it is recommended that each commercial product should indicate the minimum daily amount required for it to confer health benefit(s) based on in vitro and human studies. Besides the inherent low growth of *Bifidobacterium* sp in milk, there is a need for a reliable method for counting *Bifidobacterium* sp in the presence of yoghurt bacteria. This study evaluated the use of the media M-MRS, MRS-NNLP and RCPB pH5 aimed at counting the number of *Bifidobacterium animalis* subsp. *lactis* in the presence of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* after yoghurt fermentation. The M-MRS medium was not selective, allowing for the growth of *L. delbrueckii* subsp. *bulgaricus*. MRS-NNLP medium presented a good selectivity for *B. animalis* Bb12 with a slight reduction in the cell count of this microorganism when compared it to the standard MRS medium in pure culture. MRS-NNLP medium was considered a good option to enumerate *B. animalis* Bb12 although the reduction found in pure culture due to the low difference between the counts. The medium RCPB pH5 presented differentiated growth of *B. animalis* Bb12 in relation to the yoghurt bacteria and a cell recovery equal to that of the standard MRS, being considered the best option to enumerate *Bifidobacterium* sp in the presence of yoghurt bacteria.

Key words: count, *Bifidobacterium*, lactic bacteria, yoghurt

INTRODUCTION

According to FAO/WHO probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host. It is recommended that each product should indicate the minimum daily amount required for it to confer health benefit(s) based on in vitro (animal) and human studies (5). There are many health aspects related to the use of *Bifidobacterium* sp as follow: treatment and prevention of diarrhoea, reduction in lactose intolerance in some individuals, to increase resistance to microbial infections, potential role in cancer prevention, possible treatment of inflammatory bowel disease, alleviation of constipation, impact on immune function and reduction of serum cholesterol (9).

However, the conditions used for yoghurt manufacture are not optimal for the survival of bifidobacteria, resulting in low viability of the microorganisms in commercial products. In addition, the lack of a standard method to count these microorganisms in the presence of yoghurt cultures makes it very difficult to improve the technology for the manufacture of these products. Thus the standardisation of a method for the selective or differential count of *Bifidobacterium* sp in the presence of yoghurt cultures has become very important. Various methods for the selective count of *Bifidobacterium* sp in yoghurt, based on pure cultures, have been proposed (1-3,6-8,10-14).

The selective medium Maltose – de Man Rogosa Sharpe (M-MRS), developed for the isolation of *Lactobacillus acidophilus* in yoghurts (12) allows for the development of

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maltose-fermenting strains of *Bifidobacterium* sp. Dave and Shah (3,4) successfully used this medium to selectively count *Bifidobacterium* sp. in yoghurts.

Van der Wiel-Korstanje and Winkler (13) used the dye China Blue in CBRCA (China Blue-Blood-RCA) medium for the differential count of the predominant anaerobic microbiota in human faeces, establishing a concentration of 0.03% for the use of the dye. Subsequently, Onggo and Fleet (11) proposed the use of RCPB medium for the isolation and differentiation of *Bifidobacterium* sp, *L. delbrueckii* subsp *bulgaricus* and *S. thermophilus* in yoghurts. These species were easily differentiated in this medium due to the distinct characteristics of the colonies on account of the action of the dye, forming halos round the *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* colonies. Subsequently, Rybka and Kailasapathy (12) proposed adjusting the pH of the medium to 5 (RCPB pH5) aiming at inhibiting the non-lactic milk microbiota.

Laroia and Martin (8) indicated the use of Glucose-Blood-Liver medium associated with the inhibitors nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate in 1991. Modler and Villa-Garcia (10) stated that the formulation described by Laroia and Martin two years before, was incomplete, since the component defibrinated blood (50mL horse or sheep blood/L) had been inadvertently removed from the formulation. Subsequently Dave and Shah (3,4) proposed using the antibiotics NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) together with MRS medium to selectively count *Bifidobacterium* in yoghurts.

According to Vinderola and Reinheimer (14), few methods are efficient in correctly counting yoghurt cultures containing bifidobacteria, making quality control difficult. Despite the great number of methods proposed in the literature, not all presented good results for the counting of *Bifidobacterium* sp in the presence of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus*. For the quality assurance of probiotic products, a safe, reliable routine method is required to quantify viable *Bifidobacterium* sp in the presence of yoghurt cultures. The objective of this study was to evaluate the media RCPB pH5, M-MRS and MRS-NNLP to count *Bifidobacterium animalis* Bb12 in the presence of the lactic cultures of yoghurts after yoghurt fermentation.

MATERIAL AND METHODS

Cultures

Freeze dried pure cultures of the following microorganisms were used: *Bifidobacterium animalis* subsp *lactis* (Chr. Hansen Indústria e Comércio Ltda, Valinhos, SP), *Streptococcus thermophilus* A (VisbyVac, Série 1000, Cotia, SP) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB341 TEXEL-Rhodia, Cotia, SP). The cultures were kept frozen until the time

of the experiments, when they were activated as indicated in the following items.

Inoculum preparation and maintenance

The inoculum of *B. animalis* Bb 12 was prepared as follows (100 mL): powdered skimmed milk, brand name Molico (12.5g), and yeast extract (1g) were diluted to 85 mL with Milli-Q water and sterilised at 115°C for 10 min. (Universal Ortossintese Autoclave). One gram of glucose and 0.05g L-cysteine were diluted in 15 mL Milli-Q water and sterilised by filtering through a membrane with a pore size of 0.45 µm (Millipore®). Using lamina flow equipment, the sterile milk containing yeast extract was mixed with the solution of glucose and L-cysteine. Then *B. animalis* Bb 12 were activated at 37°C for 18hr. and then cooled in an ice bath.

For yoghurt cultures, one inoculum for *S. thermophilus* and another for *L. bulgaricus* were made by incubating the freeze dried cells of each one in recombined skim milk (11% of solids), previously sterilised at 115°C for 10min., at 37°C for 18hr. and then cooled in an ice bath.

Media for enumeration of pure cultures of *Bifidobacterium* MRS (de Man, Rogosa, Sharpe) medium

MRS medium (Oxoid) was sterilised at 121°C for 15min. and used as the standard medium for enumerating pure cultures of *B. animalis* Bb 12.

Media for enumeration of *Bifidobacterium* in the presence of yoghurt bacteria

1) Maltose-MRS (Maltose – de Man, Rogosa, Sharpe) medium

All the normal ingredients of MRS medium, with the exception of the agar and glucose, were suspended in 1L of distilled water and the pH adjusted to 6.6. Ten grams of agar were then added and the media sterilised at 121°C for 15 min. To prepare the maltose solution, 25g maltose were dissolved in 50 mL of distilled water and sterilised by passing through a 0.45 µm filter (Millipore®). In the media sterilised were added 4 mL of this maltose solution to 100 mL of the medium at a temperature of about 50°C. The media was homogenised carefully without excessive agitation, to avoid the incorporation of air.

2) MRS-NNLP medium

Commercial MRS medium (Oxoid) was sterilised at 121°C for 15min. in 96% of the total water weight of the final media. The inhibitors and the aminoacid L-cysteine were dissolved in the rest of the water (4%) and sterilised by vacuum filtration using a 0.42 µm pore membrane and then added to the sterilised MRS medium in a laminar flow at about 50°C. The inhibitors concentration in the final medium were: lithium chloride 3 mg/mL, nalidixic acid 15 µg/mL, neomycin sulphate 100 µg/mL and paramomycin sulphate 200 µg/mL. The L-cysteine concentration was 0.5 g/mL in the final medium.

3) RCPB pH5 (Reinforced Clostridial Prussian Blue at pH5) medium

Commercial RCA medium (Oxoid) was supplemented with 0,01 g/mL glucose and adjusted to pH5 according to Rubka and Kailasapathy (12). RCA medium and Prussian blue dye (IRON III Ferrocyanide, Sigma-Aldrich) were sterilised separately at 115°C for 15 min., cooled to 50°C and mixed aseptically. The Prussian blue dye was adjusted at a final concentration of 0.03%.

Products

After cooling the inoculae in an ice bath the following tests were carried out:

- A) *B. animalis* Bb12 culture was produced by incubating 1% of the inoculum of *B. animalis* Bb12 in reconstituted milk (12% solids plus 1% yeast extract);
- B) Standard yoghurt was produced after incubation of 1% of the inoculum of *S. thermophilus* and 1% of the inoculum of *L. delbrueckii* subsp *bulgaricus* in milk;
- C) A mixture of *B. animalis* Bb12 culture (A) and the standard yoghurt (B) was prepared spreading the 10⁻⁴ to 10⁻⁶ dilutions of each culture directly onto the media's surfaces (M-MRS, MRS-NNLP or RCPB pH5);
- D) The probiotic yoghurt was produced after incubation of 1% of the inoculum of *B. animalis* Bb 12 and 1% of each inoculum of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* in milk.

All products were incubated at 37°C for 4hr. and 40 min. because it was the time-temperature required for the probiotic yoghurt (D) to reach pH 4.6. The counts were carried out on the media M-MRS, MRS-NNLP and RCPB pH5 for trials B, C and D, since the MRS medium was not selective or differential. For trial A, the count was carried out in MRS medium (used as the standard to evaluate recovery capacity) and in the media M-MRS, MRS-NNLP and RCPB pH5.

Quantitative evaluation

Decimal dilutions of 1.0 mL aliquots of *B. animalis* Bb12 (A), the standard yoghurt (B) and the probiotic yoghurt (D) were prepared in peptone water (0.1%) up to 10⁻⁶ UFC/mL. For plating, 0.1mL aliquots of the 10⁻⁴ to 10⁻⁶ dilutions of the cultures to be evaluated were spread on the medium surfaces in duplicate. For the item C mixture, 0.05 mL aliquots of the 10⁻⁴ to 10⁻⁶ dilutions of *B. animalis* Bb12 and of the standard yoghurt were spread on the medium surfaces. The plates were incubated at 37°C for 72 hr in anaerobic jars (Merck), using the Anaerocult A® (Merck) anaerobiosis generator. After the incubation period, the plates were analysed for differential or selective growth, and the cell recovery capacity of *B. animalis* Bb12 was compared with the count obtained for the pure *B. animalis* Bb12 (A) in standard MRS medium. For the RCPB pH5 medium, after the period of anaerobic incubation the plates were exposed to air

for a few hours to allow for the formation of a blue halo around the *L. delbrueckii* subsp *bulgaricus* colonies. All experiments were carried out in triplicate.

Statistical analysis

The Tukey test was applied to check for differences in microbial recovery in the media studied using the Software Statistica 5.0.

RESULTS AND DISCUSSION

M-MRS medium

M-MRS is a selective medium that should inhibit the growth of *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* permitting the growth of *Bifidobacterium* sp strains. This culture medium, however, was not selective for *B. animalis* Bb12 in relation to the strains studied, because a high cell count of *L. delbrueckii* subsp *bulgaricus* (6,68 log CFU/mL) was verified and the appearance of colonies (white, with 0,5 to 1mm of diameter) was similar to that of *B. animalis* Bb12. Rybka and Kailasapathy (12) reported that some *L. delbrueckii* subsp *bulgaricus* strains could ferment maltose, and also that the presence of certain other components of the medium, such as meat extract, could favour development of this species. On the other hand, Dave and Shah (3) observed that the yoghurt bacteria did not grow in M-MRS medium, allowing for excellent recovery of *Bifidobacterium* sp, although the authors of this paper worked with other *Bifidobacterium* strains (*B. bifidum* 1900 and 1901, *B. longum* 1941 and 20097, *B. adolescentis* 1920, *B. pseudolongum* 20099, *B. breve* 1930 and *B. infantis* 1912).

MRS-NNLP medium

The MRS-NNLP medium showed good selectivity for *Bifidobacterium* sp, since *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* (B) failed to grow in this medium (Table 1). The yoghurt cultures in mixture (C) and in the probiotic yoghurt (D) also failed to grow. *B. animalis* Bb12 developed producing white, approximately 1mm diameter colonies when in pure culture (A) or in the probiotic yoghurt (D). Vinderola and Reinheimer (14) observed that *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* were able to develop in MRS-NNLP medium, although with low counts. Dave and Shah (3) obtained good performance with MRS-NNLP medium for the count of *Bifidobacterium* sp. in yoghurt and used it again to count *Bifidobacterium* sp. in a later study (4).

Table 1 shows a count of 6,04 log CFU/mL for the pure culture of *B. animalis* Bb12 in MRS-NNLP medium and 6,38 log CFU/mL in the standard media MRS. Although the difference was statistically significant at p<0.05 (Table 1), this counts are very closed in microbiology terms, so this media was considered useful for enumeration of *Bifidobacterium* sp. after yoghurt fermentation.

Table 1. Yoghurt cultures and *B. animalis* Bb12 counts on MRS-NNLP medium and on standard MRS medium.

	Count (log CFU/mL)		
	MRS medium	MRS-NNLP medium	
	<i>B. animalis</i> Bb12	Yoghurt cultures	<i>B. animalis</i> Bb12
A	6.38±0.02	-	6.04±0.03*
B	nd	<4	-
C	nd	<4	5.87±0.08
D	nd	<4	6.34±0.11

* Mean differ significantly ($p < 0.05$) from the standard count in MRS medium; A- *B. animalis* Bb12 culture; B- standard yoghurt *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus*; C- mixture of *B. animalis* Bb12 with standard yoghurt *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus* plated directly onto the medium surface; D- probiotic yoghurt with *B. animalis* Bb12 + *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus*; nd- not determined since MRS medium was not selective or differential for *B. animalis* Bb 12.

RCPB pH5 medium

B. animalis Bb12 developed producing white colonies as described in the literature (12). However, the diameter of the colonies was smaller than the 2mm described by Rybka and Kailasapathy (12), being approximately 1mm in the present study. In the standard yoghurt, *L. delbrueckii* subsp *bulgaricus* produced colonies with a blue halo and white centre, with a diameter varying from 1 to 2 mm. Rybka and Kailasapathy (12) observed that growth of *S. thermophilus* was prevented by adjusting the pH to 5. However in the present study, when only the yoghurt cultures were inoculated on the medium, *S. thermophilus* did grow, producing extremely small colonies with a slight blue halo and white centre. On the other hand, *S. thermophilus* failed to grow when the mixture of pure culture of *B. animalis* Bb12 and yoghurt cultures were inoculated directly onto the medium surface and when the probiotic yoghurt were counted (Table 2).

The factor responsible for this differentiation has yet to be completely elucidated. Onggo and Fleet (11) explained the formation of the blue halo as a function of a possible preferential metabolism of the dye by the yoghurt cultures, producing blue zones round the colonies. Another possibility could be a change in the oxido-reduction potential of the medium, caused by the *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus*. In the present study, it was discerned that the formation of the blue halo only occurred after exposing the surface of the plate to the air for a few minutes or even a few hours in some cases, which suggests that the second hypothesis is more likely. Another factor that appears to have influenced the appearance and

Table 2. Yoghurt cultures and *B. animalis* Bb12 counts on RCPB pH5 medium and on standard MRS medium.

	Count (log CFU/mL)			
	MRS medium	RCPB pH5 medium		
	<i>B. animalis</i> Bb12	<i>St</i>	<i>Lb</i>	<i>B. animalis</i> Bb12
A	6.56±0.02	-	-	6.58±0.09**
B	nd	6.39±0.12	6.53±0.08	-
C	nd	ng	6.04±0.01	6.53±0.05
D	nd	ng	7.70±0.25	7.28±0.15

** Mean did not differ significantly ($p < 0.05$) from the standard count in MRS medium; A- *B. animalis* Bb12 culture; B- standard yoghurt *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus*; C- mixture of *B. animalis* Bb12 with standard yoghurt *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus*, plated directly onto the medium surface; D- probiotic yoghurt with *B. animalis* Bb12 + *S. thermophilus* + *L. delbrueckii* subsp *bulgaricus*; Bb12 = *Bifidobacterium animalis* Bb12; *Lb* = *Lactobacillus delbrueckii* subsp. *bulgaricus*; *St* = *Streptococcus thermophilus*; nd – not determined since MRS medium was not selective or differential for *B. animalis* Bb12; ng – no growth.

intensity of blue zones was the greater acidification of the medium by the *L. delbrueckii* subsp *bulgaricus* cultures. The spreading of 6N HCl over the surface of the RCPB pH5 medium using a Pasteur pipette, considerably increased the blue tone of the medium, showing that greater acidification tended to form more intensely blue zones. To the contrary, the addition of concentrated sodium hydroxide formed a completely transparent zone on the medium, the characteristic blue colour of the medium disappearing. The Prussian blue forming reaction is well known and widely used in medicine to determine iron in cases of anaemia.

The *B. animalis* Bb12 count in RCPB pH5 medium was similar to that in standard MRS medium (Table 2). The count of the pure culture of *B. animalis* Bb12 was 6.58 log CFU/mL in RCPB pH5 medium and it was 6.56 log CFU/mL in the standard MRS medium. In the mixture, *B. animalis* Bb12 count was 6.53 log CFU/mL and in the probiotic yoghurt, *B. animalis* Bb12 count was 7.28 log CFU/mL. *S. thermophilus* only grew when resulting from the inoculation of the standard yoghurt, the count being 6.39 log CFU/mL. *L. delbrueckii* subsp *bulgaricus* developed well in all situations, but its colonies were easily distinguishable from the *B. animalis* Bb12 colonies on account of the blue halo. The RCPB pH5 medium proved to be suitable for counting *Bifidobacterium* sp in the presence of yoghurt bacteria after yoghurt fermentation, not only because of the differential growth of its colonies, but also due to the excellent cell recovery.

CONCLUSIONS

The M-MRS medium failed to show selectivity for the *B. animalis* Bb12 count in yoghurt due to the growth of *L. delbrueckii* subsp *bulgaricus*. The MRS-NNLP medium showed good selectivity for *B. animalis* Bb12 and it was considered a good option to count *B. animalis* Bb12 after yoghurt fermentation, although showing a slight reduction in the number of viable bifidobacteria cells when compared to the standard MRS medium when the tests were carried out with pure cultures. Of the culture media studied, the RCPB pH5 medium was the most indicated for the count of *B. animalis* Bb12 in the presence of yoghurt bacteria after yoghurt fermentation, due to its ease of preparation and good *Bifidobacterium* recovery. More studies are recommendable in order to test these media with another *Bifidobacterium* and yoghurt bacteria strains and also during storage of yoghurt to better evaluate these media as standard media to enumerate *Bifidobacterium* sp in yoghurt.

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RESUMO

Avaliação de meios de cultura para contagem de *Bifidobacterium animalis* na presença de bactéria no iogurte

Tem-se dado muita atenção à utilização de *Bifidobacterium* sp. em iogurtes devido às suas excelentes propriedades terapêuticas e nutricionais. Entretanto, é recomendado que, baseado em testes in vitro e em humanos, cada produto comercial indique qual a quantidade mínima diária recomendada de ingestão para que se obtenha os benefícios desejados à saúde. Além da inerente dificuldade do crescimento de *Bifidobacterium* sp em leite, há uma necessidade de padronização de um método de contagem confiável de *Bifidobacterium* sp na presença das bactérias do iogurte. Este trabalho avaliou o uso dos meios M-MRS, MRS-NNLP e RCPB pH5, visando a contagem do número de células de *Bifidobacterium animalis* Bb12 na presença de *Streptococcus thermophilus* e *Lactobacillus delbrueckii* subsp *bulgaricus* após a fermentação do iogurte. O meio M-MRS não foi seletivo, apresentando crescimento de *L. delbrueckii* subsp. *bulgaricus*. O meio MRS-NNLP apresentou boa seletividade para *B. animalis* Bb12, apesar de uma leve redução no número de células ter sido verificada quando comparado ao meio padrão MRS em cultura pura. O meio MRS-NNLP foi considerado uma

boa opção para a contagem de *B. animalis* Bb12 porque a diferença encontrada entre as contagens em cultura pura foi bastante pequena. O meio RCPB pH5 apresentou crescimento diferencial de *Bifidobacterium animalis* Bb12 em relação às bactérias do iogurte e uma recuperação de células igual ao meio padrão MRS, sendo considerado o melhor meio de cultura deste estudo para a contagem de *Bifidobacterium* sp na presença das culturas do iogurte.

Palavras-chave: contagem, *Bifidobacterium*, bactérias lácticas, iogurte

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