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Evaluation of culture media for enumeration of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium animalis* in the presence of *Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus*

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ABSTRACT

The study compared the growth capability of probiotic (*Lactobacillus acidophilus* La05, *Lactobacillus casei* Lc01 and *Bifidobacterium animalis* Bb12) and non-probiotic (*Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus*) cultures on twenty-one culture media grouped according to selectivity: non-selective agars, selective agars without antibiotics and MRS agars containing different combinations of lithium chloride, cystein, bile salts and antibiotics. Four of these media were selected for quantitative enumeration of *L. acidophilus* La05, *L. casei* Lc01, and *B. animalis* Bb12. The best culture media and incubation conditions for enumeration of the probiotic cultures were: *B. animalis*: MRS agar with dicloxacillin, 37 °C or 42 °C, anaerobiosis; *L. acidophilus*: MRS agar with bile salts, 37 °C or 42 °C, aerobiosis; *L. casei*: MRS agar with lithium chloride and sodium propionate, 37 °C or 42 °C, aerobiosis or anaerobiosis. Plating on MRS with glucose replaced by maltose, 37 °C or 42 °C, anaerobiosis, will distinguish probiotic from non-probiotic cultures. For enumeration of each probiotic in a mixed culture, the following media and incubation conditions were recommended: *B. animalis*: 4ABC–MRS, 42 °C, anaerobiosis, *L. acidophilus*: LC medium, 42 °C, aerobiosis or anaerobiosis and *L. casei*: LP–MRS, 42 °C, aerobiosis or anaerobiosis. In all experiments, differences in counts using pour plating or surface plating were not significant ($P \leq 0.05$).

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1. Introduction

Probiotic bacteria have many beneficial effects on human health, and these effects are dependent on the number of viable microbial cells that reach the human gut (Leahy, Higgins, Fitzgerald, & Sinderen, 2005). Thus, reliable determination of viability of bacteria in probiotic foods is important (Lahtinen, Gueimonde, Ouwehand, Reinikainenm, & Salminen, 2006; Saarela, Mogesen, Fonden, Matto, & Mattila-Sandholm, 2000). Traditionally, lactic acid bacteria are counted in MRS agar (De Man, Rogosa, Sharpe Agar), but this medium is not selective for enumeration of probiotic microorganisms when non-probiotic starter cultures are present. Effective isolation depends on medium formulation, fermentation conditions, time, temperature and atmosphere of incubation as well as on the type of food product under analysis (Aasen, Moretro, Katla, Axelsson, & Storro, 2000; Liew, Ariff, Raha, & Ho, 2005; Payne, Morris, & Beers, 1999; Roy, 2001; Talwalkar & Kailasapathy, 2004; Vinderola & Reinheimer, 1999, 2000).

Besides MRS agar, several other culture media have been proposed for the enumeration of probiotic and starter cultures, which include the selective *Lactobacillus casei* agar (LC agar) described by Shah and Ravula (2000), MRS agar supplemented with bile salts, sodium propionate, lithium chloride, cystein, gentamycin or dicloxacillin (Ingham, 1999; Lapierre, Undeland, & Cox, 1992; Lim, Huh, & Baek, 1995; Vinderola & Reinheimer, 2000) and MRS agar in which glucose was replaced by other carbohydrates, such as trehalose and maltose (Hull & Roberts, 1984). Bile salts are used to inhibit growth of non-intestinal microorganisms and sodium propionate and lithium chloride have been shown to be inhibitory for *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Streptococcus thermophilus* (Dave & Shah, 1996; Vinderola & Reinheimer, 2000). Supplementation with cystein was recommended by Shah (2000), because this substance reduces the redox potential, creating favorable conditions for the growth of microaerophilic microorganisms. Antibiotics, specially gentamycin and dicloxacillin, were used by Ingham (1999) and Lim et al. (1995), to inhibit certain species of lactic acid bacteria, without inhibiting the bifidobacteria.

The aim of this study was to compare the growth capability of probiotic (*L. acidophilus*, *L. casei* and *Bifidobacterium animalis*) and

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non-probiotic (*L. delbrueckii* subsp *bulgaricus* and *S. thermophilus*) bacteria on culture media divided into four groups: I. non-selective media for lactic acid bacteria, II. modified MRS agar, III. selective media without antibiotics and IV. MRS supplemented with different combinations of antibiotics. The influence of incubation conditions (temperature and atmosphere) and plating technique (pour and surface plating) on the growth were also evaluated.

2. Materials and methods

2.1. Cultures

The study was conducted with pure lyophilized cultures of *L. acidophilus* La05, *L. casei* Lc01, *B. animalis* Bb12, and the RICH yoghurt cultures (*L. delbrueckii* subsp *bulgaricus* and *S. thermophilus*), provided by Chr. Hansen S/A (Valinhos, SP, Brazil). The lyophilized cultures were maintained under −20 °C until used. Each culture was separately reactivated in 10 g/L reconstituted powdered milk (Molico, Nestlé, Sao Paulo, Brazil), sterilized at 121 °C for 15 min, and supplemented with 20 g/L glucose and 10 g/L yeast extract. The incubation was carried out at 37 °C for 24 h, under aerobiosis (Shah & Ravula, 2000).

2.2. Culture media

The study was carried out with twenty-one culture media, divided in four groups. Group I comprised non-selective media for lactic acid bacteria (MRS agar and M17 agar). Group II corresponded to four types of modified MRS agar, i.e. MRS 5.4 (MRS agar adjusted to pH 5.4 with glacial acetic acid), C–MRS (MRS agar plus 0.5 g/L cystein), T–MRS (formulated MRS agar containing 20 g/L trehalose instead of glucose) and M–MRS (formulated MRS agar containing 20 g/L maltose instead of glucose). Group III contained five different selective media without antibiotics: formulated LC agar, prepared according to Shah and Ravula (2000) (peptone 10 g/L, meat extract 4 g/L, yeast extract 1 g/L, Tween 80 1 mL, KH_2PO_4 2 g/L, sodium acetate.$3H_20$ 3 g/L, tri-ammonium citrate 1 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4.4H_2O$ 0.05 g/L, acid hydrolysate of casein 1 g/L and bacteriological agar 12 g/L, final pH 7.0), LP–MRS (MRS agar supplemented with 2 g/L lithium chloride and 3 g/L sodium propionate), LP–C–MRS (MRS agar supplemented with 0.5 g/L cystein, 2 g/L lithium chloride and 3 g/L sodium propionate), BL–MRS (agar supplemented with 1.5 g/L bile salts) and BL–C–MRS (MRS agar supplemented with 0.2 g/L bile salts and 0.5 g/L cystein). Media in Group IV were MRS agar added of different combinations of dicloxacillin, lithium chloride, cystein and gentamycin, as shown in Table 1.

MRS agar, peptone, yeast extract, meat extract and bacteriological agar were from Oxoid Ltd. (Basingstoke, Hampshire, UK). Cystein, tri-ammonium citrate, sodium propionate and sodium acetate were from Merck (Darmstadt, Germany); trehalose and maltose were from Difco (Le Pont de Claix, France); Tween 80 was from Inlab Indústria Brasileira (Sorocaba, SP, Brazil); $MgSO_4$, $MnSO_4$, acid hydrolysate of casein, lithium chloride, bile salts, dicloxacillin and gentamycin were from Sigma Chemical Co. (St. Louis, US) and K_2PO_4 was from Labsynth Produtos para Laboratórios Ltda (Diadema, SP, Brazil).

2.3. Qualitative microbiological analysis

Each reactivated culture was streaked on the surface of eight Petri plates containing 12–15 mL of the agar under evaluation. For streaking, a 2–3 mm standard loop was used. Four plates were incubated at 37 °C up to 72 h, two under aerobiosis and two under anaerobiosis. The remaining four plates were incubated at 42 °C up to 72 h, two under aerobiosis and two under anaerobiosis. For anaerobiosis, the GasPak-Anaerogen system (Oxoid Ltd., Basingstoke, UK) was used. After incubation, the plates were observed for growth of colonies and results were classified as "+" (visible growth) or "−"(absence of visible growth). The purity of the cultures on the plates was monitored through Gram staining and observation of cell morphology using microscopy (Olympus CBA, Melville, NY, USA), at 100x magnification. Each experiment was repeated three times. Final results were considered "+" or "−" when at least two of the three repetitions were "+" or "−", respectively.

2.4. Quantitative microbiological analysis

Three probiotic strains (*B. animalis* Bb12, *L. acidophilus* La05 and *L. casei* Lc01) and four culture media (MRS, MRS 5.4, LP–MRS and BL–MRS) were selected for the quantitative tests. One gram of the lyophilized cultures of *B. animalis* Bb12, *L. acidophilus* La05 or *L. casei* Lc01 was added to 9 mL of sterile saline (8.5 g/L) and submitted to serial decimal dilutions up to 10^{-9} using sterile saline as diluent. For enumeration of viable cells, eight Petri plates containing the same agar were surface-plated with 0.1 mL of each decimal dilution of the bacterial suspensions. Simultaneously, 1 mL of each decimal dilution was transferred to empty Petri plates and pour-plated with the agar under evaluation. Four surface-plated plates were incubated at 37 °C, two under aerobiosis and two under anaerobiosis (GasPak-Anaerogen system). The remaining four surface-plated plates were incubated at 42 °C, two under aerobiosis and two under anaerobiosis (GasPak-Anaerogen system). The same incubation scheme was followed for the pour-plated plates. After 72 h of incubation, the plates containing 25–250 visible colonies were selected and counted using a colony counter (CP 600 Plus, Phoenix, Brazil). The average number of colonies in the duplicates was calculated, and the results were expressed as log CFU mL^{-1}.

Based on the results of evaluation of each culture media and incubation condition, three media were selected (4ABC, LC and LP–MRS) for the enumeration of *B. animalis* Bb12, *L. acidophilus* La05 and *L. casei* Lc01 in a mixed culture; prepare mixing the three probiotic cultures in 1:1:1 proportion. The mixed culture was submitted to serial decimal dilutions up to 10^{-9} using sterile saline

Table 1
Selectivity agents in modified MRS agar belonging to the Group IV media

Culture media	Dicloxacillin (A) mg/L	Lithium chloride (B) g/L	Cystein (C) g/L	Bile salts (BL) g/L	Gentamycin (G) mg/L
ABC–MRS agar	0.5	1.1	0.5	0	0
2ABC–MRS agar	1.0	1.1	0.5	0	0
4ABC–MRS agar	2.0	1.1	0.5	0	0
A5BC–MRS agar	0.5	5.5	0.5	0	0
2A5BC–MRS agar	1.0	5.5	0.5	0	0
4A5BC–MRS agar	2.0	5.0	0.5	0	0
BL–G–MRS agar	0	0	0	0.2	30.0
BL–G–C–MRS agar	0	0	0.5	0.2	30.0
G–MRS agar	0	0	0	0	30.0
G–C–MRS agar	0	0	0.5	0	30.0

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