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Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products

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Abstract

The aim of this work was to select a set of culture media to perform the enumeration of *L. casei* when it appears together with *L. acidophilus*, bifidobacteria and bacteria of lactic acid starters, in fermented dairy products. A number of *L. acidophilus*, *Bifidobacterium* and *L. casei* strains (8 of each) were tested for their ability to grow in two selective/differential media (LP-MRS agar and B-MRS agar). The enumeration of these bacteria using the media mentioned above was also carried out in fermented dairy products available on the market. B-MRS agar and LP-MRS agar were able to inhibit the lactic acid bacteria from the starters. B-MRS agar could be useful for the selective colony count of *L. acidophilus* or *L. casei*, or for a differential enumeration when these bacteria appear together in a fermented dairy product. In the case of bifidobacteria, a selective colony count could be performed on LP-MRS agar, even when it appears with *L. casei*, because a differential cell enumeration between both organisms was possible. The three probiotic bacteria could be simultaneously enumerated from dairy products that contain them along with starter bacteria using media developed in this work.

Keywords: *Lactobacillus casei*; Bifidobacteria; *Lactobacillus acidophilus*; *S. thermophilus*; *L. delbrueckii* subsp. *bulgaricus*; Dairy products

1. Introduction

Lactobacillus acidophilus, *Lactobacillus casei* and bifidobacteria are normal inhabitants of the human intestine and numerous health benefits have been reported for them (Kailasapathy & Rybka, 1997; Salmine & Wright, 1998; Parodi, 1999). Fermented dairy products are considered to be vehicles by which consumers might receive adequate numbers of probiotic bacteria (Samona & Robinson, 1994; Gomes, Malcata, Klaver & Grande, 1995; Nighswonger, Brashears & Gilliland, 1996; Stanton et al., 1998). Counts higher than 1×10^7 CFU g^{-1} are adequate in order to ensure their probiotic effects. For dairy products consumed regularly, the minimum level suggested for probiotic viable cells in commercial products ranges from 1×10^5 CFU g^{-1} (Shah, Warnakulasuriya, Lankaputhra, Britz & Kyle, 1995) to 1×10^6 CFU g^{-1} (Samona & Robinson, 1994; Arroyo, Cotton & Martin, 1994; Rybka & Kailasapathy, 1995; Pagano, 1998). To ensure these numbers of probiotic bacteria, there is a need for rapid and reliable methods for routine enumeration. These methods are also essential to monitor the changes in their populations throughout the commercial storage of products (Arroyo, Cotton & Martin, 1994). The enumeration methodologies should not be complex or time-consuming and should offer a good cell recovery for the microorganisms (Lim, Huh & Baek, 1995). Except for *L. acidophilus* (IDF, 1995), the lack of official methodologies for accurate enumeration of *L. casei* and bifidobacteria causes difficulties in quality control (Rybka & Kailasapathy, 1996) and setting official norms.

Several media have been suggested for the enumeration of probiotic bacteria alone or in combination in commercial cultures or products (Beerens, 1990; Lapierre, Undeland & Cox, 1992; Arroyo, Cotton, & Martin, 1994; IDF, 1995; Lim, Huh & Baek, 1995; Nighswonger, Brashears & Gilliland, 1996; Rybka & Kailasapathy, 1996; Pacher & Kneifel, 1996; Dave & Shah, 1996; Silvi, Rumney & Rowland, 1996; Vinderola & Reinheimer, 1999). When enumeration of *L. acidophilus* and *B. bifidum* is required in the presence of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, the media assayed gave variable results (Dave & Shah, 1996;

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Vinderola & Reinheimer, 1999). If *Lactobacillus casei* is also present in dairy products, selective or differential enumeration of each bacteria becomes very difficult to perform due to the lack of recovery of one or more species (Lankaputhra & Shah, 1996), selectivity (Lim, Huh & Baek, 1995; Pacher & Kneifel, 1996) and/or differentiation ability among colonies (Kneifel & Pacher, 1993; Ghoddusi & Robinson, 1996; Nighswonger, Brashears & Gilliland, 1996). There are few reports about the enumeration of *L. casei* in the presence of *L. acidophilus*, bifidobacteria and lactic acid starter (Ravula & Shah, 1998) and new methods are necessary for this complex microbial system.

The aim of this work was to evaluate a set of culture media for the selective and/or differential enumeration of *L. casei* from *L. acidophilus*, bifidobacteria and lactic acid bacteria starters in fermented dairy products.

2. Materials and methods

2.1. Cultures

Pure lyophilised cultures of *Bifidobacterium bifidum* (strains BBI and BB12) and *Lactobacillus acidophilus* (strains LAI and LA5), and concentrated frozen cultures of *B. bifidum* (strain A12), *L. acidophilus* (strains A3 and A9) and *L. casei* (strains A13, A15 and A16) were supplied by local industries. Frozen cultures of *B. bifidum* (strain A8), *B. longum* (strains A1, A7 and BL), *Bifidobacterium* sp. (strain A2), *L. acidophilus* (strains N2, N3, CNRZ 1881 and CNRZ 1923) and *L. casei* (strains A14, LS, YKT, LB and CNRZ 1874) were taken from our collection. All cultures, with the exception of the collection strains, are widely used in Argentina for fermented dairy products (yoghurt, cultured milk and probiotic cheese).

2.2. Culture media

Lithium propionate MRS agar (LP-MRS) and bile MRS agar (B-MRS) were used as selective or differential media, according to the results of a previous work (Vinderola & Reinheimer, 1999). These media were reported as inhibitory for *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. To formulate selective media, MRS agar was used as a basal medium to which inhibitory compounds were added at concentrations shown in Table 1. B-MRS agar was used exactly as it was originally proposed (IDF, 1995). For LP-MRS agar, one concentration of lithium chloride and sodium propionate from several suggested (Lapierre et al., 1992) was chosen (Table 1), and these salts were added to MRS agar (commercially available) instead of the culture medium proposed (Lapierre et al., 1992). MRS agar without selective agents was chosen as a reference medium because it supported optimum growth for every strain used in this work (Vinderola & Reinheimer, 1999).

Table 1
Selective media used in this study for viable cell counts of bifidobacteria, *Lactobacillus acidophilus* and *L. casei*[a]

Agar medium	Inhibitory agent	Final composition of selective compounds in the media (% w/v)	
Bile-MRS	Bile (Sigma) (IDF, 1995)	Bile	0.15
LP-MRS	LP (Lapierre et al., 1992)	Lithium chloride	0.2
		Sodium propionate	0.3

[a]LP: Lithium chloride and sodium propionate.

2.3. Media performance

The suitability of the media was tested by plating decimal dilutions of the probiotic cultures. Thus, a sample of 1 g of pure lyophilised or frozen cultures, or 1 mL of an overnight culture of collection strains was decimally diluted in sterile peptone water (0.1%) and 0.1 mL aliquot dilutions were plated on the different media, in triplicate. Plates of B-MRS agar were incubated aerobically for 72 h at 37°C to inhibit bifidobacteria (Vinderola & Reinheimer, 1999). Plates of LP-MRS agar were incubated anaerobically (72 h at 37°C, GasPak System-Oxoid, Basingstoke, Hampshire, England). The population in colony-forming units (CFU) and the characteristics of the colonies were recorded for each medium. To confirm the identity of the colonies, catalase testing (Mac Faddin, 1980) was carried out and the cell morphology (phase contrast, 1000× Microscope Jenamed 2 CARL ZEISS) was recorded.

2.4. Statistical analysis

Experiments were replicated at least three times following a randomised block design. All data were analyzed using the one-way ANOVA procedure of SPSS. The differences among means were detected by the Duncan's multiple range test (Lizasoain & Joaristi, 1995).

2.5. Analysis of fermented dairy products

A total of nine products claimed to contain bifidobacteria, *L. acidophilus* and/or *L. casei* were purchased in supermarkets in Santa Fe (Argentina) and immediately examined microbiologically. One of the products was a soft, rindless cheese with *S. thermophilus* and *Lactococcus lactis* as lactic starters. Samples (1.0 mL) of the products (except soft cheese) were decimally diluted in sterile peptone water (0.1%) and 0.1 mL aliquot dilutions were spread over the surfaces of plates of the different media.

To enumerate thermophilic lactic starters from dairy products, lactic bacteria differential agar (LBD-agar; HI MEDIA, Bombay, India; aerobic incubation, 72 h at 37°C) was used (Vinderola & Reinheimer, 1999). The composition of LBD-agar is: enzymic digest of casein = 10 g L^{-1}; papaic digest of soyameal = 1.5 g L^{-1}; casein acid hydrolysate = 3 g L^{-1}; yeast extract = 1 g L^{-1}; fructose = 2.5 g L^{-1}; monopotassium phosphate = 2.5 g L^{-1}; bromocresol green = 0.055 g L^{-1} and agar = 15 g L^{-1}). Plates of LP-MRS and B-MRS agar were incubated as indicated previously. For soft cheese, samples were collected aseptically into sterile flasks. Portions of 20 g were homogenised with a 180 mL sterile aliquot of sodium citrate solution (2%) for 3 min in a stomacher. Decimal dilutions of the soft cheese homogenates were produced in peptone water (0.1%) and plated on the same media. To enumerate starter bacteria for cheese, Elliker agar (BIOKAR, Beauvais, France) was used for the colony count of *Lactococcus lactis* (72 h at 25°C, aerobic incubation) and LBD-agar was used for enumerating *S. thermophilus* (72 h at 37°C, aerobic incubation). Three independent experiments were carried out in each case.

3. Results and discussion

All strains of each probiotic bacteria tested in this study showed the same kind of colonies in the media described above: *L. casei* yielded round white creamy colonies on both media with diameters ranging from 1.7 to 2.4 mm (LP-MRS agar) and from 0.9 to 1.3 mm (B-MRS agar); *L. acidophilus* appeared on B-MRS agar as irregular light brown colonies ranging in diameter from 0.9 to 1.5 mm. Bifidobacteria on LP-MRS agar yielded small round colonies ranging from 0.7 to 1.2 mm in diameter.

Table 2 includes the plate counts of *Lactobacillus casei*, *L. acidophilus* and bifidobacteria obtained on B-MRS, LP-MRS and MRS agars. MRS agar was chosen as the

Table 2
Enumeration (log CFU g^{-1} unless specified otherwise) of *L. casei*, *L. acidophilus* and bifidobacteria on different media (3 d at 37°C). The values are the mean of three determinations[a]

Microorganism	Strain	Culture medium		
		MRS	MRS-Bile[b]	MRS-LP[b]
Lactobacillus casei[c]	A13	10.43 ± 0.14ba	10.50 ± 0.21a	10.44 ± 0.11a
	A14[d]	9.10 ± 0.12a	8.73 ± 0.08b	9.09 ± 0.23a
	A15	10.33 ± 0.06a	10.28 ± 0.40a	10.09 ± 0.18a
	A16	11.90 ± 0.06a	11.91 ± 0.04a	11.91 ± 0.07a
	LS[d]	9.84 ± 0.05a	9.81 ± 0.12a	9.78 ± 0.04a
	YKT[d]	9.56 ± 0.11a	9.37 ± 0.07a	9.46 ± 0.12a
	LB[d]	9.39 ± 0.16a	9.18 ± 0.14a	9.22 ± 0.10a
	CNRZ 187[d]	9.70 ± 0.16a	9.40 ± 0.23a	9.48 ± 0.26a
Lactobacillus acidophilus[c]	LAI	10.84 ± 0.13a	10.82 ± 0.06a	[e]
	LA5	10.64 ± 0.27a	10.12 ± 0.25a	[e]
	A3	9.43 ± 0.06a	9.30 ± 0.25a	[e]
	A9	11.81 ± 0.06a	11.72 ± 0.07a	[e]
	N2[d]	9.30 ± 0.34a	9.32 ± 0.18a	[e]
	N3[d]	8.31 ± 0.30a	8.02 ± 0.20a	[e]
	CNRZ 1881[d]	8.35 ± 0.07a	8.10 ± 0.09b	[e]
	CNRZ 1923[d]	8.24 ± 0.22a	7.97 ± 0.11a	[e]
Bifidobacterium bifidum[f]	BBI	10.18 ± 0.09a	[e]	9.92 ± 0.16a
	BB12	11.28 ± 0.10a	[e]	11.26 ± 0.09a
	A12	10.59 ± 0.12a	[e]	10.16 ± 0.24b
	A8[d]	9.68 ± 0.18a	[e]	9.54 ± 0.11a
Bifidobacterium longum[f]	A1[d]	8.92 ± 0.07a	[e]	8.93 ± 0.07a
	A7[d]	8.02 ± 0.29a	[e]	7.90 ± 0.09a
	BL[d]	9.40 ± 0.10a	[e]	9.29 ± 0.09a
Bifidobacterium sp.[f]	A2[d]	9.69 ± 0.12a	[e]	9.60 ± 0.13a

[a] Means in row with a common letter do not differ ($P > 0.05$).
[b] inhibitory medium for yoghurt starter bacteria (Vinderola & Reinheimer, 1999).
[c] incubation under aerobiosis.
[d] CFU mL^{-1}.
[e] $<10^5$ CFU g^{-1} or mL^{-1}.
[f] incubation under anaerobiosis.

Table 3
Enumeration of probiotic and lactic acid bacteria in Argentinian fermented dairy products

Samples	pH	Colony counts (log CFU mL^{-1})[a]					
		S. thermophilus[b]	*L. bulgaricus*[b]	*Lac. lactis*[c]	*Bifidobacte rium*[d]	*L. acidophilus*[e]	*L. casei*[d]
Full-fat yoghurt A	4.35	9.24	7.81	[g]	5.54	6.67	[f]
Full-fat yoghurt B	4.19	9.13	[f]	[g]	[f]	[f]	8.02
Fermented milk A	4.72	9.49	[f]	[g]	[f]	4.62	[f]
Fermented milk B	4.63	9.11	[f]	[g]	6.52	6.60	[f]
Yoghurt with fruits	4.25	9.46	[f]	[g]	[f]	[f]	[f]
Yoghurt	4.12	8.87	5.58	[g]	[f]	[f]	[f]
Reduced-fat yoghurt A	4.25	9.18	7.95	[g]	[f]	[f]	8.09
Reduced-fat yoghurt B	4.22	9.30	7.90	[g]	2.60	[f]	[f]
Fresco cheese [h]	5.25	9.08	[f]	4.90	8.71	8.39	8.33

[a] Mean of three determinations.
[b] On LBD agar.
[c] On Elliker agar.
[d] On LP-MRS agar.
[e] On B-MRS agar.
[f] $<10^2$ CFU mL^{-1}.
[g] Not determined.
[h] CFU g^{-1}.

reference medium for probiotic bacteria. Since the highest colony counts were obtained in this medium, it was used to compare cell recovery in the other ones. It could be concluded that both B-MRS and LP-MRS agars can be used for the enumeration of *L. casei* since no significant differences ($P < 0.05$) were detected between the counts obtained on the reference medium and on them, with the only exception of *L. casei* A14 on B-MRS agar. In this case, LP-MRS agar should be used to perform a selective or differential colony count. For *L. acidophilus* strains, only *L. acidophilus* CNRZ 1881 showed significant differences between counts performed on selective media and the reference one. Finally, no significant differences were detected for bifidobacteria counts obtained on LP-MRS agar and MRS agar, except for *B. bifidum* A12, in which case the difference showed to be small. In a previous work (Vinderola & Reinheimer, 1999) it was demonstrated that B-MRS and LP-MRS agar were able to inhibit the growth of lactic acid starter bacteria. Ravula and Shah (1998) proposed LC agar for the selective count of *L. casei* in yoghurts and fermented milk drinks. However, the basal medium is not commercially available and must be prepared from single ingredients. The basal medium for the selective media proposed in this work is a commercial version of MRS agar. Another advantage of B-MRS and LP-MRS agars is that both media allows the enumeration of *L. casei* plus bifidobacteria (LP-MRS agar) or *L. acidophilus* (B-MRS agar).

Table 3 shows the results obtained when the culture media were applied to the enumeration of probiotic bacteria in fermented dairy products. The levels of probiotic bacteria found were very variable and, in some cases, lower than the minimum level suggested. Among the probiotic bacteria, the highest cell counts corresponded to *L. casei*, suggesting a higher ability to survive in fermented milk products. Fresco cheese showed a significantly higher content of probiotic bacteria than the contents found in fermented milk products, perhaps due to the lower acid and dissolved oxygen contents of this kind of cheese (Kailasapathy & Rybka, 1997; Marth & Steele, 1998).

4. Conclusions

In a previous work (Vinderola & Reinheimer, 1999), B-MRS agar and LP-MRS agar were confirmed to be inhibitors for lactic acid bacteria from the starters. As aerobiosis is a factor that inhibits the growth of bifidobacteria, B-MRS agar was useful in the selective colony count of *L. acidophilus* or *L. casei* or in the selective and differential enumeration when these bacteria appear together in a fermented dairy product. In the case of bifidobacteria, a selective colony count could be performed on LP-MRS agar, even when it appears with *L. casei* because a differential cell enumeration between both organisms was possible. The three probiotic bacteria can be simultaneously enumerated from a product that contains them using both selective/differential media evaluated in this work.

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