Differential enumeration of *Bifidobacterium longum* and *Lactobacillus acidophilus* in the presence of *Streptococcus thermophilus*[1]

Modified (T and M-MRS) and selective (Bile and LP-MRS) media were assayed to quantify *Bifidobacterium longum* (BL) and *Lactobacillus acidophilus* (LAC) in the presence of *Streptococcus thermophilus* (Str). Microbial suspensions (isolated and mixed) were tested at 37°C/72h aerobically with T, M and Bile-MRS. All tests using LP-MRS were done anaerobically. LP-MRS was tested under four different combinations of lithium chloride and sodium propionate concentrations (0.2; 0.4; 0.5 and 0.6% of LiCl with respectively 0.3; 0.6; 0.75 and 0.9% of sodium propionate). The ability of each medium to inhibit the organisms was also tested. M-MRS allowed the growth of Str. Differential counting of LAC was possible using T and Bile-MRS, aerobically, (the latter condition being more practical and economical). Differential counting of BL was possible using LP-MRS in anaerobiosis, with a combination of 0.6g/l of LiCl and 0.9 g/l of sodium propionate.

Os meios modificados T e M-MRS e os meios seletivos Bile e LP-MRS foram avaliados na quantificação de *L. acidophilus* (LAC) e *Bif. longum* (BL) na presença de *Str. thermophilus* (Str). Suspensões microbianas (isoladas e mistas) foram utilizadas, sendo 37°C/ 72h e aerobiose as condições de incubação para estudo de T, M e Bile-MRS. Todos os ensaios com LP-MRS foram realizados em anaerobiose. O ágar LP-MRS foi avaliado pela adição de quatro diferentes combinações de concentrações de cloreto de lítio e propionato de sódio (0,2; 0,4; 0,5 e 0,6% de LiCl e, respectivamente, 0,3; 0,6; 0,75 e 0,9% de propionato de sódio). A habilidade de cada meio inibir os microrganismos também foi verificada. O ágar M-MRS possibilitou o crescimento de Str. A quantificação diferencial de LAC foi possível utilizando-se T e Bile-MRS, sendo este mais prático e econômico. A quantificação diferencial de BL foi possível através do LP-MRS com 0,6g/l de LiCl e 0,9 g/l de propionato de sódio.

[1] Milchwissenchaft 59, 5/6. 258-261. 2004

1. INTRODUCTION

The consumption of probiotics of the genera *Lactobacillus* and *Bifidobacterium* has been linked to health improvement and relief/prevention of disorders, including, among others, acute episodes of diarrhoea (bacterial or viral infection); immune enhancing; prevention of radiotherapy-related diarrhoea; alleviation of Chrohn's diseases and inhibition of superficial bladder cancer (28, 16, 15, 8, 10). According to GOMES and MALCATA (8), probiotics are defined as viable microorganisms (lactic and other bacteria and yeasts ingested as dried cells or in a fermented product), exhibiting a beneficial effect on the health of the host (upon ingestion) by improving the properties of its indigenous microflora.

Bifidobacteria were first isolated from the faeces of breast-fed infants and described in 1899-1900 by TISSIER. Before the current designation, bifidobacteria received other names, like *Bacillus bifidus* and *Lactobacillus bifidus*. This microbial group is gram-positive, catalase-negative, anaerobic and non-motile, presenting a rod shape and producing no gas (8, 28, 3).

L. acidophilus (LAC) was first named *Bacillus acidophilus* by MORO, in 1990, who isolated it from the faeces of breast-fed infants. Some characteristics of these microorganisms are: aerotolerant or anaerobic, strictly fermentative, gram-positive, non-flagellated rods or coccobacilli (8, 3).

Fermented milks containing bifidobacteria and/or LAC are produced using either single strains of these organisms alone or in combination with other lactic acid bacteria, mainly *Streptococcus* and *Lactobacillus*. Many articles have reported these kinds of fermented milk or yoghurt (19, 28, 8, 4, 5, 23, 27, 12).

Since the efficacy of added probiotic bacteria is dependent on the dose level, the viability must be maintained throughout the intended product shelf life. To monitor the maintenance of concentrations of viable probiotics, simple and inexpensive methods are desirable for use in quality assurance laboratories (10). These methods must determine the counts of *Bifidobacterium*, LAC and yoghurt bacteria (e.g. *Str. thermophilus*) and differentiate them. Additionally, fermented milk products must obey the legal standards, which establish minimal viable cell counts per gram or ml, of 10^6 (2).

Many media for the differential enumeration of *Bifidobacterium* are cited in the literature (24, 28, 14, 21, 29, 4, 20, 7, 27, 3, 13). Media for the differential enumeration of LAC have been reported by many authors (29, 4, 7, 3, 13).

Some selective media for the enumeration of bifidobacteria in the presence of lactic bacteria are formulated using many ingredients and/or their inhibitory agents must be filter-sterilized, being time-consuming to prepare (18, 29, 1, 17). However, LiCl and sodium propionate (14) can be added directly to the medium base before sterilization.

Previous experiments by the authors of this paper (31) detected the growth of Str on LP-MRS, as suggested by VINDEROLA and REINHEMER (29) for the differential enumeration of *Bifidobacterium* in the presence of yoghurt bacteria.

The aim of this research was to select methodology for the selective enumeration of LAC in the presence of Str and BL and for the selective enumeration of BL in the presence of Str and LAC*,* using a model system of peptone water suspensions of these microorganisms.

2. MATERIAL AND METHODS:

2.1. Material: Maltose and yeast extract (Merck, Ge); Meat extract, bacto peptone, bacto bile, tri-ammonium citrate, tween 80 and agar (Difco, USA); Sodium propionate, KH_2PO4; Magnesium sulphate $7xH_2O$, trehalose; (SIGMA, USA); Lithium chloride, manganese sulphate $4xH_2O$ and sodium acetate $3xH_2O$ (Synth, Brasil); Anaerobac (Probac, Brasil); MRS agar (Oxoid, En). Freeze dried cultures of *Str. thermophilus*, *L. acidophilus* and *Bif. longum,* codified respectively as TA559, LAC4 and BL (Ezal®, Texel - Rhodia Food), supplied by Rhodia Food, were used.

2.2. Media: Maltose-MRS agar (M-MRS) was prepared according to HULL and ROBERTS (9); Bile-MRS agar according to IDF (1995) as reported by VINDEROLA and REINHEIMER (29); Trehalose MRS agar (T-MRS) according to VINDEROLA and REINHEIMER (29) and Lithium chloride - sodium propionate agar (LP-MRS) according to LAPIERRE *et al.* (14), with the basal medium suggested by VINDEROLA and REINHEIMER (29).

2.3. Experimental procedures

2.3.1. Microbial suspensions: Isolated suspensions of Str (ISST), LAC (ISLA) and BL (ISBL) were prepared with 1 unit of each freeze dried culture (10^{10} to 10^{11} cell/unit) rehydrated in 9 ml 0.1% peptone water. The mixed suspension (MIX 3), was obtained by mixing equal volumes of ISST, ISLA and ISBL.

2.3.2. Testing M-MRS, T-MRS and Bile-MRS agars

Modified media: T-MRS (30) and M-MRS agar (4, 5, 9, 22); and selective media: Bile-MRS agar (29, 13) were assayed to quantify LAC in the presence of Str and BL at 37^{o}*C*/72h aerobically.

2.3.3. Testing LP-MRS agar at different concentrations of the inhibitory agents

LiCl - sodium propionate agar (LP-MRS) (29) was assayed to quantify BL in the presence of Str and LAC at 37^{o}*C*/72h anaerobically. Anaerobiosis was generated by the PROBAC® system. Concentrations of: 0.2; 0.4; 0.5 and 0.6% of LiCl and 0.3; 0.6; 0.75 and 0.9% of sodium propionate for LP-MRS, were tested.

2.3.4. Enumeration:

The suspensions described (ISSL, ISLA, ISBL and MIX3) were serial decimal diluted in 0.1% sterile peptone water and pour plated with the media cited.

2.4. Statistical analyses:

The data from the experiments with M, T and Bile-MRS agar (Table 1) were analyzed by the General Linear Model procedures of the Statistical Analysis System (SAS 8.02 TS level 02M0). A p value < 0.05 was considered to be significant.

3. RESULTS AND DISCUSSION

Viable cell counts in M-MRS, T-MRS and Bile-MRS agar under aerobiosis for Str, LAC, BL, isolated and in MIX3, are presented in Table 1.

Table 1. Viable cell counts[a] in M-MRS, T-MRS and Bile-MRS agars of *Str. thermophilus*, *L. acidophilus* and *Bif. longum*, isolated and mixed, incubated at 37°C

	Culture media		
Microorganisms	M-MRS	T-MRS	Bile-MRS
ISST[b]	6.3×10^6	---[d]	---[d]
ISLA[b]	4.9×10^9	5.5×10^9	7.5×10^9
ISBL[b]	---[e]	---[e]	---[e]
Mix 3[c]	2.2×10^9	3.5×10^8	1.7×10^8

a. results are means of 3 replicates
b. ISST, ISLA and ISBL mean Isolated suspensions of *Str. thermophilus*, *L. acidophilus* and *Bif. longum*
c. suspension obtained by mixing equal volumes of *Str. thermophilus*, *L. acidophilus* and *Bif. longum* suspensions
d. no colonies found
e. no colonies found because BL is anaerobic

From the counts presented in Table 1, it was observed that Str grew in M-MRS agar. The LAC counts obtained in M-MRS did not differ significantly from the counts of this microorganism in T-MRS and Bile-MRS agar, the counts of MIX3 in M-MRS being higher then in the other two media, probably due to the growth of Str in the M-MRS medium.

The counts of LAC from ISLA were higher than those from MIX3 when using T-MRS and Bile-MRS. This could be explained by the inhibitory effect of Str against LAC. SHAH *et al.* (28) reported an inhibitory effect of Str bacteriocin against *Bifidobacterium*. Other authors (5, 6, 11) also reported inhibitory effects of probiotic bacteria against yoghurt bacteria. VINDEROLA *et al* (30) reported the inhibitory effect of lactic and probiotic bacteria against LAC.

M-MRS was suggested by RYBKA and KAILASAPATHY (22) for the selective enumeration of LAC. DAVE and SHAH (4, 5, 6) suggested M-MRS agar for the enumeration of probiotics (LAC and bifidobacteria) in the presence of yoghurt bacteria. LANKAPUTHRA *et al*. (13) reported that *Streptococcus* from different sources, did not grow on M-MRS agar. The Chr.Hansen's Laboratorium indicates M-MRS as the medium of choice for differential enumeration of *Lactobacillus* (3). However, in our experiments, Str grew (Table 1), interfering in the selective counts of LAC.

Bile and T-MRS showed good results for selective LAC enumerations, but Bile-MRS is less expensive and time-consuming then T-MRS.

The counts of Str, BL and LAC in LP-MRS agar with different concentrations of the inhibitory agents, from dilutions of 10^{-3} up to 10^{-8}, are presented in Table 2.

Table 2. Viable cell counts[a] of *Str. thermophilus*, *L. acidophilus* and *Bif. longum* in LP-MRS at different concentration of lithium chloride and sodium propionate

		Concentration LiCl : Na propionate (g/l)							
		2 : 3		4 : 6		5 : 7.5		6 : 9	
microorganisms[b]	Dilution	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
ISST	-3	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	-[c]	-[c]
ISBL		tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]
ISST	-4	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	-[c]	-[c]
ISBL		tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]
ISST	-5	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	$9.1x10^6$	-[c]	-[c]
ISBL		tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]
ISST	-6	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	$2.2x10^7$	-[c]	-[c]	-[c]
ISBL		tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]
ISST	-7	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	-[c]	-[c]	-[c]	-[c]
ISBL		tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	tmtc[e]	$3.4x10^9$	tmtc[e]
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]
ISST	-8	$5x10^{10}$	$3x10^{10}$	$2.6x10^{10}$	$1.0x10^{10}$	-[c]	-[c]	-[c]	-[c]
ISBL		$9.1x10^9$	$7.0x10^9$	$5x10^9$	$5.8x10^9$	$5.4x10^9$	$4.6x10^9$	$4.0x10^9$	$5x10^9$
ISLA		-[c]	-[c]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]

a. Data from tests 1 and 2
b. ISST, ISLA and ISBL means Isolated suspension of *Str. thermophilus*, *L. acidophilus* and *Bif. longum* suspensions
c. no colonies found
d. test did not performed
e. tmtc = too much to count

LAPIERRE *et al*. (14) were the first to suggest the use of LiCl and sodium propionate as inhibitory agents. They tested different LiCl and sodium propionate concentrations, suggesting a combination of 2g/l of LiCl and 3g/l of sodium propionate to suppress the growth of lactic acid bacteria (Str, *L. bulgaricus*, LAC and *Lactococcus cremoris*). They also reported that most strains of streptococci were inhibited. VINDEROLA and REINHEMER (29) used MRS agar as the basal medium with the LiCl and sodium propionate concentrations suggested by LAPIERRE *et al*. (14). SHAH (25) reported that one strain of Str grew on LAPIERRE *et al.* (14) medium.

In our experiments, Str colonies grew in LP-MRS when the inhibitory agents were used at the concentrations suggested by VINDEROLA and REINHEMER (29). However the use of greater concentrations showed that LP-MRS could be used for BL selective enumeration (Table 2).

The LAC from ISLA did not grow at the inhibitory agent concentrations suggested by LAPIERRE *et al.* (14) and VINDEROLA and REINHEMER (29) (Table 2).

4. CONCLUSIONS

The Str tested here cannot be inhibited, under aerobiosis, using M-MRS agar.

T and Bile-MRS were capable of the differential enumeration of LAC in the presence of Str and BL, but Bile-MRS is less expensive and less time-consuming then T-MRS.

The LAC tested was inhibited, under anaerobiosis, in LP-MRS agar, at LiCl and sodium propionate concentrations of 2 and 3g/l, respectively.

The Str tested could not be inhibited, under anaerobiosis, in LP-MRS agar at LiCl and sodium propionate concentrations of 2 and 3g/l, respectively, but with three times these concentrations of the inhibitory agents, Str did not grow.

LP-MRS agar with LiCl and sodium propionate concentrations of 6 and 9g/l, respectively, was capable of the differential enumeration of BL in the presence of Str and LAC.

LiCl and sodium propionate have the advantage of being added before sterilization when used as inhibitory agents.

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