ENUMERATION OF *Streptococcus thermophilus* IN THE PRESENCE OF *Bifidobacterium longum* AND *Lactobacillus acidophilus* – EFFECT OF INCUBATION TEMPERATURE AND ATMOSPHERIC CONDITIONS[1]

The enumeration of *Streptococcus thermophilus* (Str) in the presence of *Lactobacillus acidophilus* (LAC) and *Bifidobacterium longum* (BL) on ST agar at 37ºC/48h/aerobically and on Lee's agar at 37ºC/48h/microaerophiliae were shown to be non-selective for Str. ST agar allowed the growth of LAC and Lee's agar allowed the growth of LAC and BL. ST agar (aerobically) and Lee's agar (aerobically and under microaerophiliae) were incubated at 30, 35, 37 and 45ºC. At 30 and 45ºC LAC was inhibited with ST agar and either LAC and BL with Lee's agar. The recovery of Str was efficient under all conditions. The tests were carried out using isolated and mixed suspensions of these microorganisms. Counts were also made with Str fermented skim milk with added probiotics. The ability of each medium to inhibit the organisms was also tested.

A enumeração de *Str. thermophilus* (Str) na presença de *L. acidophilus* (LAC) e *Bif. longum* (BL) em ágar ST (*Streptococcus thermophilus*) a 37^{o}C/ 48h em aerobiose e em ágar Lee a 37^{o}C/48h em microaerofilia mostrou-se não seletiva para Str. O ágar ST permitiu o crescimento do LAC e o ágar Lee permitiu o crescimento de LAC e BL. O ágar ST e o ágar Lee foram incubados a 30, 35, 37 e 45^{o}C, o primeiro apenas em aerobiose e, o segundo, em aerobiose e microaerofilia. A 30 e 45^{o}C, o LAC foi inibido no ágar ST e LAC e BL no ágar Lee. A recuperação de Str foi eficiente em todas as condições. Os ensaios foram realizados com suspensões isoladas e mistas dos microrganismos. Também foram realizados experimentos com leites fermentados adicionados dos probióticos liofilizados. A habilidade de cada meio de inibir os microrganismos também foi verificada.

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1. INTRODUCTION

At the beginning of the 20th century, Elie Metchnikoff suggested that the consumption of fermented milk could prolong life. Since then, interest has been shown in the microorganisms of these products, especially in bifidobacteria and *Lactobacillus* because of their capacity to treat or prevent diseases. There is considerable information in the literature about their function in human health (3, 2, 9, 16, 12, 19, 10, 24). Today many milk products contain such microorganisms (8, 17, 23, 16, 15, 18, 22, 5, 19, 25) and many may contain *Streptococcus thermophilus* (Str)*, Bifidobacterium* and *Lactobacillus* (9, 23, 17, 25, 13, 14, 16).

An important parameter in product development and quality assurance is the ability to differentiate and determine the viable count of these organisms (22) and, in addition, they must obey the legal standards, which establish minimal viable cell counts (1, 8). Different media have been suggested in the literature for the enumeration of Str (15, 6, 23, 8, 3, 26, 27, 4, 14, 20).

The aim of this research was to select a methodology for Str enumeration in the presence of LAC and BL using a model system consisting of microbial suspensions in peptone water (both alone and mixed) as well as in Str fermented milk.

2. MATERIAL AND METHODS

2.1.Material: Lactose, bacto peptone and tryptone (Difco, USA); Agar, bromo-cresol purple, K_2PO_4 and Sucrose (Merck, Ge); Yeast extract (BioBrás, Brazil); $CaCO_3$ (Ecibra, Brazil); Microaerobac (Probac®, Brazil); Skim milk powder (Molico, Nestle, Brazil). Freeze dried cultures of *Str. thermophilus, L. acidophilus* and *Bif. longum* were used, codified by the supplier, respectively, as TA559, LAC4 and BL (Ezal®, Texel-Rhodia Food). ST and Lee's agars were prepared, respectively, according to DAVE and SHAH (6) and LEE *et al.* (15).

2.2.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.Fermented milks: Sterile (115ºC/10min) reconstituted (11% total solids) skimmed milk was used for the mother cultures; fermented milks were prepared with 12% TS. The mother culture was prepared by inoculating sterile milk (10 ml test tube) with a loopful of freeze-dried Str, fermented (42ºC) to coagulation and cooled. The fermented milk (FM) (300 ml) was prepared by inoculating sterile milk with 2% mother culture, fermenting (42ºC) to pH 4.7 and chilling to 5ºC. One unit of each freeze-dried culture (BL and LAC) was added to 100g of FM for production of fermented milk with added probiotic (FMPA) with 10^8 cells/g of each microorganism.

2.4.Enumeration: The ISST, ISLA, ISLB, MIX3, FM and FMPA were diluted in 0.1% peptone water, pour plated in ST agar (aerobiosis) and spread plated on Lee's agar (aerobisis and microaerophiliae) and incubated at 30, 35, 37 and 45ºC. The microaerophilic atmosphere was generated by the Probac® system.

2.5. Statistical analyses: The experimental data was analyzed by the general Linear Model procedures of the Statistical Analysis System (SAS 8.02 TS level 02MO).

3. RESULTS AND DISCUSSION

3.1.Viable cell counts of peptone water suspensions

Table 1 presents the cell counts in ST and Lee's agars of isolated Str, LAC and BL and MIX3. LAC grew in ST agar at 37ºC forming pinpoint white colonies and, also, in Lee's agar at 37ºC (microaerophiliae and aerobiosis). BL grew in Lee's agar at 37ºC under microaerophiliae. The observed growth of LAC and/or BL, can interfere in selective counts of Str in mixed populations.

Table 1. Viable cell counts[1] in ST agar (aerobic) and on Lee's agar (aerobic and under microaerophilic conditions) of isolated *Str. thermophilus, L. acidophilus* and *Bif. longum* and of MIX3 incubated at 30, 35, 37 and 45ºC[1]

	Enumeration conditions		Incubation temperature (ºC)			
Micr. susp	**media**	**atmosphere**	**30**	**35**	**37**	**45**
ISST[2]	ST agar	aerobiosis	$2.1x10^{11}$	$1.7x10^{11}$	$2.3x10^{11}$	$1.8x10^{11}$
ISLA[2]	ST agar	aerobiosis	-[4]	$5.3 x10^{9}$	$6.6x10^{9}$	-[4]
ISBL[2]	ST agar	aerobiosis	-[5]	-[5]	-[5]	-[5]
Mix 3[3]	ST agar	aerobiosis	$2.6x10^{10}$	$2.3x10^{10}$	$5.2x10^{10}$	$3.3x10^{10}$
ISST[2]	Lee agar	microaerofiliae	$1.7x10^{11}$	$2.60x10^{11}$	$1.10x10^{11}$	$1.80x10^{11}$
	Lee agar	aerobiosis	$2.35x10^{11}$	$2.70x10^{11}$	$3.10x10^{11}$	$2.20x10^{11}$
ISLA[2]	Lee agar	microaerofiliae	-[4]	$2.50x10^{10}$	$1.00x10^{10}$	-[4]
	Lee agar	aerobiosis	-[4]	$9.40x10^{9}$	$1.10x10^{10}$	-[4]
ISBL[2]	Lee agar	microaerofiliae	-[4]	$3.00x10^{10}$	$2.70x10^{10}$	-[4]
	Lee agar	aerobiosis	-[5]	-[5]	-[5]	-[5]
Mix 3[3]	Lee agar	microaerofiliae	$2.30x10^{10}$	$2.50x10^{10}$	$2.70x10^{10}$	$2.40x10^{10}$
	Lee agar	aerobiosis	$3.00x10^{10}$	$3.70x10^{10}$	$3.10x10^{10}$	$2.90x10^{10}$

1.results are means of duplicates.
2.ISST, ISLA and ISBL signify isolated suspensions of *Str. thermophilus, L. acidophilus* and *Bif. longum*
3.suspension obtained by mixing equal volumes of *Str. thermophilus, L. acidophilus* and *Bif. longum.*
4.no colonies found.
5. .no colonies found because BL is anaerobic.

The non growth of LAC at 30 or 45ºC in ST or Lee's agars (Table 1) and the inability of BL to grow aerobically suggests the use of ST or Lee's agars, at these temperatures, aerobically, to selectively count Str.

The statistical analysis of the ISST counts in ST and Lee's agars (aerobically) showed that there was no statistical difference between these counts and those of MIX3 with respect to the incubation temperature. This suggests the use of ST or Lee's agars aerobically, at 30 and/or 45ºC, to enumerate Str either alone or in mixtures of Str, LAC and BL, since there was no significant difference between these cell counts and those at 37ºC using the reference medium. However, the recovery of cells present in the MIX3 in ST or Lee's agars (aerobically) was of the order of x10 less than with ISST under the same conditions. This fact should be taken into consideration.

The evaluation of temperature influence using Lee's agar (aerobiosis or microaerophiliae), aiming at counting viable Str cells, showed that there was no significant difference between these four temperatures in the recovery of cells present in ISST or in MIX3. These results suggest the use of Lee's agar aerobically at 30 and/or 45ºC to enumerate Str, both in the isolated form and in mixtures with

LAC and BL. The counts from MIX3 were higher aerobically than under microaerophiliae, by about 50%, based on the average values. This suggests that aerobic conditions are more appropriate.

KARAGUL-YUCEER *et al.* (14) also reported the growth of LAC with M17 agar, used to enumerate Str in a LAC containing carbonated fermented milk based beverage. M17 agar was used and/or suggested in the enumeration of Str in the presence of LAC, by authors such as VINDEROLA *et al.* (27), RYBKA and KALAISAPATHY (20, 21), MEDINA and JORDANO (18), DAVIDSON *et al.* (8) and SHAH (23).

The difference between the counts obtained for ISST and MIX3 on ST and Lee's agars is probably due to inhibition of Str by BL and LAC. JOSEPH *et al.* (11) confirmed the inhibition of five strains of Str by LAC and BL due to organic acids and not to inhibitory substances like bacteriocins. DAVE and SHAH (7) studied bacteriocin production by LAC and its inhibitory action against lactic bacteria, including Str. They reported that LAC bacteriocin production in a model system is greater than in milk and whey based media. Our observations agree with this report, since such differences were not observed with fermented milk (Table 2).

Thus, we can suggest the use of ST or Lee's agars aerobically at 30 or 45ºC to selectively quantify Str, either alone or in mixtures with LAC and BL. Compared to 45ºC, 30ºC appears to be more adequate since there is less dehydration of the culture medium during the incubation period.

3.2.Viable cell count in fermented milk

Table 2 shows the counts at 30, 35, 37 and 45ºC in ST and Lee's agars from FM and FMPA.

The temperature influence evaluation using the ST and Lee's agars aerobically for enumeration of Str present in FM and in FMPA, showed there was no significant difference in the viable cell recovery among the four temperatures and the two media. These results suggest the use of ST or Lee's agars aerobically at incubation temperatures of 30 and/or 45ºC to enumerate Str, either alone or mixed with LAC and BL.

Table 2. Viable cell counts[1] in ST agar (aerobic) and Lee's agar (aerobic and microaerophilic) of FM and FMPA at 30, 35, 37 and 45ºC[1]						
	Enumeration conditions		**Incubation temperature (ºC)**			
Micr. susp	**media**	**atmosphere**	**30**	**35**	**37**	**45**
FM[2]	ST agar	aerobiosis	$1.0x10^9$	$7.8x10^8$	$7.0\ x10^8$	$9.5x10^8$
FMPA[3]	ST agar	aerobiosis	$1.1x10^9$	$1.1x10^9$	$7.50x10^9$	$1.2x10^9$
FM[2]	Lee agar	microaerofiliae	$1.0x10^9$	$7.6x10^8$	$7.1x10^8$	$8.1x10^8$
	Lee agar	aerobiosis	$8.3x10^8$	$7.4x10^8$	$6.6x10^8$	$7.0x10^8$
FMPA[3]	Lee agar	microaerofiliae	$1.3x10^9$	$1.9x10^9$	$1.7x10^9$	$7.70x10^8$
	Lee agar	aerobiosis	$9.4x10^8$	$1.6\ x10^9$	$1.6\ x10^9$	$9.2x10^8$

1.results are means of triplicate determinations.
2.FM is milk fermented by *Str. thermophilus.* 3.FMPA is FM with added freeze dried *L. acidophilus* and *Bif. longum*

The temperature influence evaluation using Lee's agar aerobically and at microaerophiliae for enumeration of Str present in FM and in FMPA, showed that there was no significant difference in the viable cell recovery among incubation conditions tested. These results suggest the use of Lee's agar (aerobiosis or microaerophiliae) incubated at 30 and/or 45ºC to enumerate Str*,* either alone or mixed with LAC and BL.

The results above permit the suggestion to use ST or Lee's agars aerobically, or Lee's agar at microaerophiliae at 30 or 45ºC to enumerate Str either alone or mixed with BL or LAC*.* However, aerobic conditions are cheaper and more practical, and at 30ºC, culture media dehydration during the incubation period is lower.

4. CONCLUSIONS

It was possible to inhibit growth of the LAC strain tested at 30 and 45ºC, aerobically or at microaerophiliae, using ST or Lee's agars. Inhibition of BL was possible at 30 and 45ºC aerobically, using ST or Lee's agar.

The Str counts obtained from ISST in ST agar (aerobisis) and Lee's agar (aerobisis or microaerophiliae) incubated at 30 and 45ºC, were not significantly different from those obtained at 37ºC under the same conditions.

The counts obtained from either ISST or MIX3 on Lee's agar, were slightly larger, aerobically than under microaerophiliae.

The counts obtained from MIX3 were smaller than those from ISST, reflecting a possible inhibition of Str by BL and/or LAC in this model system. This was not observed with FM and FMPA.

The Str counts obtained for either FM or FMPA in ST agar (aerobisis) and Lee's agar (aerobisis or microaerophiliae) incubated at 30 and 45ºC, were not significantly different from those obtained at 37ºC under the same conditions.

Inhibition of LAC and BL was obtained under the following conditions: ST agar/30 and 45ºC/aerobiosis; Lee's agar/30 and 45ºC/both atmosphere conditions, the former being more economical and practical.

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