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Short communication

## Development of a selective culture medium for bifidobacteria, Raffinose-Propionate Lithium Mupirocin (RP-MUP) and assessment of its usage with Petrifilm™ Aerobic Count plates

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### ABSTRACT

This study aimed to develop a selective culture media to enumerate bifidobacteria in fermented milk and to assess this medium when used with Petrifilm™ AC plates. For this purpose, *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus thermophilus* strains were tested to verify their fermentation patterns for different carbohydrates. All bifidobacteria strains were able to use raffinose. Based on these characteristic, a selective culture medium was proposed (Raffinose-Propionate Lithium Mupirocin, RP-MUP), used with Petrifilm™ AC plates, and was used to enumerate bifidobacteria in fermented milk. RP-MUP performance was assessed by comparing the results with this medium to reference protocols and culture media for bifidobacteria enumeration. RP-MUP, whether used or not with Petrifilm™ AC, presented similar performance to TOS-MUP (ISO 29981), with no significant differences between the mean bifidobacteria counts ( $p < 0.05$ ) and with high correlation indices ( $r = 0.99$ ,  $p < 0.05$ ). As an advantage, reliable results were obtained after just 48 h of incubation when RP-MUP was used with Petrifilm™ AC, instead of the 72 h described in the ISO 29981 protocol.

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## 1. Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO, 2001, 2006). The genus *Bifidobacterium* has been highlighted in this context, including several species with proven probiotic activity which are often added to fermented milk (Tamime, 2002). After the addition of a probiotic culture to a food, it is necessary that the population be monitored to ensure that its minimum concentration is maintained throughout the shelf life of the product, which ensures its functionality (Shah, 2000).

Many culture media have been developed for the selective enumeration of bifidobacteria, being basically composed of the medium de Man, Rogosa & Sharpe (MRS) supplemented with different antimicrobial compounds or their combinations (Dave and Shah, 1996; Leuschner et al., 2003; Lima et al., 2009). In 2010, the International Organization for Standardization published ISO 29981, a protocol for the enumeration of bifidobacteria in fermented milk which employs a culture medium containing transgalactosylated oligosaccharide (TOS) and lithium mupirocin as an inhibitor (ISO, 2010).

To monitor populations of bifidobacteria, food industries require methodologies that, besides using selective culture media, have fast and practical execution. In this regard, the Petrifilm™ system (3M Microbiology, St. Paul, MN, USA) represents a viable alternative and is routinely used with dairy products. Petrifilm™ Aerobic Count (AC) plates have been used with selective agents for the enumeration of different groups of lactic acid bacteria and bifidobacteria (Gonçalves et al., 2009; Miranda et al., 2011; Nero et al., 2006; Ortolani et al., 2007).

Jasson et al. (2010) described the need for standardization and performance testing of alternative methods for the enumeration of microorganisms such as bifidobacteria in foods. Therefore, this study aimed to develop a culture medium based on TOS-MUP and to evaluate its use with Petrifilm™ AC. The performance was assessed for the selective enumeration of bifidobacteria in fermented milk.

## 2. Material and methods

### 2.1. Microorganisms

Microorganisms, culture media for storage and recovery, and the incubation conditions used in this study are described in Table 1. All microorganisms were maintained in their culture media supplemented with 20% glycerol (v/v) at  $-20$  °C. At the moment of

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**Table 1**  
Microorganisms used in this study and culture media used for purification and growth conditions.

Genus	Species	Specification <sup>a</sup>	Culture media, stock <sup>b</sup>	Culture media <sup>b</sup> , purification	Incubation				
<i>Bifidobacterium</i>	—	CIRMBIA 1387	MRS, L-cysteine (0.5 g/L)	MRS-ABC:Dicloxacin (0.5 mg/L), lithium chloride (1.1 g/L), e L-cysteine (0.5 g/L)	37 °C, 72 h, anaerobiosis (GasPak)				
	—	CIRMBIA 1394							
	<i>animalis</i>	CIRMBIA 1388							
	<i>animalis</i>	CIRMBIA 1389							
	<i>animalis</i>	CIRMBIA 1390							
	<i>animalis</i>	CIRMBIA 1392							
	<i>animalis</i> subsp. <i>animalis</i>	CIRMBIA 1335							
	<i>animalis</i> subsp. <i>lactis</i>	Bb12, Chr. Hansen							
	<i>animalis</i> subsp. <i>lactis</i>	CIRMBIA 1340							
	<i>animalis</i> subsp. <i>lactis</i>	BI-07, Du Pont							
	<i>lactis</i> subsp. <i>lactis</i>	SAB 440A, Clerici-Sacco							
	<i>longum</i>	CIRMBIA 1391							
	<i>longum</i>	CIRMBIA 1395							
	<i>longum</i> subsp. <i>longum</i>	LGM P-17500, CSL							
	<i>Lactobacillus</i>	<i>acidophilus</i>				SAB 440A, Clerici-Sacco	MRS	MRS, pH 5.2	37 °C, 48 h
		<i>acidophilus</i>				ATCC 4356			
		<i>acidophilus</i>				LA-5, Chr. Hansen			
	<i>brevis</i>	ATCC 367							
	<i>casei</i>	CIRMBIA 667							
	<i>casei</i>	CIRMBIA 767							
	<i>casei</i>	CIRMBIA 769							
	<i>casei</i>	CIRMBIA 771							
	<i>casei</i> subsp. <i>alactosus</i>	CCT 1376							
	<i>casei</i> subsp. <i>casei</i>	CCT 1465							
	<i>casei</i> subsp. <i>rhamnosus</i>	CCT 0842	MRS	MRS, pH 5.2					
	<i>casei</i> subsp. <i>rhamnosus</i>	CCT 5835							
	<i>casei</i> subsp. <i>rhamnosus</i>	CCT 6645							
	<i>paracasei</i>	CCT 0566							
	<i>paracasei</i>	CCT 7501							
	<i>delbrueckii</i> subsp. <i>bulgaricus</i>	Yo-Mix, Du Pont							
	<i>delbrueckii</i> subsp. <i>delbrueckii</i>	CCT 3744							
	<i>delbrueckii</i> subsp. <i>lactis</i>	ATCC 4797							
	<i>fermentum</i>	ATCC 23271							
	<i>fermentum</i>	ATCC 9338							
	<i>helveticus</i>	ATCC 12046							
	<i>leichmanii</i>	ATCC 7830							
	<i>paracasei</i> subsp. <i>paracasei</i>	ATCC 335							
	<i>plantarum</i>	ATCC 10012							
	<i>plantarum</i>	ATCC 8014							
	<i>rhamnosus</i>	ATCC 7469							
	<i>rhamnosus</i>	ATCC 9595							
<i>Streptococcus</i>	<i>thermophilus</i>	SAB 440A, Clerici-Sacco	M17, lactose 5 g/L	M17, lactose 5 g/L	37 °C, 24 h				
	<i>thermophilus</i>	Yo-Mix, Du Pont							
	<i>thermophilus</i>	ST066, Clerici-Sacco							

<sup>a</sup> CIRMBIA: Centre de ressources biologiques dédié aux bactéries d'intérêt alimentaire culture collection, INRA, Rennes, France; ATCC: American Type Culture Collection, Manassas, VA, EUA, CCT: Fundação André Tosello culture collection, Campinas, SP, Brazil; Christian Hansen A/S, Hørsholm, Denmark; Du Pont, Palo Alto, CA, USA; Clerici-Sacco Group, Cadorago, Italy; CSL: Centro Sperimentale del Latte, Zelo Buon Persico, Italy.

<sup>b</sup> MRS: Man, Rogosa & Sharpe (Becton–Dickson and Company – BD, Franklin Lakes, NJ, USA); L-cysteine (Sigma–Aldrich Co., St. Louis, MO, USA); M17 (Oxoid Ltd., Basingstoke, England); dicloxacin (Sigma–Aldrich); lithium chloride (Merck KGaA, Darmstadt, Germany); GasPak (BD).

use, aliquots of stored cultures were streaked in their culture media added to bacteriological agar (Oxoid Ltd., Basingstoke, England; 15 g/L) and incubated as outlined in Table 1. Isolated colonies of each microorganism were then transferred to growth media and incubated (Table 1) until they reached turbidity similar to scale 1 MacFarland, which corresponds to approximately  $3 \times 10^8$  colony-forming units per mL (CFU/mL). These cultures were used in the experiments described in the following sections.

## 2.2. Carbohydrate fermentation patterns

One milliliter aliquots of the cultures were centrifuged at  $14,000 \times g$  with subsequent removal of the supernatant and suspension of the bacterial pellet in 1 mL of 0.85% NaCl solution (w/v). Bromocresol purple broth (Becton–Dickson and Company, Franklin Lakes, NJ, USA) was prepared and supplemented with solutions of different carbohydrates (fructose, glucose, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, sucrose, sorbitol and xylose, all from Sigma–Aldrich Co., St. Louis, MO, USA), to obtain 11

different culture media with a final concentration of 20 g/L for each carbohydrate. The pH was adjusted to 7.0 with 1 N NaOH.

One hundred microliter aliquots of each supplemented broth were distributed into wells of microtiter plates and then inoculated with 10  $\mu$ L of each microorganism culture. The sets were incubated at 37 °C for 72 h under anaerobic conditions (GasPak EZTM Gas Generating Container Systems, BD), with the change in color of the culture medium (purple to yellow) indicating the ability of the inoculated microorganism to ferment the added carbohydrates. This experiment was conducted in duplicate.

The results were grouped considering the genera tested by calculating the frequencies of microorganisms able to ferment different carbohydrates added to the culture medium.

## 2.3. Performance of culture media for selective enumeration of bifidobacteria

All bacterial cultures were ten-fold diluted in 0.85% NaCl (w/v) to a concentration of approximately  $10^2$  CFU/mL. Then, the selected

dilutions were pour-plated in duplicate in the following selective culture media:

- ✓ TOS-MUP: TOS-Propionate agar base (Merck KGaA, Darmstadt, Germany) supplemented with lithium mupirocin solution (MUP, Merck, 50 mg/L) (ISO, 2010);
- ✓ RP-MUP: culture medium proposed as an alternative for enumeration of bifidobacteria, comprising a culture medium called Raffinose-Propionate base broth (RP) supplemented with bacteriological agar (Oxoid, 15 g/L) and MUP solution (Merck, 50 mg/L). For preparation of the RP broth, the TOS-Propionate broth had its TOS component substituted by raffinose to a final concentration of 5 g/L.
- ✓ MRS-NNLP: MRS agar (BD) supplemented with filter-sterilized (Millex 0.22 mm, Millipore, Bedford, MA, EUA) solutions of nalidixic acid (Sigma–Aldrich, 15 mg/L), neomycin sulfate (Calbiochem, San Diego, CA, EUA, 100 mg/L), lithium chloride (Merck KGaA, Darmstadt, Germany, 3.0 g/L), paromomycin sulfate (Sigma–Aldrich, 200 mg/L), and L-cysteine hydrochloride (Sigma–Aldrich, 0.5 g/L) (Dave and Shah, 1996);
- ✓ MRS-ABC: prepared as described in Table 1 (Christian-Hansen, 2005);

As a control for the populations of *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus thermophilus*, the same dilutions were pour-plated in duplicate in MRS-C, MRS and M17 agars, respectively. MRS-NNLP, MRS-ABC, TOS-MUP, RP-MUP and MRS-C plates were incubated at 37 °C for 72 h in anaerobiosis (GasPak EZ™, BD), and MRS and M17 plates were incubated at 37 °C for 48 h. After incubation, the colonies formed in each culture medium were counted and the results expressed as CFU/mL.

For each microorganism, the counts obtained in the selective culture media were converted into proportional values using as reference the counts obtained in the control culture media. These values were grouped according to the genera of the tested microorganisms and compared using ANOVA and Tukey test to verify significant differences ( $p < 0.05$ ) using the software XLSTAT 2010.2.03 (Addinsoft, New York, NY, USA).

#### 2.4. Evaluation of RP-MUP associated with Petrifilm™ AC plates (3M Microbiology) for selective enumeration of bifidobacteria in fermented milk

Reconstituted skimmed milk (RSM) was prepared by adding skimmed milk powder (Molico, Nestlé Brazil, São Paulo, SP, Brazil) to sterile distilled water (100 g/L), subjecting the mix to heat treatment (90 °C for 5 min), and then cooling to 41 °C before using the milk to produce the following fermented milk:

- ✓ FM 01: yoghurt produced with YoMix starter culture (Du Pont, Palo Alto, CA, USA, composed of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) and Bb12 (Chr. Hansen, Hørsholm, Denmark, *Bifidobacterium animalis* subsp. *lactis*);

- ✓ FM 02: fermented product produced with the cultures ST066 (Clerici-Sacco Group, Cadorago, Italy, *S. thermophilus*), *Lactobacillus casei* subsp. *casei* (CCT 1465) and Bb12 (Chr. Hansen, *B. animalis* subsp. *lactis*);
- ✓ FM 03: fermented product produced with the cultures ST066 (Clerici-Sacco, *S. thermophilus*), *Lactobacillus rhamnosus* (ATCC 7469) and Bb12 (Chr. Hansen, *B. animalis* subsp. *lactis*);
- ✓ FM 04: fermented product produced with SAB 440A starter culture (Clerici-Sacco, composed of *S. thermophilus*, *Lactobacillus acidophilus* and *B. lactis* subsp. *lactis*).

The commercial starter cultures used (YoMix, ST066, SAB 440A and Bb12) were prepared according to the instructions of the manufacturers; after fermented milk production, the bifidobacterium final concentrations ranged from  $10^6$  (SAB 440A) to  $10^9$  CFU/g (Bb12). Cultures of *L. casei* subsp. *casei* CCT 1465 and *L. rhamnosus* ATCC 7469 were inoculated in the fermented milk to obtain final concentrations of approximately  $10^6$  CFU/g. After inoculation of the RSM, aliquots of 200 mL of each of the products were distributed into sterile flasks, incubated at 42 °C for 4.5 h and then stored at 4 °C. The fermented milk preparations were produced and analyzed in triplicate.

Samples from fermented milk were collected immediately after production and again after 15 and 28 days of production. Each sample was serially diluted on the decimal scale with one-quarter strength Ringer solution (Merck) and two dilutions were selected and pour-plated in duplicate on TOS-MUP, RP-MUP, MRS-NNLP and MRS-ABC agars, prepared as described above. Additionally, the same samples were ten-fold diluted using RP-MUP broth and the same previously selected dilutions were plated in triplicate on Petrifilm™ AC (3M Microbiology). All plates were incubated at 37 °C for 72 h under anaerobic conditions (GasPak EZ™, BD). The Petrifilm™ AC plates were incubated for 24, 48 and 72 h. After incubation, the colonies formed were counted and the results for each fermented milk were expressed as CFU/g. The counts obtained were converted into  $\log_{10}$  and compared by analysis of variance and Tukey test to check for differences between the various culture media and incubation periods evaluated ( $p < 0.05$ ). The values were subjected to linear regression to check the correlation between counts ( $p < 0.05$ ) using the software XLSTAT 2010.2.03 (Addinsoft).

### 3. Results and discussion

#### 3.1. Carbohydrate fermentation profile

All tested *Bifidobacterium* spp. strains were able to ferment glucose, lactose, raffinose and sucrose. According to Scardovi (1986), only *B. bifidum*, *B. cuniculi* and *B. minimum* are unable to ferment raffinose among representatives of the genus *Bifidobacterium*; however, these species were not included in this study. Scalabrini et al. (1998) demonstrated the ability of *Bifidobacterium* spp. to ferment raffinose naturally present in a water soluble soybean extract. Zavaglia et al. (1998) tested the ability of bifidobacteria isolated from feces to use different carbohydrates, and

**Table 2**  
Relative mean counts of *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus thermophilus* using MRS-ABC, MRS-NNLP, TOS-MUP and RP-MUP media. Incubation at 37 °C in anaerobiosis for 72 h.

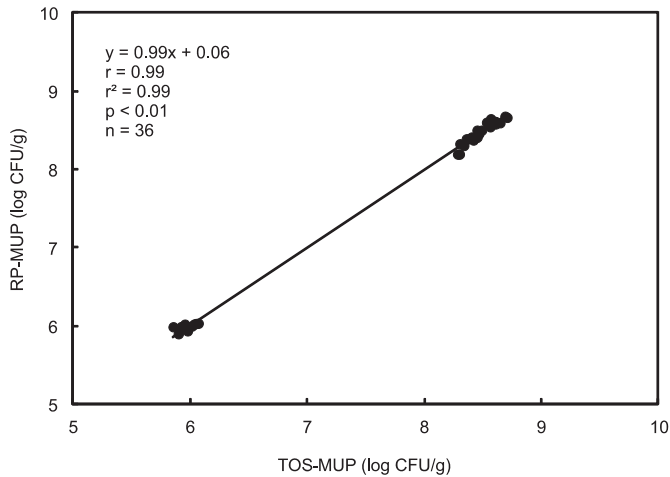
Group	n	Control	MRS-ABC	MRS-NNLP	TOS-MUP	RP-MUP	ANOVA
Bifidobacteria	11	100.0 <sup>a</sup>	94.68 <sup>a</sup>	61.81 <sup>b</sup>	86.75 <sup>ab</sup>	86.51 <sup>ab</sup>	$F_{(4,50)} = 4.888, p = 0.002$
Lactobacilli	26	100.0 <sup>a</sup>	59.97 <sup>b</sup>	10.68 <sup>c</sup>	0.0 <sup>c</sup>	0.0 <sup>c</sup>	$F_{(4,125)} = 79.020, p < 0.0001$
Streptococci	3	100.0 <sup>a</sup>	41.71 <sup>ab</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>	$F_{(4,10)} = 6.822, p = 0.006$

n = number of strains; Control: MRS-C for bifidobacteria, MRS for lactobacilli, M17 for streptococci; \* Analysis of Variance (ANOVA). Relative mean counts with different letters indicate statistical differences in the respective line (Tukey test,  $p < 0.05$ ). F = ANOVA, df = degrees of freedom, p = significance level.

**Table 3**Means ( $\pm$ standard deviation) of counts of bifidobacteria inoculated into fermented milk obtained by different culture media and incubation times.

Fermented milk	n	TOS-MUP	RP-MUP	Petrifilm™ AC associated to RP-MUP			ANOVA <sup>a</sup>
				24 h	48 h	72 h	
FM 01	9	8.37 $\pm$ 0.07 <sup>a</sup>	8.43 $\pm$ 0.11 <sup>a</sup>	8.39 $\pm$ 0.09 <sup>a</sup>	8.38 $\pm$ 0.08 <sup>a</sup>	8.34 $\pm$ 0.08 <sup>a</sup>	$F_{(4,37)} = 0.543, p = 0.705$
FM 02	9	8.55 $\pm$ 0.08 <sup>a</sup>	8.54 $\pm$ 0.09 <sup>a</sup>	8.61 $\pm$ 0.03 <sup>a</sup>	8.53 $\pm$ 0.09 <sup>a</sup>	8.55 $\pm$ 0.11 <sup>a</sup>	$F_{(4,37)} = 0.844, p = 0.507$
FM 03	9	8.59 $\pm$ 0.08 <sup>a</sup>	8.58 $\pm$ 0.07 <sup>a</sup>	8.58 $\pm$ 0.09 <sup>a</sup>	8.49 $\pm$ 0.10 <sup>a</sup>	8.51 $\pm$ 0.08 <sup>a</sup>	$F_{(4,32)} = 2.407, p = 0.070$
FM 04	9	5.98 $\pm$ 0.07 <sup>a</sup>	5.98 $\pm$ 0.05 <sup>a</sup>	5.78 $\pm$ 0.05 <sup>b</sup>	6.00 $\pm$ 0.08 <sup>a</sup>	5.97 $\pm$ 0.07 <sup>a</sup>	$F_{(4,37)} = 12.39, p < 0.001$
Total	36	7.85 $\pm$ 1.12 <sup>a</sup>	7.84 $\pm$ 1.12 <sup>a</sup>	7.81 $\pm$ 1.24 <sup>a</sup>	7.83 $\pm$ 1.09 <sup>a</sup>	7.82 $\pm$ 1.11 <sup>a</sup>	$F_{(4,158)} = 0.006, p = 1.0$

<sup>a</sup> Analysis of Variance (ANOVA). Mean counts with different letters indicate statistical differences in the respective line (Tukey test,  $p < 0.05$ ).  $F$  = ANOVA, df = degrees of freedom,  $p$  = significance level.



**Fig. 1.** Dispersion of counts of bifidobacteria inoculated in fermented milks and obtained by plating on selective culture media (TOS-MUP, Merck, and RP-MUP), incubated at 37 °C for 72 h under anaerobic conditions (GasPak, BD).

observed that raffinose was not fermented by most isolates of *B. bifidum*. Roy et al. (1997) developed a selective medium for bifidobacteria (RAF 5.1) whose carbon source was raffinose, and were able to selectively enumerate cultures of *Bifidobacterium* spp. in cheese, including *B. bifidum*.

Considering the other microorganisms examined, only one strain of *Lactobacillus* (*L. acidophilus*, SAB 440A) was able to ferment raffinose. The ability of *L. acidophilus* to ferment raffinose was demonstrated by Gueimonde et al. (2004), although this characteristic varies with the strain evaluated (Kandler and Weiss, 1986). Among the carbohydrates tested, raffinose had better performance as an enriching agent in culture media for the selection of

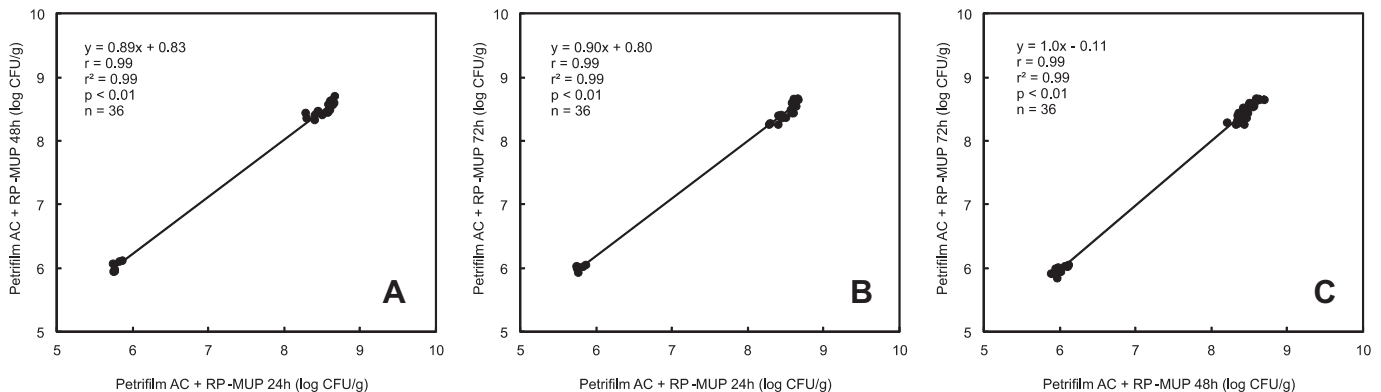
bifidobacteria, and was considered for this purpose in the later stages of the study.

### 3.2. Performance of culture media for selective enumeration of bifidobacteria

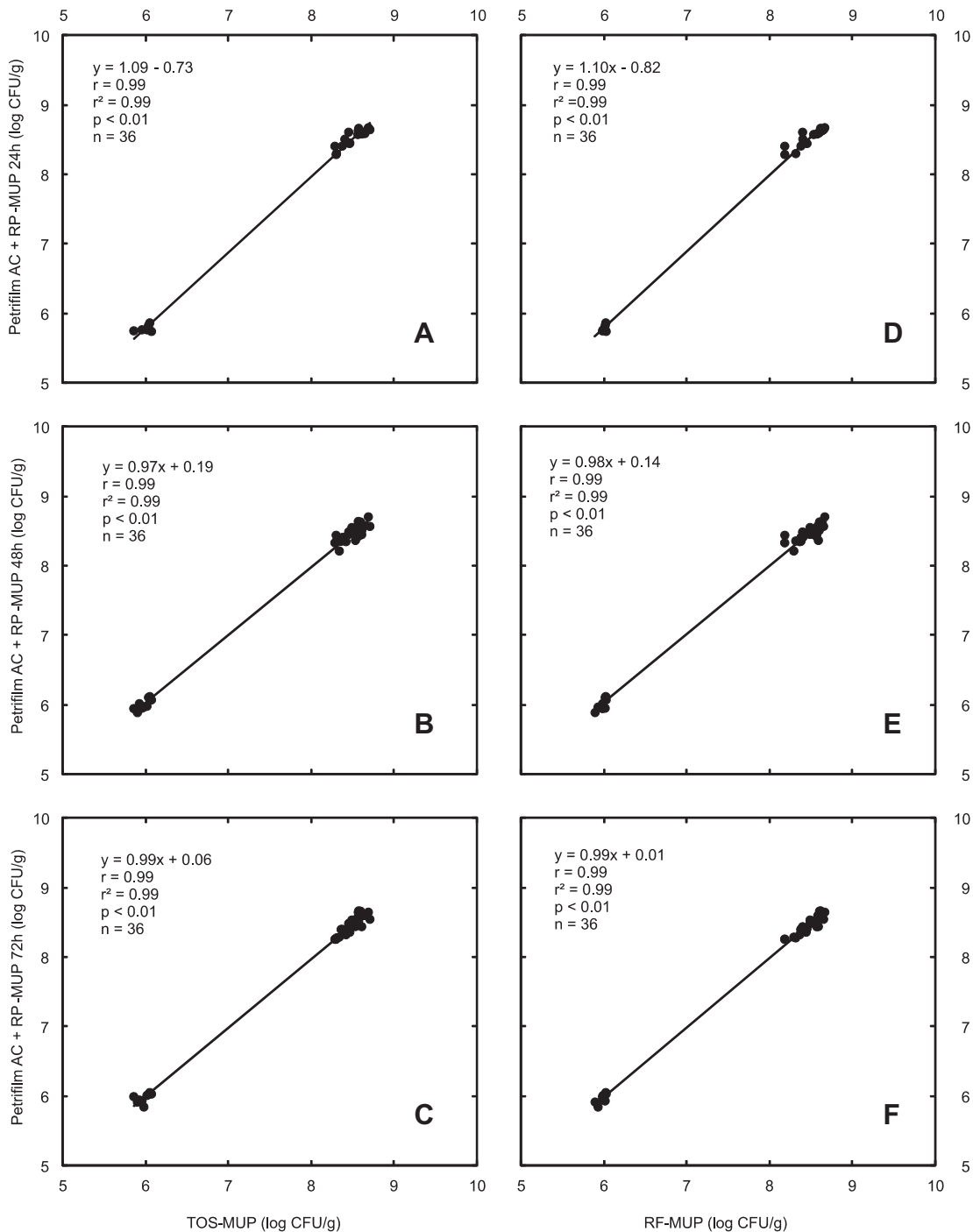
The proportions of counts of each genera of microorganisms in the selective culture media with comparisons to their respective controls are presented in Table 2. Except for MRS-NNLP, all culture media evaluated for counting *Bifidobacterium* spp. presented no significant differences compared to control in MRS-C ( $p > 0.05$ ). Considering the data for *Lactobacillus* spp., all culture media showed significant differences compared to control in MRS ( $p < 0.05$ ), indicating their potential selectivity; however, only TOS-MUP and RP-MUP demonstrated a total absence of counts for cultures of this genus. Strains of *S. thermophilus* showed no counts on MRS-NNLP, TOS-MUP and RP-MUP and, despite having lower scores on the MRS-ABC compared to control in M17, this difference was not significant ( $p > 0.05$ ).

MRS-ABC was developed for the selective enumeration of *Bifidobacterium* spp. in dairy products (Christian-Hansen, 2005). One of its principal selective agents is dicloxacillin, which has been described as a substance with potential use in selective culture media for the enumeration of bifidobacteria at concentrations ranging between 2 and 4 mg/L (D'Aimmo et al., 2007; Sozzi et al., 1990). However, in MRS-ABC, dicloxacillin is originally added at a concentration of 0.5 mg/L, which may explain the low selectivity of the culture medium with respect to *Lactobacillus* spp. and *S. thermophilus* (Table 2). Lima et al. (2009) modified the concentration of dicloxacillin in MRS-ABC to 2 mg/L and observed adequate selectivity for *Bifidobacterium* spp.

Although it is described as a culture medium usually used for the enumeration of bifidobacteria, MRS-NNLP allowed deficient development of colonies of such microorganisms while allowing



**Fig. 2.** Dispersion of counts of bifidobacteria inoculated in fermented milks and obtained by plating on Petrifilm™ AC (3M Microbiology) with RP-MUP, incubated at 37 °C for 24, 48 and 72 h under anaerobic conditions (GasPak, BD). A: comparison between counts at 24 and 48 h, B: 24 and 72 h, C: 48 and 72 h.



**Fig. 3.** Dispersion of counts of bifidobacteria inoculated in fermented milks and obtained by plating on TOS-MUP (Merck), RP-MUP, and Petrifilm™ AC (3M Microbiology) with RP-MUP, incubated at 37 °C for 24, 48 and 72 h under anaerobic conditions (GasPak, BD). Graphs on the left side: counts obtained by TOS-MUP and Petrifilm™ AC with RP-MUP after 24 h (A), 48 h (B) and 72 h (C). Graphs on the right side: counts obtained by RP-MUP and Petrifilm™ AC with RP-MUP after 24 h (D), 48 h (E) and 72 h (F).

the formation of colonies of other species of lactic acid bacteria (Darukaradhya et al., 2006; Dave and Shah, 1996; Lim et al., 1995; Miranda et al., 2011; Moriya et al., 2006). These shortcomings of MRS-NNLP were also observed in this study (Table 2), reinforcing the limitations on its use as a medium to be considered for monitoring bifidobacteria in fermented milk.

TOS-MUP and RP-MUP were the only culture media that did not enable colony formation by *Lactobacillus* spp. and *S. thermophilus*, indicating their selective potential for *Bifidobacterium* spp. in mixed cultures with these microorganisms (Table 2). Only *B. animalis*

subsp. *animalis* CIRM BIA 1335 showed no colony formation in these two media. TOS-MUP was developed from a collaborative study (IDF, 2007) which determined its inclusion as a selective medium for *Bifidobacterium* spp. in ISO 29981 (ISO, 2010). Mupirocin is its selective agent, and has been used in different culture media used for the isolation and enumeration of bifidobacteria (Leuschner et al., 2003; Rada, 1997; Simpson et al., 2004; Turroni et al., 2009).

Besides mupirocin, TOS-MUP contains TOS obtained by the transformation of lactose by the enzyme  $\beta$ -galactosidase. Obtaining this compound requires specific equipment (ISO, 2010; Toba et al.,



1985) and therefore the use of raffinose as an alternative source of carbohydrate has been proposed, due to previously obtained results. RP-MUP was then developed, and showed performance similar to TOS-MUP for the selective enumeration of *Bifidobacterium* spp. (Table 2).

### 3.3. Evaluation of RP-MUP associated with Petrifilm™ AC plates (3M Microbiology) for the selective enumeration of bifidobacteria in fermented milk

The mean counts obtained for bifidobacteria with different culture media and incubation times are shown in Table 3. Only the counts of bifidobacteria obtained from FM 04 in Petrifilm™ AC associated with RP-MUP after 24 h of incubation were significantly lower than the others ( $p < 0.01$ ). Counts of bifidobacteria obtained in other media and at different incubation times showed no significant differences ( $p > 0.05$ ). These results indicate that the proposed alternative protocols for the enumeration of bifidobacteria in fermented milk (RP-MUP and RP-MUP associated with Petrifilm™ AC plates with incubation for 24, 48 and 72 h) showed no significant differences when compared to the results obtained by following the ISO 29981 protocol, which uses TOS-MUP.

The equivalence between counts on TOS-MUP and RP-MUP can be confirmed by the distribution of the data presented in Fig. 1. The correlation parameters presented indicate the equivalence of these culture media for the enumeration of bifidobacteria in fermented milk.

An advantage of the Petrifilm™ system is a reduction in the incubation time needed to obtain the final results. The mean counts of bifidobacteria obtained in this system associated with RP-MUP after different incubation times (24, 48 and 72 h) showed no significant differences between them; Fig. 2 shows the distribution of the data. Although all correlation coefficients are considered excellent ( $r = 0.99$ ), the additional correlation parameters indicate deficiencies of equivalence when the counts obtained at 24 h are considered ( $a$ , slope; and  $b$ , intercept). Additionally, the colonies formed after 24 h of incubation in Petrifilm™ AC associated with RP-MUP tended to be of a reduced size, making them difficult to count properly. The need for an incubation time greater than 24 h for the appropriate enumeration of lactic acid bacteria and bifidobacteria in Petrifilm™ AC combined with selective culture media has been described in similar studies (Miranda et al., 2011; Nero et al., 2008).

Finally, Fig. 3 shows the distribution of the counts for bifidobacteria in fermented milk obtained by conventional methods of enumeration using TOS-MUP and RP-MUP compared with the results obtained by Petrifilm™ AC associated with RP-MUP, after 24, 48 and 72 h of incubation. Again, it can be seen that although the correlation coefficients for all comparisons are considered excellent ( $r = 0.99$ ), the additional correlation parameters tend to approach the ideal value ( $a = 1.0$ ,  $b = 0.0$ ) as the incubation time with Petrifilm™ AC increased from 24 to 72 h.

These analyses demonstrate the equivalence between TOS-MUP and RP-MUP culture media, as well as the possibility of combining them with Petrifilm™ AC plates to obtain reliable counts of bifidobacteria in fermented milk. In this context, the use of the Petrifilm™ system is an advantage to the food industry for its ability to obtain results in less time than described in ISO 29981 (ISO, 2010), and with greater convenience for implementation of the analyses, as well as preparation and disposal of laboratory material (Jasson et al., 2010).

## 4. Conclusions

Considering these results, raffinose was identified as the only carbohydrate fermented exclusively by all strains of *Bifidobacterium*

spp. evaluated, and just for a single *L. acidophilus* strain. This result led to the development of an alternative culture medium for the enumeration of bifidobacteria, RP-MUP, which showed similar selectivity to TOS-MUP and better performance than MRS-NNLP and MRS-ABC. Finally, the association of RP-MUP with Petrifilm™ AC plates permitted the selective enumeration of bifidobacteria in fermented milk, obtaining final results in less time than the protocol described in ISO 29981. This represents a viable alternative to the food industry for monitoring these products.

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