



Short communication

Enumeration of bifidobacteria using Petrifilm™ AC in pure cultures and in a fermented milk manufactured with a commercial culture of *Streptococcus thermophilus*

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ABSTRACT

Bifidobacteria are probiotic microorganisms that are widely used in the food industry. With the aim of using of Petrifilm™ Aerobic Count (AC) plates associated with selective culture media, aliquots of sterile skim milk were inoculated separately with four commercial cultures of bifidobacteria. These cultures were plated by both the conventional method and Petrifilm™ AC, using the culture media NNLP and ABC. The cultures were incubated under anaerobiosis at 37 °C for 24, 48 and 72 h. No significant differences ($p > 0.05$) were observed between the obtained counts at 48 and 72 h. Bifidobacteria counts in ABC were usually higher than in NNLP, independent of the plating method. Subsequently, fermented milk was prepared with a *Streptococcus thermophilus* strain, and aliquots were inoculated with the same bifidobacteria. Then, the fermented milks were submitted to microbiological analysis for bifidobacteria enumeration using the same culture media and methodologies previously described, incubated under anaerobiosis at 37 °C for 48 h. Again, bifidobacteria counts in ABC were higher than in NNLP, with significant differences for some cultures ($p < 0.05$). The counts obtained by both methodologies presented significant correlations ($p < 0.05$). The results indicate the viability of Petrifilm™ AC as an alternative method for bifidobacteria enumeration when associated to specific culture media, specially the ABC.

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1. Introduction

Bifidobacterium spp. are Gram positive, anaerobic microorganisms, sharing the morphological characteristics of rods, often bifurcate, Y-shaped or swollen extremities. They were initially isolated in 1899 from infant stools (Arunachalam, 1999). These microorganisms are closely related to lactic acid bacteria (LAB), presenting some key differences. They have a high content of G + C % in their DNA, and the fermentation pattern of glucose has the ratio of 3 mol of acetate and 2 mol of lactate for every 2 mol of glucose. The enzyme fructose-6-phosphate phosphoketolase (F6PPK), which is specific to *Bifidobacterium* spp., is responsible for this alternative fermentation pathway (Doleyres and Lacroix, 2005).

These microorganisms are frequently described as probiotics, and this potential application was first described in 1958 (Ballongue, 2004; Fuller, 1989). Probiotic microorganisms provide many important benefits to consumers, including nutritional effects, vitamin production, lactose intolerance suppression, the anti-infection resistance to pathogenic bacteria, antitumor effects and the prevention of digestive tract infectious diseases (Binns and Lee, 2010; Gismondo et al., 1999; Fuller, 1989). Beyond these beneficial effects, the probiotic microorganisms added to food must be resistant to low pH and high concentrations of bile salts, and they must be stable in industry processes and storage (Shah, 2000). Many strains of *Bifidobacterium animalis* subsp. *lactis* exhibit these characteristics, being well adapted to survive in large numbers during the manufacturing process and storage up to consumption, enabling their use as commercial cultures by the food industry especially in the production of fermented milks (Gueimonde et al., 2004).

To ensure the probiotic effect of the *Bifidobacterium* spp. cultures, the inoculated food must have a minimal concentration of viable cells, usually between 10^6 and 10^7 CFU/g (Lourens-Hattingh

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and Viljoen, 2001; Sanders et al., 1996; Shah, 2000). Therefore, the enumeration of these cultures and the monitoring of probiotic products during storage is important to ensure the health effects desired by consumers. Many culture media have been developed for the selective enumeration of probiotics, including *Bifidobacterium* spp. For the selectivity of *Bifidobacterium* spp. in foods fermented by other starter cultures, culture media containing NNLP (nalidixic acid, neomycin sulfate, lithium chloride, paromomycin sulfate) (Laroia and Martin, 1991) and dicloxacillin (Lima et al., 2009) are generally used. In addition, L-cysteine hydrochloride is usually added as an enrichment component because it decreases the redox potential of the culture media (Roy, 2001). However, researchers have described variations in the accurate enumeration of *Bifidobacterium* spp. and the overall performance of these media in different types of fermented milks (Champagne et al., 2009; Darukaradhy et al., 2005; Ingham, 1999; Lapiere et al., 1992; Laroia and Martin, 1991; Lim et al., 1995; Lima et al., 2009; Roy, 2001; Shah, 2000; Tharmaraj and Shah, 2003; Vinderola and Reinheimer, 1999).

Alternative methods for the enumeration of microorganisms, such as the Petrifilm™ system (3M Microbiology, St. Paul, MN, USA), are excellent alternatives for the food industry because they enable less laboratory work and reduce the space needed for incubation. Petrifilm™ Aerobic Count (AC) plates are routinely used for the enumeration of mesophilic bacteria, but they can also be employed for lactic acid bacteria enumeration (Nero et al., 2006, 2008; Ortolani et al., 2007; Gonçalves et al., 2009). Therefore, the purpose of this study was to evaluate the adequacy of Petrifilm™ AC plates associated with various culture media in enumerating commercial cultures of bifidobacteria in sterile skim milk and in a fermented milk produced with a commercial culture of *Streptococcus thermophilus*.

2. Material and methods

2.1. Commercial cultures

Four lyophilised commercial *Bifidobacterium* spp. cultures were used: *B. animalis* subsp. *lactis* Bb 12 (B1, Chr. Hansen A/S, Hørsholm, Denmark), *B. lactis* subsp. *lactis* SAB 440A (B2, Clerici-Sacco Group, Cadorago, Italy), *B. lactis* subsp. *lactis* BI-07 (B3, Danisco A/S, Copenhagen, Denmark) and *B. longum* subsp. *longum* LGM P-17500 (B4, Centro Sperimentale del Latte, Zelo Buon Persico, Italy). Moreover, one *S. thermophilus* culture (ST 066, Clerici-Sacco Group) was used for fermented milk production, being previously distributed in doses for 3 L of milk and stored at $-18\text{ }^{\circ}\text{C}$ until the moment of use.

2.2. Culture media for the enumeration of *Bifidobacterium* spp.

First, de Man-Rogosa-Sharpe broth (MRS, BD - Becton, Dickson and Company, Franklin Lakes, NJ, USA) was conventionally prepared and supplemented with different substances and solutions to obtain two types of culture media: NNLP and ABC. These culture media were used in association with Petrifilm™ AC (3M Microbiology) or added with the bacteriological agar (1.5%, Merck KGaA, Darmstadt, Germany) for *Bifidobacterium* spp. enumeration.

For NNLP, the solution was prepared with lithium chloride (3 g, Merck KGaA), nalidixic acid (15 mg, Sigma–Aldrich Co., St. Louis, MO, USA), neomycin sulfate (100 mg, Calbiochem, San Diego, CA, USA), paromomycin sulfate (200 mg, Sigma–Aldrich Co.) and L-cysteine hydrochloride (0.5 g, Sigma–Aldrich Co.), diluted in distilled water (100 mL), filter sterilized (Millex 0.22 μm , Millipore, Bedford, MA, USA), and added to MRS broth at 10% (Laroia and Martin, 1991; Shah, 2000).

ABC medium was obtained according to the protocol of Chr-Hansen A/S (Chr-Hansen A/S, 2005). Three solutions were prepared: (A) dicloxacillin (10 mg, Sigma–Aldrich Co.) in 100 mL of distilled water; (B) lithium chloride (2 g, Merck KGaA) in 18 mL of distilled water; and (C) L-cysteine hydrochloride (10 g, Sigma–Aldrich Co.) in 100 mL of distilled water. Solutions A and B were filter sterilized (Millex 0.22 μm , Millipore), and solution C was sterilized by heat (121 $^{\circ}\text{C}$ for 15 min). ABC media were finally obtained with the addition of the prepared solutions to the MRS broth at the following final concentrations: A at 0.5%, B at 1.0% and C at 0.5%.

2.3. Establishment of the incubation time for the enumeration of *Bifidobacterium* spp.

Pasteurized skim milk was submitted to heat treatment (90 $^{\circ}\text{C}$ for 5 min). It was then added separately with the cultures B1, B2, B3 and B4 (estimated concentrations between 10^5 e 10^7 CFU/mL) and distributed in sterile flasks in triplicate. Aliquots of the samples of inoculated milks were submitted to serial dilutions in the decimal scale using NNLP and ABC media. Considering the estimated concentrations of the inoculated cultures, two dilutions were selected and immediately plated onto Petrifilm™ AC (3M Microbiology) and onto NNLP and ABC agar in duplicate, using standard pour-plate techniques. All plates were incubated at 37 $^{\circ}\text{C}$ under anaerobiosis (GasPak EZ™ Gas Generating Container Systems, BD), and the formed colonies were enumerated after 24, 48 and 72 h. The obtained results were expressed as CFU/mL.

2.4. *Bifidobacterium* spp. enumeration in fermented milk

Pasteurized skim milk was submitted to heat treatment (90 $^{\circ}\text{C}$ for 5 min), cooled to 41 $^{\circ}\text{C}$, inoculated at 10^7 CFU/mL with *S. thermophilus* (ST 066, Clerici-Sacco Group), and fermented at 39 $^{\circ}\text{C}$ for 4 h. After fermentation, B1, B2, B3 and B4 were added at 10^7 CFU/g in separate aliquots of fermented milks and distributed in sterile flasks of 200 mL. The fermented milks were produced in three repetitions and stored between 4 and 10 $^{\circ}\text{C}$ for a period of 28 days.

Immediately after storage and every seven days thereafter, one sample of each fermented milk was submitted to microbiological analysis for the enumeration of the *Bifidobacterium* spp. cultures. The analysis used NNLP and ABC media associated with Petrifilm™ AC and the conventional pour-plate methodology, as previously described. The final counts were obtained after 48 h of incubation, and the results were expressed as CFU/g. In addition, colonies formed in each culture media were randomly selected (10%) and submitted to Gram staining.

2.5. Data analysis

The counts for each *Bifidobacterium* spp. culture obtained by the different culture media and methodologies were converted to \log_{10} . The results obtained by the different incubation times were compared to verify the significant differences by ANOVA and Tukey ($P < 0.05$), and linear regression was used to verify the equivalence of the results ($p < 0.05$). The same statistical tests were used to compare the performance of the culture media and methodologies for the enumeration of *Bifidobacterium* spp. from the fermented milk produced with the commercial culture of *S. thermophilus*. All analyses were performed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

The results of the *Bifidobacterium* spp. counts obtained by the use of different incubation times and two different types of culture media and plating methods are shown in Table 1. After 24 h of incubation, the formation of colonies was not observed in most of the samples analysed by the two different types of culture media in both the conventional procedure and the Petrifilm™ AC plates. The few colonies that were present appeared small in size (pin pointed, smaller than 0.5 mm of diameter) and slightly stained, hindering a precise visualization and enumeration. After 48 h, the adequate visualization of the formed colonies was possible, enabling a reliable count. Finally, after 72 h, a small variation of the visible colonies was observed. Regardless of the culture media/methodology, the results obtained between 48 and 72 h did not show significant differences ($p > 0.05$, except for B3 using NNLP associated with Petrifilm™ AC) (Table 1). In the situations where enumeration was possible at 24 h, significant differences were observed in the counts at 48 and 72 h ($p < 0.05$) in most of the strains and culture media (Table 1). According to Laroia and Martin (1991), the incubation time of NNLP must be between 48 and 72 h; however, Shah (2000) and Tharmaraj and Shah (2003) recommend 72 h. Lima et al. (2009) used ABC media with an incubation time of 72 h, which is also recommended by Christian Hansen S/A (Chr-Hansen, 2005), a B1 culture manufacturer. However, according to the obtained results, significant differences between *Bifidobacterium* spp. enumeration after 48 or 72 h of incubation were generally not observed, regardless of the strain, culture media or employed methodology. This allows the adoption of 48 h for the incubation and adequate enumeration of the culture samples. The equivalence of the obtained counts after 48 and 72 h of incubation was confirmed with the correlation results, which showed significant values in every possible combination ($p < 0.05$), including 24 h of incubation with ABC (Table 2). Considering the obtained results in this step, the

Table 2

Correlation parameters between the counts of *Bifidobacterium* spp. cultures artificially inoculated in sterile skim milk and enumerated by 4 distinct culture media/procedures after incubation under anaerobiosis conditions at 37 °C for 24, 48 and 72 h. Comparisons were made between reading times for each culture medium.

Culture media	Reading time comparisons (x : y)*	n	r	r ²	P
ABC	24 h : 48 h	9	0.97	0.93	0.000
	24 h : 72 h	9	0.96	0.92	0.000
	48 h : 72 h	12	1.00	1.00	0.000
ABC + Petrifilm	24 h : 48 h	9	0.93	0.87	0.000
	24 h : 72 h	9	0.90	0.81	0.001
	48 h : 72 h	12	1.00	1.00	0.000
NNLP	48 h : 72 h	12	0.99	0.98	0.000
NNLP + Petrifilm	48 h : 72 h	11	0.99	0.98	0.000

n = number of samples considered for analysis; r = correlation index; r² = coefficient of determination; p = level of significance.* for NNLP and NNLP + Petrifilm no results were found at 24 h of incubation.

incubation time of the ABC and NNLP culture media was standardized to 48 h for the enumeration of the *Bifidobacterium* spp. cultures in fermented milk.

Although similarities have been observed between the enumerations carried out at different incubation times, significant differences were found between the results obtained for the different culture media ($p < 0.05$) (Table 1). Generally, the counts obtained with ABC were higher than those obtained with NNLP for B1 (48 and 72 h), B2 (48 h), B3 (48 and 72 h) and B4 (48 and 72 h). However, considering all cultures, this difference was only observed between the counts obtained after 24 h of incubation. The results suggest an interference of the selective agents used in NNLP in the adequate recovery of the tested cultures and in the formation of visible colonies (Roy, 2001). These problems were likely reduced when the counts of all cultures were evaluated together.

Table 1

Mean counts of *Bifidobacterium* spp. cultures artificially inoculated in sterile skim milk and enumerated by 4 distinct culture media/procedures after incubation under anaerobiosis conditions at 37 °C for 24, 48 and 72 h.

Culture	Culture media	Incubation time			Statistical test*
		24 h	48 h	72 h	
B1	ABC	6.96 ^{aA}	6.99 ^{aA}	7.00 ^{aA}	$F_{(2,6)} = 0.2, P = 0.797$ $F_{(2,6)} = 0.4, P = 0.700$ $F_{(1,4)} = 0.2, P = 0.666$ $F_{(2,5)} = 53.7, P = 0.000$
	ABC + Petrifilm	6.88 ^{aA}	6.94 ^{aA}	6.94 ^{aA}	
	NNLP	nc	6.44 ^{aC}	6.47 ^{aC}	
	NNLP + Petrifilm	5.24 ^{bB}	6.71 ^{aB}	6.71 ^{aB}	
	Statistical test	$F_{(2,5)} = 70.2P = 0.000$			
B2	ABC	nc	5.53 ^{aA}	5.57 ^{aA}	$F_{(3,8)} = 22.7, P = 0.000$ $F_{(1,4)} = 0.04, P = 0.853$ $F_{(1,4)} = 0.00, P = 1.000$ $F_{(1,4)} = 4.00, P = 0.116$ $F_{(1,2)} = 1.58, P = 0.335$
	ABC + Petrifilm	nc	5.46 ^{aA,B}	5.46 ^{aA}	
	NNLP	nc	4.70 ^{aB}	5.02 ^{aA}	
	NNLP + Petrifilm	nc	5.45 ^{aA,B}	5.65 ^{aA}	
	Statistical test	$F_{(3,7)} = 5.4, P = 0.031$			
B3	ABC	7.39 ^{aA}	7.45 ^{aA}	7.46 ^{aA}	$F_{(3,7)} = 2.26, P = 0.173$ $F_{(2,6)} = 0.36, P = 0.709$ $F_{(2,6)} = 0.95, P = 0.437$ $F_{(1,4)} = 0.17, P = 0.700$ $F_{(1,4)} = 16.43, P = 0.015$
	ABC + Petrifilm	7.33 ^{aA}	7.42 ^{aA}	7.40 ^{aA}	
	NNLP	nc	6.89 ^{aB}	6.93 ^{aC}	
	NNLP + Petrifilm	nc	7.12 ^{aB}	7.14 ^{bB}	
	Statistical test	$F_{(3,8)} = 28.2, P = 0.000$			
B4	ABC	6.99 ^{bA}	7.15 ^{aA}	7.17 ^{aA}	$F_{(3,8)} = 32.23, P < 0.001$ $F_{(2,6)} = 16.80, P = 0.003$ $F_{(2,6)} = 2.78, P = 0.140$ $F_{(1,4)} = 0.01, P = 0.923$ $F_{(1,4)} = 0.53, P = 0.509$
	ABC + Petrifilm	6.80 ^{aA}	7.02 ^{aA,B}	7.03 ^{aA,B}	
	NNLP	nc	6.83 ^{aB}	6.84 ^{aB}	
	NNLP + Petrifilm	nc	6.77 ^{aB}	6.87 ^{aB}	
	Statistical test	$F_{(1,4)} = 3.09, P = 0.153$			
All	ABC	7.11 ^{aA}	6.78 ^{aA}	6.80 ^{aA}	$F_{(3,8)} = 11.81, P = 0.003$ $F_{(2,30)} = 0.74, P = 0.486$ $F_{(2,30)} = 0.58, P = 0.565$ $F_{(1,22)} = 0.07, P = 0.789$ $F_{(2,21)} = 5.69, P = 0.011$
	ABC + Petrifilm	7.01 ^{aA}	6.71 ^{aA}	6.71 ^{aA}	
	NNLP	nc	6.22 ^{aA}	6.31 ^{aA}	
	NNLP + Petrifilm	5.24 ^{bB}	6.61 ^{aA}	6.68 ^{aA}	
	Statistical test	$F_{(2,17)} = 45.69, P < 0.001$			
			$F_{(3,43)} = 1.21, P = 0.317$	$F_{(3,43)} = 0.99, df = 3, P = 0.403$	

*All presented statistics: Analysis of Variance (ANOVA). Mean counts with distinct lower case indicate significant differences in a same row (Tukey test, $P < 0.05$). Mean counts with distinct upper case indicate significant differences in a same column (Tukey test, $p < 0.05$). nc = no counts recorded; F = ANOVA test; df = degrees of freedom; P = level of significance.

Table 3

Mean counts of four *Bifidobacterium* spp. cultures from experimentally produced fermented milk (produced with a commercial culture of *Streptococcus thermophilus* and stored at 4 and 10 °C for 28 days) obtained by four distinct culture media/procedures after incubation under anaerobiosis at 37 °C for 48 h.

Culture	Culture media/procedures				Statistics
	ABC	ABC + Petrifilm	NNLP	NNLP + Petrifilm	
B1	7.01 ^a	6.88 ^{ab}	6.27 ^c	6.48 ^{bc}	$F_{(3,108)} = 7.88, P < 0.001$
B2	6.34 ^a	6.38 ^a	5.29 ^b	5.74 ^b	$F_{(3,104)} = 14.45, P < 0.001$
B3	7.24 ^a	7.33 ^a	6.72 ^b	7.01 ^{ab}	$F_{(3,114)} = 8.27, P < 0.001$
B4	7.30 ^a	7.20 ^{ab}	6.92 ^b	6.98 ^b	$F_{(3,114)} = 4.73, P = 0.004$
all	6.97 ^a	6.95 ^a	6.31 ^c	6.62 ^b	$F_{(3,452)} = 20.15, P < 0.001$

*All presented statistics: Analysis of Variance (ANOVA). Mean counts with distinct lower case indicate significant differences in a same row (Tukey test, $P < 0.05$). nc = no counts recorded; F = ANOVA test; df = degrees of freedom; P = level of significance.

Bifidobacterium spp. counts obtained by the different culture media and methodologies in the prepared fermented milk are shown in Table 3. As observed previously (Table 1), the counts obtained using NNLP were lower than those obtained with ABC media; however, they did not present significant differences when different plating methods and methodologies were considered (Petrifilm™ AC or conventional). Similar results were observed by Fachin et al. (2008), who obtained lower counts of pure cultures of *Bifidobacterium* spp. by NNLP than by MRS. This difference pattern was observed individually for all cultures; however, when all results were taken into consideration, significant differences were observed between the counts obtained by the conventional method and the Petrifilm™ AC technique using NNLP (Table 3). This result can be derived from a higher dilution of the selective agents of NNLP with the gelling and nutritive agents of Petrifilm™ AC. Thus, the reduced selectivity of the culture media and the visible formation of colonies lead to a more precise enumeration. Regardless of the differences observed, the *Bifidobacterium* spp. counts obtained by conventional plating and Petrifilm™ AC showed significant correlations ($P < 0.05$) and were considered good for both NNLP and ABC (Fig. 1). Similar results were obtained by Gonçalves et al. (2009), who enumerated yogurt starter cultures using specific culture media associated with Petrifilm™ AC and conventional methodology. Using the MRS culture medium, Nero et al. (2008) also found good correlation indexes between these methodologies in the enumeration of different starter cultures from commercial samples of fermented milks.

A total of 591 colonies obtained from NNLP and ABC culture media and plate methodologies were characterized according Gram staining, being the majority (545) presenting the morphology compatible with *Bifidobacterium* spp., indicating their good selectivity in the tested fermented milk. A low frequency of the colonies presented microscopic morphology compatible with *Streptococcus* (46), indicating its poor ability to develop colonies in NNLP and ABC media. Considering this result, the differences observed between the culture media and methodologies were most likely not due to the formation of colonies from the commercial culture of *S. thermophilus*. However, the ability of this specie to develop in NNLP media has already been described (Vinderola and Reinheimer, 1999). Despite some evidences of the selectivity of NNLP for *Bifidobacterium* spp. (Shah, 2000), it is necessary to evaluate the ability of forming colonies in these culture media (NNLP and ABC) by other starter cultures usually employed in the production of fermented milks, such as *Lactobacillus* spp. and *Lactococcus* spp.

Despite the similarities observed between the counts, some particularities must be considered before using the Petrifilm™ system to enumerate *Bifidobacterium* spp. in fermented milks. Despite the possible reduction of selectivity, some *Bifidobacterium* spp. cultures may not be able to properly reduce the 2,3,5-trypheinitetrazolium dye that is used as an indicator in Petrifilm™ AC plates. This deficiency has already been described (Gonçalves et al., 2009; Nero et al., 2006, 2008; Ortolani et al., 2007) and can be considered a limiting factor in the enumeration of certain cultures and LAB species by Petrifilm™ AC, even when associated with selective culture media. In a similar study, the Bb12 (B1) culture inoculated in sterile milk did not form visible colonies in Petrifilm™ AC (Champagne et al., 2009); however, the inoculated area showed a diffuse pinkish colour, as occasionally observed in this study. The acidity of the fermented milk, which naturally occurs in the development of *S. thermophilus* culture, can also be associated with the differences observed between the evaluated enumeration methodologies (Ferrati et al., 2005; Gonçalves et al., 2009).

Despite some limitations, the obtained results indicate the viability of the use of Petrifilm™ AC plates associated with selective culture media for the enumeration of *Bifidobacterium* spp. in milks fermented by the *S. thermophilus* strain used in this study. For this purpose, the ABC culture media showed better performance. It demonstrated higher recovery of the different cultures of *Bifidobacterium* spp. after 48 h of incubation.

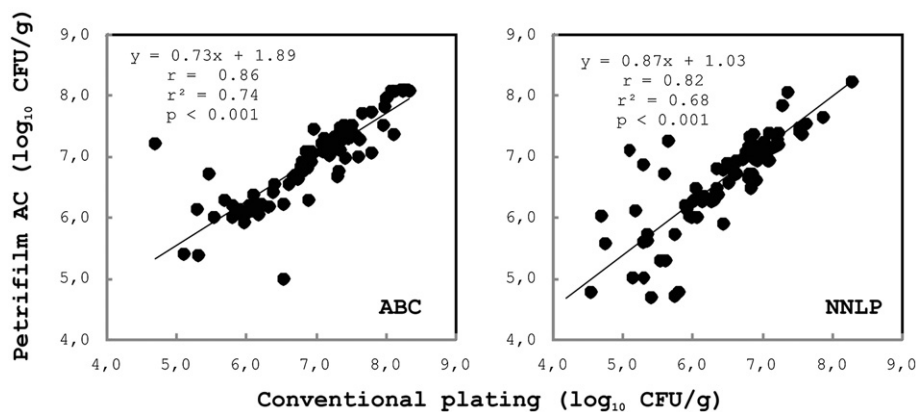


Fig. 1. Correlation parameters between the counts of *Bifidobacterium* spp. cultures from fermented milks (produced with a commercial culture of *Streptococcus thermophilus*) stored at 4 and 10 °C and obtained by four distinct culture media and procedures after incubation under anaerobiosis conditions at 37 °C for 48 h n = number of samples considered for analysis; r = correlation index; r^2 = coefficient of determination; p = level of significance.

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