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Adequacy of Petrifilm™ Aerobic Count plates supplemented with de Man, Rogosa & Sharpe broth and chlorophenol red for enumeration of lactic acid bacteria in salami

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ABSTRACT

The present study aimed to assess the performance of alternative protocols to enumerate lactic acid bacteria (LAB) in salami. Fourteen cultures and two mixed *starter* cultures were plated using six protocols: 1) Petrifilm™ Aerobic Count (AC) with MRS broth and chlorophenol red (CR), incubated under aerobiosis or 2) under anaerobiosis, 3) MRS agar with CR, 4) MRS agar with bromocresol purple, 5) MRS agar at pH 5.7, and 6) All Purpose Tween agar. Samples of salami were obtained and the LAB microbiota was enumerated by plating according protocols 1, 2, 3 and 5. Regression analysis showed a significant correlation between the tested protocols, based on culture counts ($p < 0.05$). Similar results were observed for salami, and no significant differences of mean LAB counts between selected protocols (ANOVA, $p > 0.05$). Colonies were confirmed as LAB, indicating proper selectivity of the protocols. The results showed the adequacy of Petrifilm™ AC supplemented with CR for the enumeration of LAB in salami.

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1. Introduction

Fermented meat products are widely consumed by the population due to their characteristic sensory properties. Originally, the fermentation process for meat products was developed empirically to preserve these foods for long periods, allowing their storage and distribution to different regions. On an industrial scale, this process allows the preservation of meat products and slows spoilage due to the addition of salts, low pH and water activity (Toldrá, 2010). Thus, specific *starter* cultures are added in order to promote changes in sensory properties (such as color, flavor and texture) and eventually promote the control of spoilage and pathogenic microorganisms, due to the production of antimicrobial substances (Hammes & Hertel, 1998; Talon & Leroy, 2014).

The main *starter* cultures used for the production of fermented meat products are microorganisms from the group of lactic acid bacteria (LAB) and *Staphylococcus* coagulase negative. *Pediococcus* spp. and *Lactobacillus* spp. are usually employed by the food industry with this purpose, and their main technological characteristics are the production of lactic acid from carbohydrates, leading to the acidification, and the production of aromatic compounds (Talon & Leroy, 2014). Furthermore, specific LAB strains may be added as protective cultures due to their ability to produce antimicrobial substances (Barbosa, Todorov, Jurkiewicz, & Franco, 2015; Fontana, Cocconcelli, Vignolo, & Saavedra, 2015; Toldrá, 2010). Coagulase negative *Staphylococcus* (CNS), such as *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus equorum* and *Staphylococcus saprophyticus*, contribute to the development and stability of the typical red color of fermented meat products, due to the production of the enzyme nitrate reductase (Landeta, Curiel, Carrascosa, Muñoz, & de las Rivas, 2013; Lauková, Simonová, & Strompfová, 2010). Also, CNS contribute to the development of other sensory properties, such as texture and flavor, due to the production of specific enzymes used in the metabolism of proteins and lipids (Berdague, Monteil, Montel, & Talon, 1993; Hammes & Hertel, 1998; Olesen, Meyer, & Stahnke, 2004; Sondergaard & Stahnke, 2002).

Considering the complex microbiota of fermented meat products, adequate monitoring is mandatory to assess quality and safety during production and storage. However, the conventional methodologies for the enumeration of *starter* cultures in foods have limitations regarding practicality and selectivity, which limits the accurate quantification of some groups, especially LAB. Alternatively, Petrifilm™ Aerobic Count (Petrifilm™ AC, 3M Microbiology, St. Paul, MN, USA) plates have been used for the enumeration of LAB in fermented foods, by the usage of selective agents in specific culture media for different microbial groups. Many scientific studies have been published considering this alternative, but focused on dairy products (Champagne, Gardner, Piette, & St-Gelais, 1994; Colombo, Oliveira, Carvalho, & Nero, 2014; McGregor, Traylor, Gough, Hazlett, & Bird, 1995; Miranda, Carvalho, & Nero,

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2014; Miranda, Neto, Freitas, Carvalho, & Nero, 2011; Nero et al., 2006; Ortolani, Viçosa, Beloti, & Nero, 2007; Pattison, Geornaras, & von Holy, 1998). Up to date, only one technical report has been published demonstrating the usage of Petrifilm™ AC associated with selective and differential substances to enumerate LAB in meat products (Fisher et al., 2011). Thus, the present study aimed to assess the performance of an alternative protocol based on Petrifilm™ AC plates associated with de Man, Rogosa & Sharpe (MRS) broth and chlorophenol red to enumerate LAB in salami.

2. Material and methods

2.1. Alternative protocols for LAB enumeration

2.1.1. Microorganisms

Table 1 presents the *starter* mixes and microorganisms used in the present study. *Starter* mixes were prepared according to the manufacturer instructions and subjected to ten-fold dilution in MRS broth (Oxoid Ltd., Basingstoke, England) until an approximate concentration of 10^8 colony forming units per mL (CFU/mL). Reference and wild strains were stored in MRS broth (Oxoid) added to glycerol at 30% (v/v) at −20 °C before use, when aliquots were transferred to MRS broth (Oxoid), incubated at 35 °C overnight and ten-fold diluted in MRS broth (Oxoid) until an approximate concentration of 10^8 CFU/mL.

2.1.2. Protocols for microorganism enumeration

The obtained cultures of *starter* mixes and reference and wild strains were subjected to six different protocols for LAB enumeration. Each culture was plated individually, in three independent repetitions. The tested protocols were:

- Protocol 1: cultures were ten-fold diluted in MRS broth (Oxoid) supplemented with a solution of chlorophenol red (Dinâmica Ltda, Diadema, SP, Brazil) (21 mg/100 mL). Selected dilutions were plated in Petrifilm™ AC plates (3M), and incubated at 35 °C under aerobiosis (Champagne et al., 1994; Fisher et al., 2011) for 24, 48 and 72 h;
- Protocol 2: the same dilutions selected in Protocol 1 were plated in Petrifilm™ AC plates (3M), and incubated at 35 °C under anaerobiosis (GasPak, BD — Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 24, 48 and 72 h. These procedures followed the protocol described by Fisher et al. (2011);
- Protocol 3: cultures were ten-fold diluted in MRS broth (Oxoid), and selected dilutions were pour plated in duplicate in MRS agar (Oxoid) supplemented with a solution of chlorophenol red (Dinâmica) (21 mg/100 mL), and incubated at 35 °C under anaerobiosis (GasPak, BD) for 24, 48 and 72 h. These procedures followed the protocol described by Fisher et al. (2011), adapted to conventional plating procedures;
- Protocol 4: the same dilutions selected in Protocol 3 were pour plated in duplicate in MRS agar (Oxoid) supplemented with a solution of bromocresol purple (BD) (1.6 g/100 mL) and incubated at 35 °C under anaerobiosis (GasPak, BD) for 24, 48 and 72 h. This protocol was described by Fisher et al. (2011), as a reference for enumerating LAB in meat;
- Protocol 5: the same dilutions selected in Protocol 3 were pour plated in duplicate in MRS agar (Oxoid) with the pH adjusted to 5.7, and incubated in aerobiosis at 30 °C for 24, 48 and 72 h, as described in ISO 15214/1998 (ISO, 1998). This is the reference protocol for LAB enumeration indicated by the International Standardization Organization (ISO, Geneva, Switzerland);
- Protocol 6: the same dilutions selected in Protocol 3 were pour plated in duplicate in All Purpose Tween (APT) agar (BD) supplemented with sucrose at 2% (w/v) and bromocresol purple at 1.6% (w/v), and incubated in aerobiosis at 25 °C for 24, 48 and 72 h, as described by Hall, Ledenbach, and Flowers (2001). This is the reference protocol for LAB enumeration indicated by the American Public Health Association (APHA, Washington, DC, USA).

After the incubation periods, colonies that had formed on the plates were enumerated and the results were expressed as CFU/mL.

2.1.3. Statistical analyses

The obtained counts of *starter* mixes and wild or reference strains were converted to $\log_{10}$, and then subjected to a descriptive analysis, in order to check their individual behavior according to the tested protocols and incubation periods. Also, the counts were compared by regression analysis to assess the correlation between the adopted protocols and the incubation periods ($p < 0.05$). The analyses were conducted using Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA).

2.2. Alternative protocols for LAB enumeration in salami

2.2.1. Salami samples and LAB enumeration

Thirty vacuum packed samples of salami were obtained from retail sites, which were produced by food industries inspected by the Brazilian Ministry of Agriculture. Samples were collected considering the addition of LAB as *starter* cultures and production from different batches, based on the given data on their package.

A portion of 25 g of each sample was collected under sterile conditions, added to 225 mL of sterile peptone water (Oxoid) at 0.1% (w/v), and homogenized for 5 min in a Stomacher. Then, samples were ten-fold diluted in MRS broth (Oxoid) and MRS broth supplemented with chlorophenol red (Dinâmica) (21 mg/100 mL), and plated for LAB enumeration according to protocols 1, 2, 3 and 5, as described in Section 2.1.2.

LAB counts were expressed as $\log_{10}$ CFU/g, being the mean values calculated and compared by ANOVA in order to check significant differences of tested protocols and incubation period ($p < 0.05$). Finally, LAB counts obtained were compared by regression analysis to check the correlation indexes between the tested protocols and the incubation period ($p < 0.05$). The analyses were conducted using Statistica 6.0 software (StatSoft Inc.).

Table 1
Starter mixes and microorganisms used in study.

Group	Identification/genus	n	Details/species (n)	Reference[a]
Starter mix	TEXEL AS 308	1	Mixed culture composed by: *Lactobacillus sakei*, *Staphylococcus carnosus*, *S. xylosus*	DuPont®
	TEXEL Prism 1	1	Mixed culture composed by: *L. sakei*, *S. vitulinus*, *S. xylosus*	DuPont®
Strains	*Lactobacillus*	9	*L. casei* (4)	CNRZ 313, CNRZ 1244, CNRZ 1874, CNRZ 1393
			L. paracasei (3)	CCT 7501, ATCC 10746, ATCC 335
			L. plantarum (2)	ATCC 8014, ATCC10012
	Lactococcus	3	*L. lactis* (1)	Wild strain
			L. lactis subs. *lactis* (2)	Wild strain
	Pediococcus	1	*P. pentosaceus*	Wild strain
	Staphylococcus	1	*S. xylosus*	ATCC 29971

[a] DuPont®: Danisco®, Madison, VA, EUA; CNRZ: Centre National de Recherches Zootechniques, Jouy-en-Josas, France; CCT: Coleção de Culturas Tropical, Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas, SP, Brazil; ATCC: American Type Culture Collection, Manassas, VA, USA.

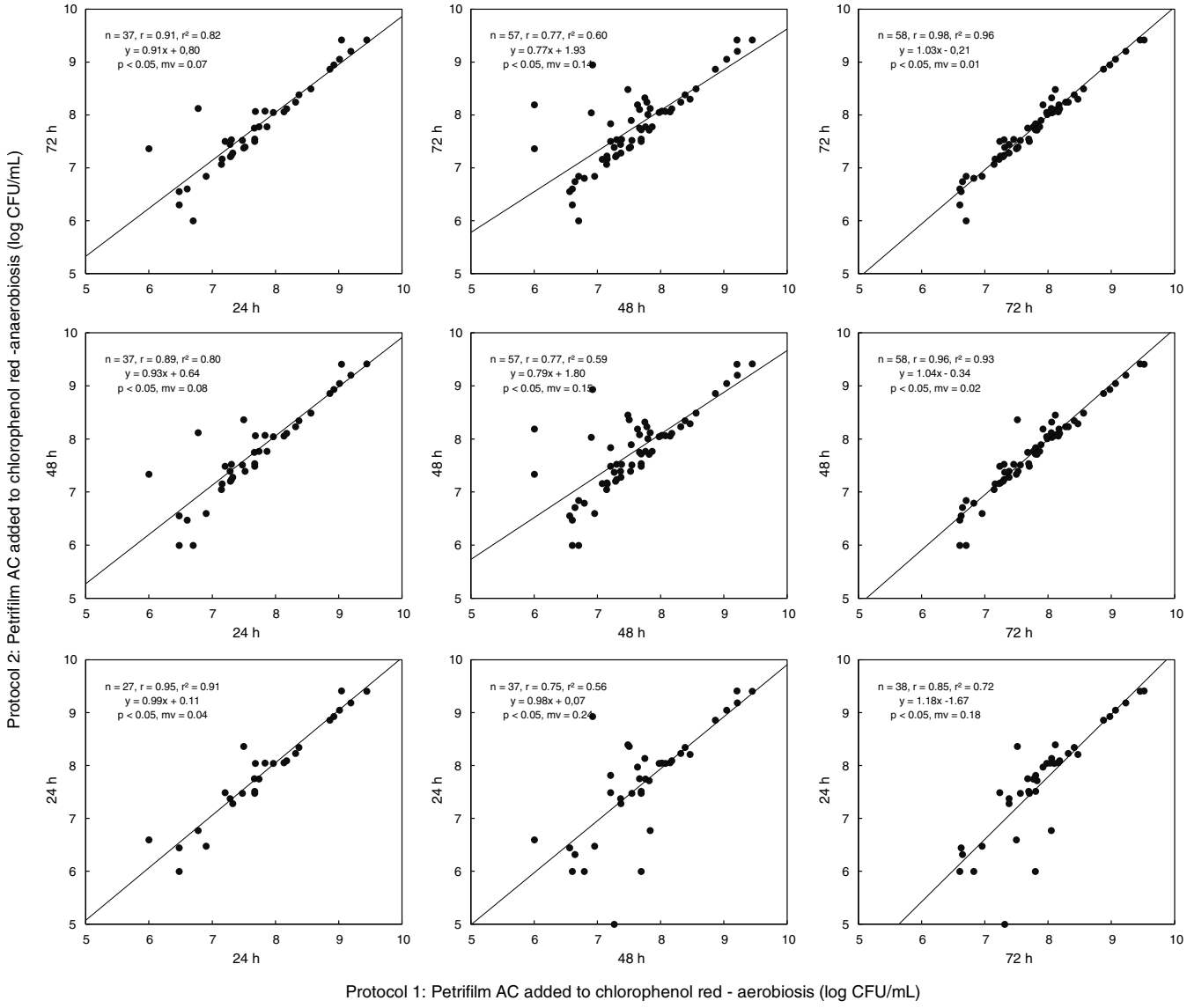


Fig. 1. Correlation parameters between the counts of microorganisms and *starter* mixes in Petrifilm™ AC associated with MRS broth supplemented with chlorophenol red in different conditions of incubation (protocol 1: aerobiosis; protocol 2: anaerobiosis) and after different incubation times (24, 48 and 72 h). n = number of microorganisms considered for analysis; r = correlation index; r^2 = coefficient of determination; p = level of significance.

2.2.2. Protocols specificity

The specificity of the tested protocols to enumerate LAB was assessed by identification of selected colonies that grown in the plates. A total of 850 representative colonies were selected from the tested protocols, streaked on MRS agar (Oxoid), incubated at 35 °C for 24 h, and subjected to Gram staining and to catalase production test. Considering the obtained results and the origin of colonies, 73 isolates were selected and subjected to DNA extraction using the Kit Wizard Genomic DNA Purification (Promega Corp., Madison, WI, USA), followed by genetic grouping by rep-PCR using the primer GTG_5 (Gevers, Huys, & Swings, 2001; Versalovic, Schneider, De Bruijn, & Lupski, 1994). PCR reactions contained 12.5 μL of GoTaq Green Master Mix 2× (Promega), 1 μL of the primer at 50 pmol, 2 μL of DNA and DNA-free water (Promega) until a final volume of 25 μL. PCR amplification conditions were: initial step at 95 °C for 30 s, 30 cycles of annealing at 40 °C for 30 s and 65 °C for 8 min, and a final extension at 65 °C for 16 min (Dal Bello et al., 2010). Rep-PCR products were electrophoresed in a 2% agarose gel for 4 h at a constant voltage of 120 V in 0.5× TBE, and gels were stained using GelRed (Biotium Inc., Hayward, CA, USA). The rep-PCR profiles

Table 2
Mean counts (MC), number of obtained data (n), standard deviation (SD), standard error (SE) and confidence intervals (−95% and +95%) of lactic acid bacteria from salami samples obtained by four enumeration protocols after incubation for 24, 48 and 72 h (results in log CFU/g).

Protocol[a]	Incubation time	MC[b]	n	SD	SE	−95%	+95%
1	24 h	6.44	22	1.30	0.28	5.86	7.01
	48 h	6.68	29	1.21	0.22	6.22	7.14
	72 h	6.73	29	1.20	0.22	6.27	7.18
2	24 h	6.38	27	1.49	0.29	5.79	6.97
	48 h	6.67	30	1.29	0.24	6.19	7.15
	72 h	6.81	30	1.14	0.21	6.39	7.24
3	24 h	6.94	19	1.24	0.28	6.34	7.53
	48 h	6.63	28	1.37	0.26	6.10	7.17
	72 h	6.77	30	1.28	0.23	6.29	7.24
5	24 h	6.67	17	1.05	0.25	6.14	7.21
	48 h	6.63	29	1.49	0.28	6.07	7.20
	72 h	6.88	29	1.29	0.24	6.39	7.37

[a] As detailed description of protocols in Section 2.1.2
[b] ANOVA: $F_{(11,307)} = 0.38$, $p = 0.963$

were recorded and analyzed using BioNumerics 6.6 software (Applied Maths, Kortrijk, Belgium). The similarity among profiles was calculated using the Dice correlation (1% for optimization, and 5% for tolerance), and an average linkage dendrogram was obtained.

Considering the genetic profiles and a similarity tax of 80%, 25 isolates were selected and subjected to PCR for amplification of the 16S rRNA gene region, using the primers P1V1 and P4V4 (Klijn, Weerkamp, & Devos, 1991). PCR reactions contained 25 μL of GoTaq Green Master Mix 2× (Promega), 1 μL of each primer (10 pmol), 2 μL of DNA and DNA-free water until a final volume of 50 μL. PCR conditions were the same as those described by Klijn et al. (1991), and PCR products were purified and sequenced at Macrogen Inc. (Seoul, South Korea). The obtained sequences were compared using the National Center for Biotechnology Information (NCBI) software Basic Alignment Search Tool (BLAST) for the identification of isolates.

3. Results and discussion

3.1. Alternative protocols for LAB enumeration

Supplementary Table presents the descriptive analysis of the obtained counts of *starter* mixes and wild and reference strains after plating individually according the six tested protocols, in three repetitions. Based on these results, it can be observed that some cultures presented difficult to develop visible colonies after of incubation, most often after 24 h, determining some missing data that did not allow proper statistical comparison based on mean values.

Considering the results obtained with Petrifilm™ AC, independent of the incubation conditions (protocols 1 and 2), the cultures presented red colonies surrounded by yellow halos, except for mix AS 308 that showed red colonies without acidification halos, and *Lactococcus* cultures, that showed slightly red colonies with yellow halos. Fisher et al. (2011) evaluated only the performance of Petrifilm™ AC plates under aerobic conditions, and observed that the initial appearance of acid zones in these plates was an accurate early indicator of the presence of LAB, as these halos were always observed surrounding typical red colonies. Fisher et al. (2011) also reported the presence of gas associated with the colonies on Petrifilm™ AC plates, representing another advantage over conventional procedures, as this allows for the preliminary identification of hetero-fermentative LAB cultures present in food samples. Nero et al. (2006) showed that some LAB species have an impaired ability or are not able to reduce the dye 2,5,5-triphenyl tetrazolium chloride (TTC) present in Petrifilm™ AC plates, such as *Leuconostoc mesenteroides*, *Streptococcus thermophilus* and *Lactobacillus casei*, which can jeopardize the reliability of the obtained counts. However, the *starter* mixes and the reference and wild strains included

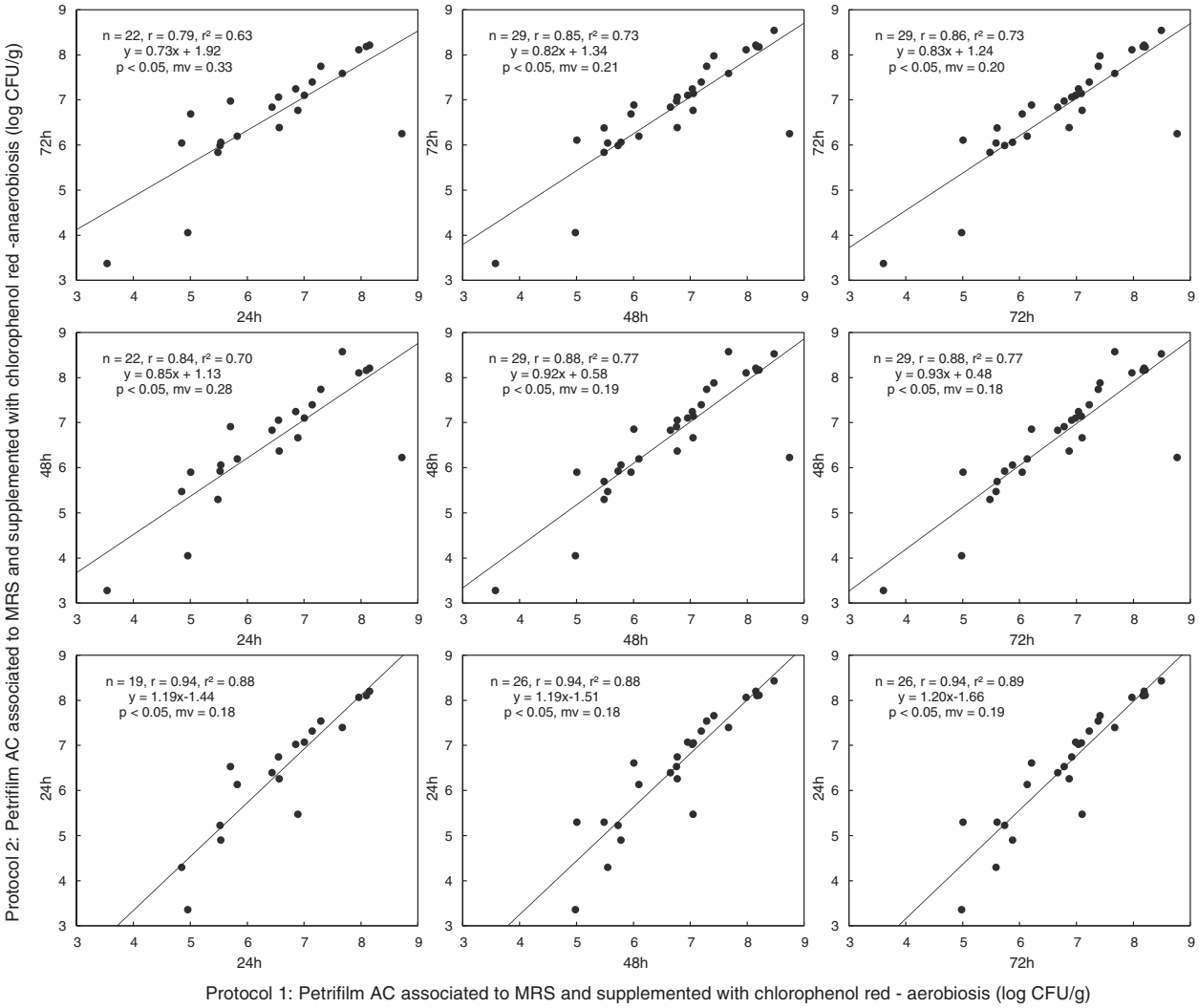


Fig. 2. Correlation parameters between the counts of lactic acid bacteria from salami samples obtained by Petrifilm™ AC associated with MRS broth supplemented with chlorophenol red in different conditions of incubation (protocol 1: aerobiosis; protocol 2: anaerobiosis) and after different incubation times (24, 48 and 72 h). n = number of microorganisms considered for analysis; r = correlation index; r^2 = coefficient of determination; p = level of significance.

in the present study did not present such a limitation in Petrifilm™ AC plates. Champagne et al. (1994) also did not observe this limitation for the enumeration of purified cultures of *Lactococcus lactis* in Petrifilm™ AC associated with MRS broth.

After 24 h of incubation, AS 308 and *S. xylosus* showed no colonies on the plates containing MRS agar at pH 5.7 (protocol 5, ISO 15214/1998) (Supplementary Table). The tested microorganisms presented small colonies on the plates from this protocol, representing a practical difficulty in allowing the precise enumeration of colonies. Considering protocol 3, based on MRS agar supplemented with chlorophenol red, the colonies presented a white-yellow color with yellow halos, suggesting the production of acids, and allowing the precise identification of acidifying microorganisms. Although no evident differences were observed in the counts of *starter* mixes and reference and wild strains obtained in protocols 4 (MRS supplemented with bromocresol purple) and 6 (APT) when compared with other protocols, technical difficulties were observed during analysis. In protocol 4, colonies were present with a very small size, leading to difficult proper visualization. Protocol 6 presented colonies with low consistency that tended to coalesce, forming clumps: the addition of Tween 80 in the formulation of APT (protocol 6) may be responsible for this characteristic.

The distribution of the counts for the *starter* mixes and the reference and wild strains obtained by protocols 1 and 2 with different incubation periods are shown in Fig. 1. The results indicate a significant correlation between the protocols, suggesting equivalence between the counts of *starter* mixes and the reference and wild strains obtained under aerobiosis and anaerobiosis ($p < 0.05$). The linear regression parameters also indicated a significant correlation between the counts obtained after 24 h of incubation ($p < 0.05$), indicating the viability of these protocols for rapid enumeration of these microorganisms (Fig. 1). The convenience of using Petrifilm™ AC plates for the enumeration of pure cultures, due to the possibility of reducing the incubation time, has been described in similar studies on LAB and bifidobacteria (Fisher et al., 2011; Miranda et al., 2011).

3.2. Alternative protocols for LAB enumeration in salami

Based on the data obtained in the previous stage of the study, protocols 4 and 6 were not included in the evaluation of alternative protocols for LAB enumeration in salami samples. The mean counts of the LAB obtained from salami samples using protocols 1, 2, 3 and 5 are presented in Table 2. No significant differences were observed among the tested protocols, independent of the period and conditions of incubation ($p > 0.05$). These results confirm the good performance of the alternative protocols for LAB enumeration of *starter* mixes and reference and wild strains, highlighting the possibility of fasting the incubation period and optimizing the time for final results. Similar results were observed by Fisher et al. (2011) in food samples, including meat

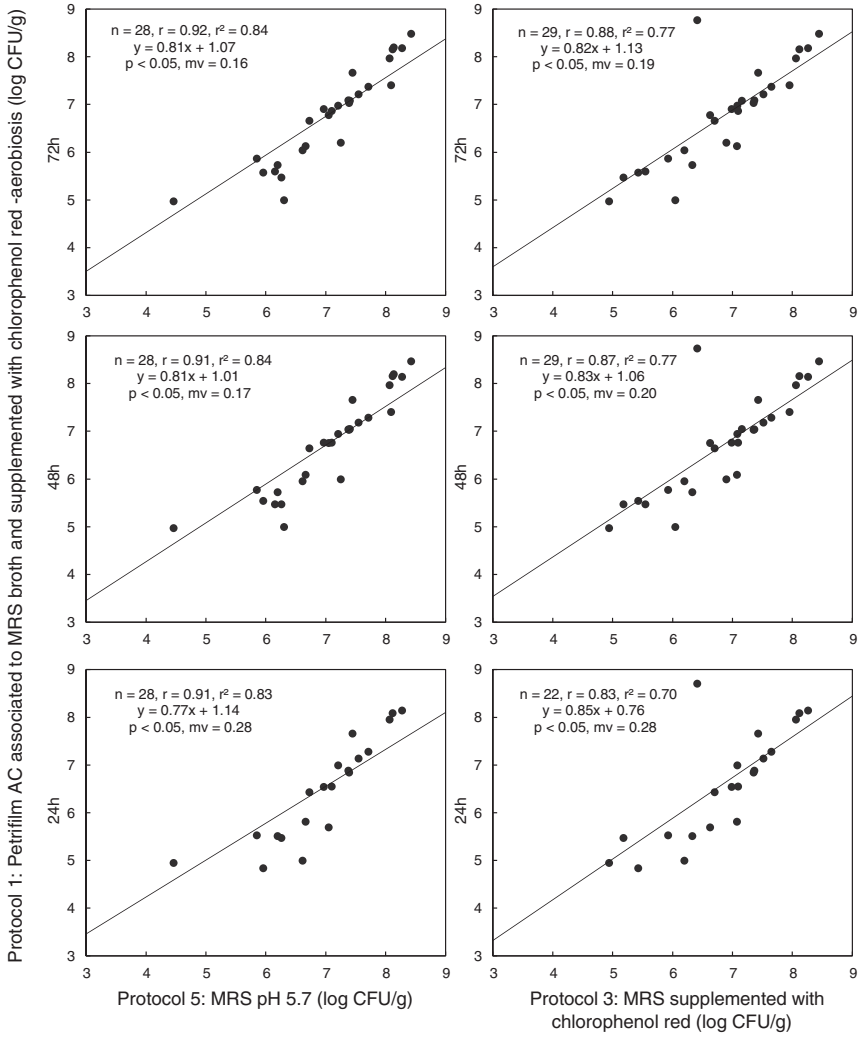


Fig. 3. Correlation parameters between the counts of lactic acid bacteria from salami samples obtained by Petrifilm™ AC associated with MRS broth and supplemented with chlorophenol red (incubated in aerobiosis, protocol 1) after different incubation times (24, 48 and 72 h) compared with two conventional plating protocols: MRS at pH 5.7 (protocol 5) and MRS supplemented with chlorophenol red (protocol 3). n = n = number of samples considered for analysis; r = correlation index; r^2 = coefficient of determination; p = level of significance.

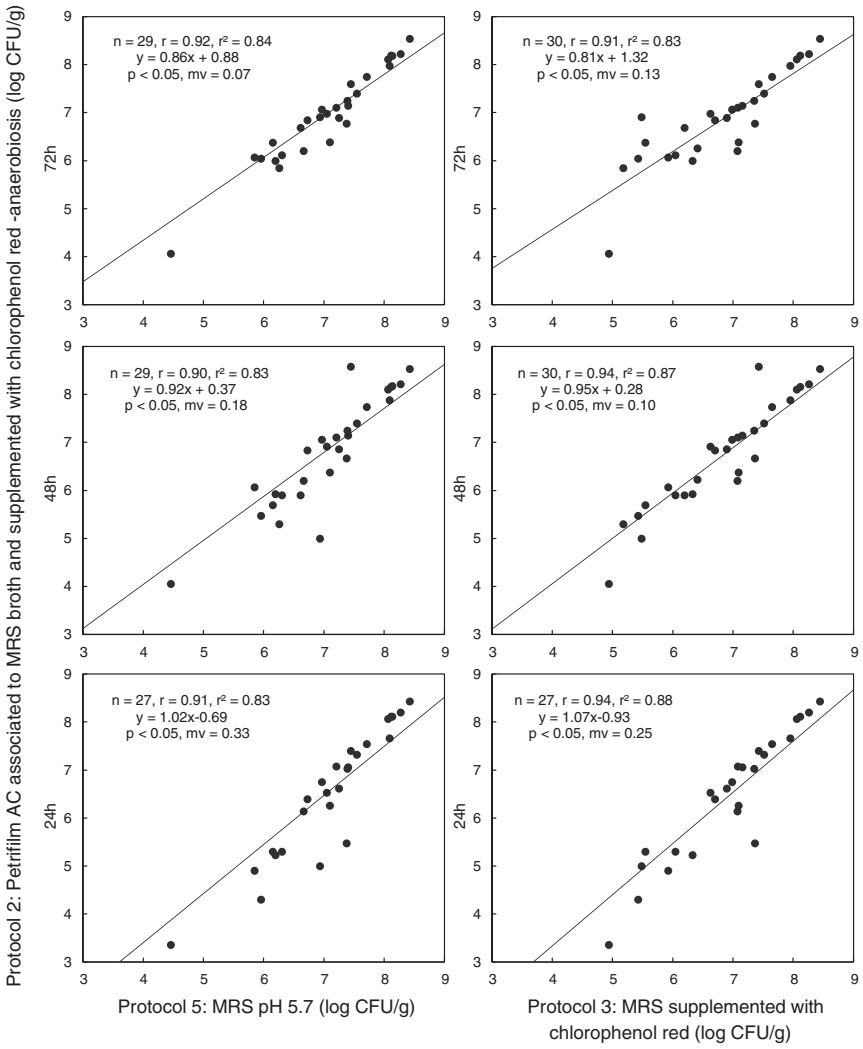


Fig. 4. Correlation parameters between the counts of lactic acid bacteria from salami samples obtained by Petrifilm™ AC associated with MRS broth and supplemented with chlorophenol red (incubated in anaerobiosis, protocol 2) after different incubation times (24, 48 and 72 h) compared with two conventional plating protocols: MRS at pH 5.7 (protocol 5) and MRS supplemented with chlorophenol red (protocol 3). n = n = number of samples considered for analysis; r = correlation index; r^2 = coefficient of determination; p = level of significance.

Table 3
Morphological characteristics of 850 colonies obtained of salami samples plated in Petrifilm™ AC supplemented with MRS broth and chlorophenol red and incubated in aerobiosis (protocol 1), Petrifilm™ AC supplemented with MRS broth and chlorophenol red and incubated in anaerobiosis (protocol 2), MRS agar supplemented with chlorophenol red and incubated in aerobiosis (protocol 3) and MRS agar pH 5.7 (protocol 5).

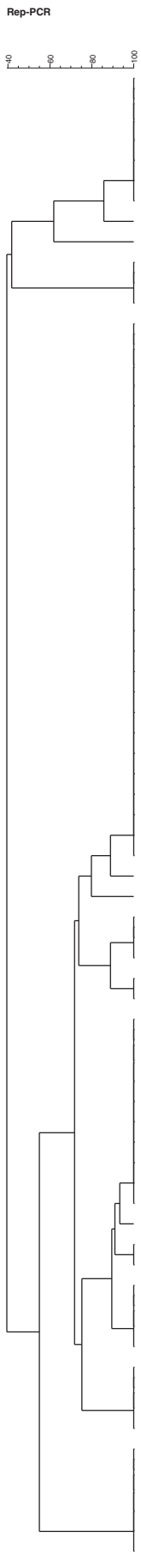
Morphology	Catalase	Protocol 1	Protocol 2	Protocol 3	Protocol 5
Cocci Gram +	Positive	16	0	11	5
	Negative	146	104	154	189
Bacilli Gram +	Negative	40	46	58	81
Total		202	150	223	275

samples, and by Miranda et al. (2011) in fermented milk supplemented with bifidobacteria.

Fig. 2 shows the dispersion of LAB counts from salami samples obtained by protocol 1 and protocol 2, considering different periods of incubation. As observed in the first step of the study (Fig. 1), all correlation indexes were significant ($p < 0.05$), indicating equivalence between the counts obtained using Petrifilm™ AC plates supplemented with chlorophenol red and incubated under aerobic and anaerobic conditions, independent of the period of incubation (24, 48 or 72 h). These results confirm the viability of using the Petrifilm™ AC plates under aerobic conditions by a low incubation period (24 h), allowing proper enumeration of LAB in salami and representing an advantage to the monitoring of these microorganisms.

The dispersion between the counts obtained by conventional methodologies (protocol 3 and protocol 5, considering only the counts obtained after 72 h of incubation, and set as reference protocols) and the counts obtained with Petrifilm™ AC (protocol 1 and protocol 2, considering incubation for 24, 48 and 72 h) are shown in Figs. 3 and 4. All correlation indexes were significant ($p < 0.05$), indicating equivalence between the alternative and conventional reference protocols. Petrifilm™ AC incubated under aerobiosis presented better performance when compared with the data obtained by the conventional procedure using MRS agar at pH 5.7 (Fig. 3), while Petrifilm™ AC incubated under anaerobiosis presented equivalent performance when compared with the counts obtained from all tested protocols (Fig. 4). These results confirm the equivalence between the LAB counts in salami obtained by different protocols,

Fig. 5. Similarity indexes, genetic profiles obtained by rep-PCR, isolates identification, original sources and Gram staining morphology of 73 isolates obtained from salami samples, and identification of 25 selected isolated obtained by sequencing of a region of the 16s RNA gene. Codes for culture medium: PAC: Petrifilm™ AC, CR: chlorophenol red; MRS: de Man, Rogosa & Sharpe.



Id.	Culture medium	Morph.	Identification
63	MRS - 5.7	bacilli	
65	PAC - CR - Anaerobiosis	bacilli	
66	MRS - CR	bacilli	
67	MRS - 5.7	bacilli	Lactobacillus paraplantarum
69	PAC - CR - Anaerobiosis	bacilli	
71	MRS - CR	bacilli	
73	MRS - 5.7	bacilli	
21	MRS - 5.7	bacilli	Lactobacillus paraplantarum
72	MRS - 5.7	bacilli	Lactobacillus sakei
6	MRS - CR	bacilli	
7	MRS - 5.7	bacilli	Lactobacillus curvatus
11	PAC - CR - Anaerobiosis	bacilli	Lactobacillus brevis
1	MRS - 5.7	cocci	
3	PAC - CR - Anaerobiosis	cocci	
5	PAC - CR - Aerobiosis	cocci	Pediococcus pentosaceus
9	PAC - CR - Anaerobiosis	cocci	Pediococcus pentosaceus
12	PAC - CR - Aerobiosis	cocci	Pediococcus pentosaceus
22	PAC - CR - Anaerobiosis	cocci	
23	PAC - CR - Anaerobiosis	cocci	
24	PAC - CR - Aerobiosis	cocci	Pediococcus pentosaceus
25	MRS - CR	cocci	
26	PAC - CR - Anaerobiosis	cocci	
27	MRS - CR	cocci	
29	PAC - CR - Aerobiosis	cocci	
30	PAC - CR - Aerobiosis	cocci	
33	MRS - 5.7	cocci	
34	MRS - CR	cocci	
35	PAC - CR - Aerobiosis	cocci	
36	MRS - 5.7	cocci	
37	PAC - CR - Anaerobiosis	cocci	
38	MRS - CR	cocci	
39	PAC - CR - Anaerobiosis	cocci	
41	PAC - CR - Anaerobiosis	cocci	
42	MRS - CR	cocci	
43	PAC - CR - Anaerobiosis	cocci	
48	PAC - CR - Anaerobiosis	cocci	
54	MRS - CR	cocci	Pediococcus pentosaceus
55	MRS - CR	cocci	
58	PAC - CR - Anaerobiosis	cocci	
4	MRS - CR	bacilli	Lactobacillus versmoldensis
2	PAC - CR - Aerobiosis	cocci	Pediococcus pentosaceus
13	PAC - CR - Anaerobiosis	bacilli	
14	MRS - CR	bacilli	Lactobacillus sp
17	MRS - 5.7	bacilli	
8	MRS - CR	cocci	
16	MRS - CR	cocci	Pediococcus acidilactici
28	MRS - CR	bacilli	
44	PAC - CR - Aerobiosis	bacilli	
45	MRS - CR	bacilli	Lactobacillus sakei
47	MRS - 5.7	bacilli	
49	MRS - CR	bacilli	
50	MRS - 5.7	bacilli	Lactobacillus sakei
51	MRS - 5.7	bacilli	
52	PAC - CR - Anaerobiosis	bacilli	
53	PAC - CR - Aerobiosis	bacilli	Lactobacillus sakei
64	PAC - CR - Aerobiosis	bacilli	
46	MRS - 5.7	bacilli	Lactobacillus sakei
15	PAC - CR - Anaerobiosis	cocci	Pediococcus pentosaceus
40	MRS - 5.7	cocci	Pediococcus pentosaceus
60	PAC - CR - Aerobiosis	cocci	Pediococcus pentosaceus
61	MRS - CR	cocci	
68	PAC - CR - Aerobiosis	bacilli	
70	PAC - CR - Aerobiosis	bacilli	Lactobacillus sakei
10	MRS - 5.7	bacilli	
19	MRS - 5.7	bacilli	
20	MRS - CR	bacilli	Lactobacillus curvatus
31	MRS - CR	bacilli	
18	MRS - CR	bacilli	
32	PAC - CR - Aerobiosis	bacilli	Lactobacillus plantaram
56	MRS - 5.7	bacilli	
57	PAC - CR - Aerobiosis	bacilli	Lactobacillus plantarum
59	MRS - CR	bacilli	
62	PAC - CR - Aerobiosis	bacilli	

and highlight the good performance of Petrifilm™ AC plates for LAB enumeration after 24 h of incubation.

Although no similar studies have been published for meat products, the adequate performance of Petrifilm™ AC associated with selective culture media for LAB enumeration has already been demonstrated with different fermented dairy products (Champagne et al., 1994; Gonçalves, Freitas, Nero, & Carvalho, 2009; Nero, Rodrigues, Viçosa, & Ortolani, 2008; Ortolani et al., 2007). As observed in the present study, the results demonstrate the equivalence between alternative methods and the conventional procedures for the enumeration of LAB cultures, providing scientific data that allow its use for monitoring the microbial quality of these products, and indicating viable and reliable alternatives for the food industry. Furthermore, the protocols using Petrifilm™ AC demonstrated adequate performance for the enumeration of LAB present in salami samples after 24 h of incubation, suggesting an additional advantage over conventional protocols due to a reduction in the time necessary to obtain the final results. This advantage of a shorter incubation time required for Petrifilm™ AC plates for the enumeration of LAB cultures, including *starter* and probiotic organisms, has been observed in previous studies, and can be considered an important factor supporting its utilization by the food industry for monitoring the quality of fermented products (Fisher et al., 2011; Miranda et al., 2011).

Although the counts showed equivalence, it is important to assess the specificity of the tested protocols, in order to demonstrate that the visible colonies were from the target microorganisms that were meant to be enumerated. The morphological characteristics of the isolated colonies from the plates of the four tested protocols and after the incubation periods are presented in Table 3. Based on the obtained data, it can be verified that all microorganisms that formed visible colonies in the culture media used in the tested protocols presented characteristics consistent with the typical LAB used for salami production. Previous studies demonstrated that the autochthonous microbiota of artisanal salami and fermented meat products was composed mainly of *Lactobacillus* species and other LAB (Aquilanti et al., 2007; Federici et al., 2014; Połka, Rebecchi, Pisacane, Morelli, & Puglisi, 2015; Samelis, Maurogenakis, & Metaxopoulos, 1994; Sawitzki et al., 2007; Silvestri et al., 2007); the results of this study support the role of these microorganisms as part of the salami microbiota, as well as Gram positive and catalase positive cocci (Table 3). The typical microbiota of salami is composed of LAB (mainly *Lactobacillus* and *Pediococcus*) and Gram positive and catalase positive cocci (*Staphylococcus* and *Kocuria*), groups responsible for microbial reactions that occur simultaneously during fermentation (Toldrá, 2010). The molecular analysis of selected LAB isolates confirmed the identification of typical LAB isolated from salami samples (*Lactobacillus* and *Pediococcus*) (Fig. 5). These results confirm the adequate selectivity of the tested protocols for LAB enumeration in salami samples.

Petrifilm™ plates present advantages, once it is an easy-to-use method for the enumeration and detection of LAB in a variety of foods. This method is simple, reduces costs in terms of material preparation, eliminates the necessity for the production and sterilization of culture media, requires less space for incubation and storage, and eliminates costly materials needed to create and maintain an anaerobic environment (Fisher et al., 2011; Fung, 2014; Nero et al., 2006). Even being necessary to prepare sterile material for some steps of Petrifilm™ plating, the indicated advantages are important for efficient quality control in the food industry, and allow for the optimization of monitoring LAB populations during the production process and ripening of fermented meats.

These results demonstrate the viability of using Petrifilm™ AC plates supplemented with MRS broth and chlorophenol red for the selective enumeration of LAB in salami samples, incubated under aerobiosis or anaerobiosis and after 24 h of incubation. This applicability was confirmed by the equivalence of the counts obtained by this protocol with conventional and standardized procedures of enumeration, and also by the selectivity of the enumerated cultures, indicating a viable and reliable alternative for the food industry regarding monitoring this bacterial population.

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