Characterization of spoilage bacteria in pork sausage by PCR–DGGE analysis

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Abstract

To investigate microbial diversity and identify spoilage bacteria in fresh pork sausages during storage, twelve industrial pork sausages of different trademarks were stored at 4 °C for 0, 14, 28 and 42 days, 80% relative humidity and packaged in sterile plastic bags. Microbiological analysis was performed. The pH and water activity (a_w) were measured. The culture-independent method performed was the Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The culture-dependent method showed that the populations of mesophilic bacteria and Lactic Acid Bacteria (LAB) increased linearly over storage time. At the end of the storage time**,** the average population of microorganisms was detected, in general, at the level of 5 log cfu g^{-1}. A significant ($P < 0.005$) increase was observed in pH and a_w values at the end of the storage time. The PCR-DGGE allowed a rapid identification of dominant communities present in sausages. PCR-DGGE discriminated 15 species and seven genera of bacteria that frequently constitute the microbiota in sausage products. The most frequent spoilage bacteria identified in the sausages were *Lactobacillus sakei* and *Brochothrix thermosphacta.* The identification of dominant communities present in fresh pork sausages can help in the choice of the most effective preservation method for extending the product shelf-life.

Keywords: microbial diversity; *Lactobacillus sakei*; *Brochothrix thermosphacta*.

1 Introduction

Fresh sausages are highly perishable and serve as substrates for several spoilage and pathogenic microorganisms due to their high water content and abundance of essential nutrients (COCOLIN et al., 2004). Spoilage can be defined as any change in a food product that makes it unacceptable to the consumer from a sensory point of view. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as textural changes (degradation of polymers) or as off-flavors (GRAM et al., 2002). In the case of meat and meat products, microbial spoilage leads to the development of off-flavors, oxidative rancidity, discoloration, gas production and, often, slime formation (LLOYD-PURYEAR et al., 1991; COCOLIN et al., 2004).

Knowledge of the Specific Spoilage Organisms (SSOs) can ultimately be used to predict the shelf-life of a product, to aid the microbiological inspections and to design new preservation or production methods (HANSEN; HUSS, 1998). Due to the limitations of conventional microbiological methods, molecular methods, independent of cultivation, have become a very important tool and Denaturing Gradient Gel Electrophoresis (DGGE) is perhaps the most commonly used (ERCOLINI, 2004; IACUMIN; MANZANO; COMI, 2012). Many scientists have used this technique to monitor the dynamics of microbial populations and to characterize the dominant spoilage bacteria in pork meat and pork meat products (COCOLIN et al., 2004; HU et al., 2009; JIANG et al., 2010). However, there are few studies that have characterized the spoilage bacteria in pork sausages in Brazil using molecular methods. Further investigation is necessary to obtain a more complete understanding of the microbial species in products responsible for spoilage. Therefore, the objective of this study was to characterize spoilage bacteria in fresh pork sausages by culture-dependent methods and Polymerase Chain Reaction (PCR)-DGGE analysis, as well as by monitoring pH and water activity (a_w) values of the sausages during the time of storage.

2 Materials and methods

2.1 Samples and storage

Sealed packages of industrial pork sausages from twelve different trademarks (line "fresh sausages") were collected from commercial establishments in the state of Minas Gerais, Brazil. The sausages analyzed contained as basic ingredients: pork meat, pork fat, water, salt, monosodium glutamate, sugar, pepper, ascorbic acid and sodium nitrite/nitrate. Samples were transported in isothermal boxes under refrigeration. In the laboratory, sausages were portioned aseptically, packaged in sterile plastic bags (Cryovac, Brazil; O_2 transmission rate, 30 cm^3 m^{-2} atm^{-1} 24 h^{-1} at 20 °C) and stored at 4 °C with 80% relative humidity for a total of 42 days. At 0, 14, 28 and 42 days, samples of sausages were subjected to molecular analysis, and triplicate samples were used for microbiological, pH and a_w analyses.

2.2 Microbiological analysis, pH and a_w measurements

Ten grams of each sausage sample were homogenized in 90 ml 0.1% peptone, pH 7.00 (Difco Laboratories, Detroit, Mich.) in a Stomacher (Mayo Homogenius HG 400, Brazil).

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Decimal dilutions (10^{-1} to 10^{-10}) were prepared and total aerobic mesophilic bacteria on Plate Count Agar (PCA, Merck) for 48 h at 37 °C; and Lactic Acid Bacteria (LAB) on Man-Rogosa-Sharpe (MRS) agar (Merck) at pH 6.5 for 48 h at 30 °C were evaluated.

The pH values were determined by homogenizing 10 g of sausage in 100 ml distilled water using a pH meter PHS-3B (Labmeter Model PH, China). The a_w values were measured from 5 g of sausage using an AquaLab model 3 TE (Braseq, Brazil).

2.3 DNA extraction and PCR-DGGE analysis

Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The bacterial community DNA was amplified with the primers 338fgc and 518r (OVREAS et al., 1997). The DGGE analyses were performed using a BioRad DCode Universal Mutation Detection System (BioRad, CA, USA) as described by Ramos et al. (2010). Single pieces of DGGE bands were excised with a sterile scalpel, and the DNA from each band was eluted in 30 µL of sterile water overnight at 4 °C and amplified at the same condition. The PCR products were sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were compared to those in GenBank database using the BLAST algorithm (NCBI, USA). Gels were analyzed using the Diversity Database program for determining the diversity of amplicons. The hierarchical clustering was performed using the program Systat 8.0, based on similarity matrices generated by the method of agreement (simple matching), using the algorithm of Ward and Euclidean distance.

2.4 Statistical analysis

A randomized block design with three replicates was used for the microbiological analyses and pH and a_w measurements. The treatments were arranged in a 12 X 4 factorial design: 12 sausages of different trademarks and 4 time points (0, 14, 28 and 42 days) to monitor the process of deterioration of sausages. The parameters bacterial count, pH and a_w were subjected to analysis of variance (ANOVA), and the means were compared by a Scott-Knott test. The quantitative data were analyzed using regression in relation to storage time. Data were considered significantly different when the *P* values were below 0.05. The statistical analysis was performed using the software SISVAR® 4.5 (Lavras, Brazil).

3 Results

3.1 Microbiological analysis and pH and a_w measurements

Bacterial counts throughout the storage are shown in Table 1. There was a significant interaction ($P < 0.05$) between the sausages and time of evaluation of mesophilic bacteria and LAB populations. The population of mesophilic bacteria increased linearly over the storage time, as observed by the regression equation for the sausages in the study. At the end of the storage time, the largest population was detected in PCA agar for sausage of brand 12 (6.72 log cfu g^{-1}). For the other brands of sausage, the population was, in general, detected at the level of 5 log cfu g^{-1}. For the LAB counts, no colonies (<1 log cfu g^{-1}) were observed in sausages of brands 4, 5, 6, 9 and 11 at day zero. In sausage 9, LAB colonies were not detected at day 14 either. However, the LAB population increased linearly over time in all of the sausages analyzed, as explained by the first-degree equations (Table 1).

The results of pH and a_w measurements are listed in Table 2. There was a significant interaction ($P < 0.05$) between the sausages and the time of pH and a_w evaluation. The initial pH values ranged from 5.60 to 6.97 for all the samples. In sausages 1, 2, 3, 4, 5, 6, and 10, the pH increased linearly over storage time in accordance with the regression equation for each sausage (Table 2). According to the quadratic equations (Table 2), the pH values of samples from sausages 7, 8 and 12 showed a reduction up to days 26, 26 and 15, respectively, with minimum values of 6.05, 5.23 and 5.22, followed by an increase from these time points until the end of the evaluation time (42 days). The water activity (a_w) values showed a significant increase ($P < 0.05$). Sausages 3, 4 and 5 showed no changes in the a_w values, which remained at 0.97 over the entire study period.

3.2 Direct analysis of microbial diversity in sausages by DGGE

The results from the DGGE analysis were obtained by amplifying the V3 region of the 16S rRNA gene. Individual bands observed in the DGGE profiles, named A to V, were excised from acrylamide gels, re-amplified for sequencing and identified (Table 3). A high microbial diversity at the beginning of the storage was observed, which was indicated by the presence of multiple bands. The spoilage microbiota identified in the brands of sausages is shown in Table 4.

The Figure 1 illustrates the cluster dendrogram, which grouped all sausage samples according to the composition similarity of microbial communities based on the presence or absence of amplicons detected by DGGE. The cluster was divided in two groups (G1 and G2), it was possible to observe a microbial diversity between the brands of sausage studied, as well as between the time of storage even in the same brand.

4 Discussion

Fresh sausages are highly perishable because of their characteristic pH and a_w values. The microbiology of fresh sausages has only been characterized by the presence of mesophilic, psychrotrophic microorganisms and pathogens so far. Thus, more detailed studies focusing on the ecology of fresh sausages and the investigation of the population dynamics of these products should be performed (COCOLIN et al., 2004). The current study confirmed, by culture-dependent methods, that the LAB population gradually increased and later became the dominant bacterial population. However, as reported by Hu et al. (2009), the population of LAB could not be detected (<1 log cfu g^{-1}) using culture-dependent methods at day zero for some samples of meat products. Nevertheless, using PCR–DGGE analysis, LAB populations were found in the initial stage of storage, similar to observed in our study. Thus, in accordance with Iacumin, Manzano and Comi (2012), PCR-DGGE has

Table 1. Population log cfu g^{-1} values of mesophilic bacteria and LAB over different storage times at 4 °C for fresh industrial pork sausage samples of twelve different trademarks.

Sausage	Mesophilic bacteria[1] (log cfu g^{-1})					LAB[2] (log cfu g^{-1})				
	Time (Days)				Equation	Time (Days)				Equation
	0	14	28	42		0	14	28	42	
1	2.30^{a}	3.66^{b}	3.94^{c}	4.52^{d}	0.05 x + 2.566 R^2 = 90.60%	7.63^{a}	7.81^{b}	8.33^{c}	8.82^{d}	0.029 x + 7.533 R^2 = 96.42%
2	2.75^{a}	3.36^{b}	4.103^{c}	5.35^{d}	0.061 x + 2.608 R^2 = 97.18%	5.52^{a}	6.33^{b}	7.62^{c}	8.07^{d}	0.063 x + 5.545 R^2 = 97.09%
3	2.53^{a}	3.15^{b}	4.66^{c}	5.63^{d}	0.077 x + 2.375 R^2 = 97.79%	5.88^{a}	6.51^{b}	6.66^{c}	7.09^{d}	0.027 x + 5.966 R^2 = 94.85%
4	2.20^{a}	3.34^{b}	4.37^{c}	5.53^{d}	0.078 x + 2.209 R^2 = 99.94%	0.00^{a}	2.64^{b}	3.33^{c}	4.32^{d}	0.098 x + 0.526 R^2 = 90.84%
5	2.51^{a}	3.36^{b}	4.63^{c}	5.76^{d}	0.078 x + 2.412 R^2 = 99.39%	0.00^{a}	2.51^{b}	3.09^{c}	3.39^{d}	0.077 x + 0.635 R^2 = 80.97%
6	2.08^{a}	3.37^{b}	4.79^{c}	5.86^{d}	0.091 x +2.111 R^2 =99.71%	0.00^{a}	2.63^{b}	2.79^{c}	3.64^{d}	0.079 x + 0.602 R^2 =82.64%
7	2.73^{a}	3.53^{b}	4.35^{c}	5.10^{d}	0.056 x + 2.736 R^2 = 99.97%	5.26^{a}	6.45^{b}	6.65^{c}	7.33^{d}	0.046 x + 5.463 R^2 = 92.19%
8	2.39^{a}	3.09^{b}	4.52^{c}	5.09^{d}	0.068 x + 2.340 R^2 = 97.25%	2.98^{a}	3.42^{b}	4.59^{c}	5.49^{d}	0.062 x + 2.814 R^2 = 97.32%
9	2.21^{a}	2.80^{b}	3.00^{c}	3.09^{c}	0.020 x+ 2.350 R^2 = 85.78%	0.00^{a}	0.00^{a}	2.39^{b}	2.58^{c}	0.072 x – 0.278 R^2 = 82.94%
10	2.26^{a}	2.63^{b}	2.93^{c}	4.14^{d}	0.042 x + 2.101 R^2 = 88.75%	4.34^{a}	4.63^{b}	5.27^{c}	6.61^{d}	0.053 x + 4.095 R^2 = 90.57%
11	2.16^{a}	2.54^{b}	2.64^{b}	2.91^{c}	0.017 x + 2.209 R^2 = 95.74%	0.00^{a}	2.10^{b}	2.62^{c}	3.42^{d}	0.077 x + 0.417 R^2 = 90.73%
12	3.09^{a}	4.40^{b}	5.56^{c}	6.72^{d}	0.086 x + 3.135 R^2 = 99.92%	6.12^{a}	7.31^{b}	8.09^{c}	8.78^{d}	0.062 x + 6.260 R^2 = 98.22%

For each row, mean values with different letters are significantly different ($P < 0.005$) according to the Scott–Knott test. [1]SE=0.0698. [2]SE= 0.0065.

Table 2. pH and A_w measurements over different storage times at 4 °C for fresh industrial pork sausage samples of twelve different trademarks.

Sausage	pH[1]					A_w[2]				
	Time (Days)				Equation	Time (Days)				Equation
	0	14	28	42		0	14	28	42	
1	5.60^{a}	5.91^{b}	7.20^{c}	8.16^{d}	0.064 x + 5.374 R^2 = 95.41%	0.96^{a}	0.97^{b}	0.97^{b}	0.97^{b}	*
2	6.16^{a}	6.96^{b}	7.20^{c}	8.26^{d}	0.047 x + 6.162 R^2 = 95.03%	0.97^{a}	0.97^{a}	0.97^{a}	0.98^{b}	*
3	6.97^{a}	7.32^{b}	8.00^{c}	8.56^{d}	0.039 x + 6.895 R^2 = 98.5%	0.97^{a}	0.97^{a}	0.97^{a}	0.97^{a}	*
4	6.52^{b}	6.11^{a}	7.84^{c}	8.20^{d}	0.049 x + 6.147 R^2 = 75.1%	0.97^{a}	0.97^{a}	0.97^{a}	0.97^{a}	*
5	6.61^{b}	6.29^{a}	7.62^{c}	8.41^{d}	0.048 x + 6.222 R^2 = 80.43%	0.97^{a}	0.97^{a}	0.97^{a}	0.97^{a}	*
6	6.64^{b}	6.27^{a}	7.33^{c}	8.20^{d}	0.041 x + 6.249 R^2 =75.87%	0.97^{a}	0.97^{a}	0.97^{a}	0.98^{b}	*
7	6.70^{b}	6.36^{a}	6.35^{a}	7.17^{c}	0.001x^2-0.052x+6.73 R^2 = 97.22%	0.92^{a}	0.92^{a}	0.92^{a}	0.93^{b}	*
8	6.66^{c}	5.64^{a}	5.98^{b}	7.22^{d}	0.003x^2 -0.106x+6.64 R^2 = 99.3%	0.96^{a}	0.96^{a}	0.97^{b}	0.97^{b}	0.0003 x + 0.959 R^2 = 80.00%
9	6.86^{b}	6.38^{a}	7.60^{c}	7.76^{d}	*	0.94^{a}	0.94^{a}	0.95^{b}	0.96^{c}	0.0005 x – 0.937 R^2 = 89.09%
10	6.73^{a}	6.96^{b}	7.95^{c}	8.43^{d}	0.043 x + 6.604 R^2 = 95.14%	0.96^{a}	0.96^{a}	0.96^{a}	0.97^{b}	*
11	6.61^{b}	6.27^{a}	6.71^{c}	6.79^{d}	*	0.96^{a}	0.96^{a}	0.96^{a}	0.97^{b}	*
12	5.92^{b}	5.35^{a}	5.94^{c}	8.03^{d}	0.003x^2 -0.093 x+5.94 R^2 = 99.86%	0.96^{a}	0.96^{a}	0.96^{a}	0.97^{b}	*

For each row, mean values with different letters are significantly different ($P < 0.005$) according to the Scott–Knott test. [1]SE= 0.0064. [2]SE= 5.807. *There was no fit of the equation to observed data.

been successfully applied to study the microbial biodiversity of complex environments.

In relation to total aerobic mesophilic counts, it was possible to establish that the sausages analyzed were of high quality. This is because the mesophilic population was ≤ 6 log cfu g^{-1}, which is indicative of good manufacturing practices because the products used were raw and not heat treated. According to Gram et al. (2002), the level of microorganisms detected, "total count", can be used to predict the shelf life of the product.

Even in the presence of high LAB populations, the pH values increased linearly during storage time in seven different sausages sampled. This fact can be explained because it is well established that glucose, lactic acid, and certain amino acids followed by nucleotides, urea and water-soluble proteins are catabolized by almost all the bacteria of the meat microbiota and consequently there was a production of alkaline radicals (ammonia and amines), contributing to increase pH values (NYCHAS et al., 2008). LAB species are able to produce decarboxylases, enzymes with proteolytic activity that generate amines and increase the matrix pH values (BOVER-CID et al., 2005). The a_w values did not decrease in any sample, which according to Borch, Kant-Muemansb and Blixt (1996) contributes to the stability of LAB.

According to the cluster based on DGGE analysis (Figure 1), two groups were found (G1 and G2). The G1 group included most of the samples, including all sausages at time zero, except

Table 3. Species identification of the DGGE band sequences of the V3 region of the 16S rRNA gene of the total bacterial community DNA directly extracted from the sausage samples.

Bands	Closest relatives	ID[a] (%)	Accession No.
A	*Lactobacillus plantarum*	99	JF756323.1
B	*Lactobacillus algidus*	99	GU430799.1
C	*Lactobacillus curvatus*	98	AB289024.1
D	*Lactobacillus sakei*	98	AY383042.1
E	*Carnobacterium divergens*	98	JF756331.1
F	*Brochothrix thermosphacta*	98	JF756334.1
G	*Lactobacillus fuchuensis*	98	AB289024.1
H	*Bacillus lichenformis*	97	HM640420.1
I	*Bacillus subtilis*	97	EU130453.1
J	*Janthinobacterium lividum*	98	HQ003440.1
K	*Psychrobacter immobilis*	97	HQ698589.1
L	*Pseudomonas fluorescens*	99	HM597248.1
M	*Paenibacillus sp*	100	HM161756.1
N	*Leuconostoc mesenteroides*	98	FR852570.1
O	*Psychrobacter sp*	99	GQ169116.1
P	*Lactobacillus brevis*	99	JF720006.1
Q	*Weisella paramesnteroides*	98	HQ721270.1
R	*Enterococcus sp*	98	JF799879.1
S	*Microbacterium*	100	AF390085.1
T	*Bacillus sp*	97	HQ620634.1
U	*Vibrio sp*	98	AB038029.1
V	*Alcaligenes sp*	98	AY346136.1

[a]ID represents the identity with the sequences in the GenBank databases.

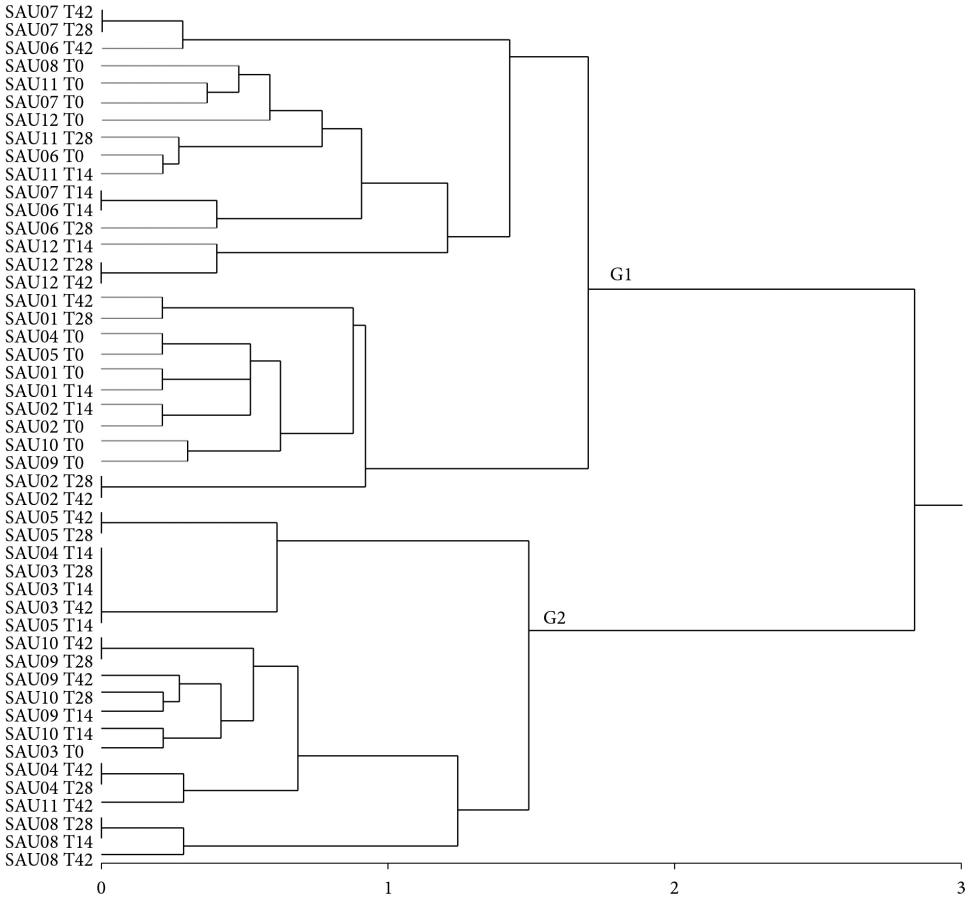

Figure 1. Cluster analysis of 16s rRNA amplicons assessed by DGGE analysis for 12 Brazilian fresh pork sausages during 42 days of storage.

Table 4. Succession of bacteria identified in 12 Brazilian fresh sausage brands during 0, 14, 28 and 42 days of storage.

Bands	Sausage brands during 42 days of storage																							
	1				2				3				4				5				6			
	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42
A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+
B	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-	+
D	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	-
E	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
G	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	-	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
K	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-
L	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-
M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Bands	Sausage brands during 42 days of storage																							
	7				8				9				10				11				12			
	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42	0	14	28	42
A	+	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+
B	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-
C	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
D	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
E	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
F	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
J	-	+	+	+	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
K	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	-	-	-
L	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	-	+	+	+
M	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
N	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
Q	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
T	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

[1]ID = percentage of identity with the sequences in the Genbank databases. + = detected and - = non-detected.

brand 3. Samples of all times evaluated of sausages 6, 7 and 12 are grouped in the G1. This group is characterized by a diversity of bacteria. The G2 group is characterized by the presence of *L. sakei, P. immobilis, P. fluorescens, J. lividum* and *B. thermosphacta* in almost all samples in the group. All samples of sausage 3 were included in this group. The microbial community has changed during 42 days of storage even under refrigeration, for the different sausages studied in this work. Different bacteria species were identified by amplification of DGGE bands.

Although *Lactobacillus* were not detected in the time zero of storage according to the culture dependent method, it was possible to identify their presence by DGGE analysis. The Table 4 lists the succession of bacteria species detected in 12 Brazilian fresh pork sausages during 42 days of storage. *L. sakei* was identified as the predominant spoilage bacterium by PCR–DGGE. *Lactobacillus sakei* is an autochthonous species in sausages (BONOMO; RICCIARDI; SALZANO, 2011). Kesmen et al. (2012) also reported as result of the PCR-DGGE analysis of sucuk, Turkish fermented sausage, *L. sakei* as one of the microorganisms dominant in the product. This species produces ropy slime that confers a strong competitive ability to this species (BJÖRKROTH; KORKEALA, 1997). A specific spoilage phenomenon of commercial significance, characterized by long, stretchy, polysaccharide ropes between sausages or sausage slices, was also detected. *L. sakei* strains play a major role in this spoilage phenomenon. *Lactobacillus curvatus* has also been shown to be a common species in sausages (KORKEALA; BJÖRKROTH, 1997; ALBANO et al., 2008). This species was detected in samples from sausages 1, 2, 3, 4, 5 and 11. *Lactobacillus plantarum* was detected in sausages 1, 5, 6, 7, 8, 9 and 11. Nguyen et al. (2013) also detected by PCR-DGGE *L. plantarum* and *L. brevis* in Nem chua, a traditional fermented meat product of Vietnam. Among LAB, *L. sakei*, *L. curvatus* and *L. plantarum* are the most widely described species in sausages, as also reported by Parente, Griego and Crudele (2001).

Lactobacillus algidus was detected in four brands of sausages (1, 2, 9 and 10). A previous study reported *L. algidus* as a psychrophilic, predominant strain isolated from vacuum-packaged meat stored at 2 °C for 3 weeks (KATO et al., 2000). In relation to the band identified as *L. fuchuensis* (or *L. sakei*), this species is phylogenetically close to but distinct from *L. sakei* and also appears to be associated with (vacuum) packaged meat (SAKALA et al., 2002). Solely based on the DGGE analysis of 16S rDNA amplicons, this band should thus be assigned to *L. sakei* and/or *L. fuchuensis*. It is possible that the use of housekeeping genes in DGGE-based population fingerprinting could result in a higher taxonomic resolution for the separation of closely related species such as *L. sakei* and *L. fuchuensis* (AUDENAERT et al., 2010). Other LABs were detected in this research: *Leuconostoc mesenteroides* and *Weissella paramesenteroide* in three and one sample, respectively (Table 3). These microorganisms are generally present in sausages and meat products (ALBANO et al., 2008; NGUYEN et al., 2013). In general, the control of growth of spoilage LAB in processed meats is difficult because these bacteria are psychrotrophic, microaerophilic and resistant to nitrite, salt and smoke (FRANZ; VON HOLY, 1996).

Janthinobacterium lividum was detected in 11 samples and was present for more than one evaluation time, similar to *B. thermosphacta*. *J. lividum* was reported by Nichas et al. (2008) and Cavill et al. (2011) as the genus of spoilage bacteria commonly found in meat and processed meat. *Brochotrix thermosphacta*, a Gram-positive, facultative anaerobe spoilage microorganism, was previously reported by the use of the PCR-DGGE method in Córdoba sausage, an artisanal Argentinian fermented sausage (FONTANA; VIGNOLO; COCCONCELLI, 2005).

Pseudomonas fluorescens was another species identified in our study and was present in eight sausages. *Pseudomonas* has been demonstrated as one of the dominant spoilage microbiota in chilled pork (LI et al., 2006).

The detection of unknown species of *Psychrobacter* in two sausages (6 and 7) and *P. immobilis* in eight (3, 4, 5, 8, 9, 10, 11 and 12), corroborates with the data reported by Gennari et al. (1992) and Albano et al. (2008) who reported the presence of *Psychrobacter* species in sausage products. Although it has been reported as a spoilage bacterium of low importance in meat, *P. immobilis* is a lipolytic species and might be a cause of incidental infections (LLOYD-PURYEAR et al., 1991).

The genus *Bacillus* and the species *B. licheniformis* and *B. subtilis* were found in our study. However, this genus was detected in few sausage brands, *B. subtilis* was detected only in sausage 2, during 28 and 42 days of storage; *Bacillus* sp. and *B. licheniformis* were detected in sausage 8 and 1, respectively, both after 42 days of storage. *Alcaligenes* and *Vibrio* were detected at the end of the study, when the pH became alkaline in the sausages. The genera *Enterococcus*, *Microbacterium* and *Paenibacillus* were detected in few samples from different sampling times. These genera are commonly associated with the spoilage of processed meats (NYCHAS et al., 2008).

5 Conclusions

Samples of the sausages showed good sanitary hygienic quality, as the microbiota was composed of only spoilage microorganisms. *Enterococcus*, which could be an indicator of fecal contamination, was detected in only one sample. PCR-DGGE allowed for the discrimination of 15 species and seven genera of bacteria that frequently compose the microbiota in sausage products. The most frequent spoilage bacteria identified in the sausages were *L. sakei* and *B. thermosphacta*. This is the first time that the microbial community present in Brazilian pork sausages is assessed by DGGE analysis. The knowledge and identification of dominant communities of bacteria in sausages can help in the choice of the most effective preservation method for extending the product shelf-life and in the evaluation of the sanitary hygienic quality of sausages produced in industry.

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