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Isolation and characterization of *Lactobacillus plantarum* BLS29 as a potential probiotic starter culture for pork sausage production

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The aim of this study was to isolate and characterize lactic acid bacteria (LAB) from naturally fermented pork sausages and test them for use as a probiotic starter culture in the production of fermented sausages. LAB (n=26) were isolated from natural fermented pork sausages. Isolates were identified using 16S rDNA or Internal Transcribed Spacer (ITS) region sequencing. After that, strains were characterized based on technological, functional and safety proprieties. A LAB strain was chosen and its survival was tested in simulated gastric juice and intestinal juice. Results indicate that *Lactobacillus plantarum* (n=15) was the predominant species in fermented sausage, followed by *Enterococcus faecium* (n=8), *Lactobacillus brevis* (n=1), *Enterococcus durans* (n=1) and *Enterococcus hirae* (n=1). *L. plantarum* BLS29 strain was selected because it was not able to produce CO_2 and was able to multiply at temperatures ranging from 15 to 37°C, usually practiced during the sausage cure process. BLS29 showed better growth capacity than other isolated LABs when exposed to curing salts, and also demonstrated antimicrobial activity against foodborne pathogens. According to our findings, BLS29 can be a promising strain to be used as a probiotic starter culture for the production of fermented pork sausage.

Key words: Probiotics, *Lactobacillus plantarum,* fermented pork sausage.

INTRODUCTION

The demand for new food products has greatly influenced the development of new fermented meat products, even though these products have been considered unhealthy because of the high saturated fat, additives and spice

contents (Arihara, 2006; De Vuyst et al., 2008; Macedo et al., 2012). According to Fiorentini et al. (2010) and Raigorodsky (2011), the evolution of fermented sausages is relatively old, having emerged from the need of using all body parts of animals as food. The production of fermented sausages was introduced in Brazil by Italian immigrants and, more recently, gave rise to small factories, transforming the economy, especially in Southern Brazil. Salamis and sausages are raw embedded, cured, fermented, matured and dry, and may or may not be smoked (Terra et al., 2004; Ordoñez Pereda, 2005). In Brazil, these products are classified into eight different classes, according to the composition of meat and fat, type of raw material used and seasoning (Brazil, 2000). Among these classes are the salamis that are fermented meat products with moisture content below 40%.

Functional foods are defined as: 'foods that contain some health-promoting component(s) beyond traditional nutrients'. One way in which foods can be modified to become functional is by the addition of probiotics (Soccol et al., 2010). Probiotics are defined as live micro-organisms which, when consumed in adequate amounts as part of the diet, may play an important role in respiratory, immune, and gastrointestinal functions and have a significant effect on the clearance of infectious diseases in children and lactose intolerance (FAO/WHO, 2001).

Foods containing probiotic are considered functional foods and present great commercial interest because its consumption has increased expressively. Although Metchnikoff (1907) has described the benefits of microorganisms to consumers' health, it was only in the last 30 years that the study of probiotics has been intensified, especially in the selection and characterization of probiotic species (FAO/WHO, 2001). Numerous studies claim that probiotic species contribute to the reduction of cholesterol levels in the blood, prevention and treatment of food-related allergies, assist in the treatment of infections caused by *Helicobacter pylori*, prevention of cancer and modulation of intestinal flora (Arihara, 2006; El-Ghaish et al., 2011; Yoon et al., 2013).

Probiotics have been extensively used in dairy product production, but few studies have demonstrated its usage in fermented meat products (Erkkilä et al., 2001; Arihara, 2006; De Vuyst et al., 2008). In 1998, Germany was the first country to produce salami containing three probiotic strains of intestinal lactic acid bacteria (*Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp.). This study highlights the promise usage and development of new probiotic meat products (Hammes and Hertel, 1998). The development of sausage containing probiotics able to provide health benefits and that ensure the safety of the product is of great interest. Generally, probiotic bacteria of the genus *Lactobacillus* are capable to produce bacteriocins, inhibiting the growth of Gram-positive bacteria like *Staphylococcus aureus* and *Listeria monocytogenes* (Incze, 1998; Guetouache and Guessas, 2015). Some species of LAB are used as starter cultures, contributing to the sensory quality and with the microbiological safety of industrial fermented products (Leroy et al., 2006). One way to obtain both characteristics is to use the new generation of 'functional starter cultures' (De Vuyst, 2000). Wild strains are naturally dominant in natural fermentation and show a high capacity for metabolic formation of flavor. Natural selected strains are interesting because frequently they produce antimicrobial compounds, enhancing the competitiveness against pathogens (Ayad et al., 2000; Maldonado et al., 2002).

In recent years, the study of functional cultures has aroused great attention (Erkkilä et al., 2001; Arihara, 2006; De Vuyst et al., 2008), either using the addition of free probiotics (Pennachia et al., 2006; Leroy et al., 2006) or using encapsulated forms (Muthukurasami and Holley, 2006). The market for probiotics has been very promising, and are rare products containing meat probiotics (De Vuyst, 2000; Pennacchia et al., 2004; Pennacchia et al., 2006; Leroy et al., 2006; De Vuyst et al., 2008). This market has been dominated mainly by dairy products, but these cannot be consumed by people with lactose intolerance. In this sense, meat products containing probiotics may have competitive advantage to dairy products (Erkkila et al., 2001; Arihara, 2006; Macedo et al., 2012). Most of the probiotic bacteria have been isolated from human or animals intestinal tracts, milk or vegetables, however the use of bacteria isolated from meat may contribute to a better sensory quality of the meat fermented product produced (De Almeida Junior et al., 2015). Considering the need to find new strains with probiotic characteristics that can be used as functional starter cultures, while preserving the technological and sensory characteristics of the sausages, the present study aimed to isolate LAB from fermented pork sausages and to characterize potential probiotic starter cultures for the use in sausage production.

MATERIALS AND METHODS

Isolation of LABs

Microorganisms were isolated from fermented pork sausages produced without the addition of starter cultures from three different batches, produced in a commercial manufacturing plant in the State of Santa Catarina, Southern Brazil. Samples of 25 g of pork

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sausage were diluted in 225 ml of 0.1% peptone water and were homogenized inside sterile plastic bags with a stomacher (Easy Mix, AES Chemunex, Rennes, France). One aliquot of 1000 µl of each dilution was inoculated on MRS agar (Man Rogosa and Sharpe, Acumedia, Baltimore EUA), using pour-plate method, and plates were incubated at 37°C for 48 h using anaerobic jars (Permution, Curitiba, Brazil). After incubation, different colonies were randomly selected and checked for its purity, being analyzed after Gram staining. All selected isolates were stored at -20°C in 30% (v / v) glycerol and propagated into tubes containing 5 ml of MRS broth, before next experiments.

Molecular Identification of Isolates

Genomic DNA of isolates was extracted and the 16S rDNA genes were amplified by PCR using the following universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). For those samples whose identification by 16S rDNA was not possible or inaccurate, the 16S-23S rDNA intergenic spacer region (ITS) was amplified using bacterium specific universal primers 13BF (5'-GTGAATACGTTCCCGGGCCT-3') and 6R (5'-GGGTTYCCCCRTTCRGAAAT-3') (where Y=C or T, R=A or G) (Gurtler and Stanisich, 1996). The sequencing reactions were carried out using the same primers previously used in both PCR reactions (Ludwig Biotec, Porto Alegre, Brazil). The 16S and ITS sequences obtained were submitted to the BLAST search program of the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov) in order to search for homologous sequences. BioEdit (Hall, 1999) was used to edit sequences and for the construction of 16S and ITS contigs.

CO_2 and exopolysaccharides production

The production of carbon dioxide gas (CO_2) was determined using MRS broth inside glass tubes with inverted Durham tubes. Ammonium citrate was replaced by ammonium sulfate in order to induce glucose use for fermentation. The tubes were incubated during 48 h at 37°C and gas production was verified from the glucose fermentation. Exopolysaccharides production was determined checking the growth of the strains on MRS agar, substituting glucose by 5% of sucrose. The plates were incubated for 7 days at 25°C and checked for exopolysaccharides production by negative staining (Hugas et al., 1993).

Acidification capacity

The acidification capability of isolates was determined using tubes containing 10 ml of MRS broth (pH 6.6) without KH_2PO_4, but containing ammonium citrate, 0.3% of sodium citrate (Dinamica, São Paulo, Brazil) (Hugas et al., 1993). The pH level was evaluated after 48 h of incubation at 37°C (Tec-5 electrode Mettler Toledo, Tecnal, Piracicaba, Brazil). The mean value of three pH measurements was calculated.

Tolerance to sodium chloride and sodium nitrite

Isolates were checked according to their tolerance for 5 and 8% NaCl (Vetec, Duque de Caxias, Brazil), to the tolerance of 3% NaCl associated with $NaNO_2$ (Vetec, Duque de Caxias, Brazil) and of 200 ppm for check the ability of growing in MRS broth after 72 h, at 37°C incubation (Hugas et al., 1993). Growth was determined by the McFarland scale.

Growth at different temperatures

The capacity of one probiotic growing at different temperatures is a very important property for the production of sausages. The temperatures of growing were investigated using MRS broth incubated for 72 h at 15, 37 and 45°C. The growth at 4°C for seven days was tested as well (Dalla Santa, 2008) and checked with MacFarland scale.

Determination of antagonistic activity

The antagonistic activity of each LAB isolate was determined by the presence of a growth inhibition zone of a specific foodborne indicator strain around the spot of LAB. For assessment of antagonistic activity, 5 µl of overnight LAB cultures were spotted on MRS agar plates and incubated at 37°C, for 24 h, in microaerophilic conditions. After this period, the plates were treated with chloroform, during 30 min, and residual chloroform was evaporated, with the lids open for another 30 min. Then, 10^8 colony forming units (CFU)/ml of the pathogen in BHI soft-agar (0.75% w/v) (Himedia, Mumbai, India) were inoculated using the overlay method. LAB isolates were tested against *Salmonella enterica* serovar Enteritidis SE86 (one of most important foodborne pathogen in the State of Rio Grande do Sul, Southern Brazil) (Tondo and Ritter, 2012), *Salmonella* Typhimurium (ATCC 14078), *Staphylococcus aureus* (ATCC 14458), *Escherichia coli* O157:H7 (ATCC 43888), *Bacillus cereus* (ATCC 33010), and *Listeria monocytogenes* (ATCC 6477). After incubation under anaerobic conditions, the diameters of the inhibition zones were determined with a caliper (Martins et al., 2010).

Tolerance to simulated gastric juice and intestinal juice

The BLS29 strain was tested for its tolerance to the upper gastrointestinal tract by using simulated gastric and intestinal juices, according to Meira et al. (2012) with modifications. The simulated gastric juice solution was daily prepared with 3 mg/ml of pepsin (Sigma Aldrich, St. Louis, USA), 0.5% NaCl and acidified with HCl until pH 4.0, 3.0, 2.5, and 2.0. The simulated intestinal juice consisted of 1 mg/ml of pancreatin (Sigma Aldrich, St. Louis, USA), 0.5% NaCl, with or without 5 g/l of porcine bile, and pH 8.0 (Sigma Aldrich, St. Louis, USA). The solutions were sterilized using 0.22 µm membrane filter. After two propagations, *L. plantarum* BLS29 cultures incubated 24 h in MRS broth were concentrated by centrifugation (12,000 *g* for 5 min), washed twice in phosphate buffer pH 7.0 and suspended in 0.5% NaCl solution. Two hundred microliters (200 µl) of the cell suspension were incubated at 37°C in a bath (De Leo, Porto Alegre, Brazil) in the presence of 1.0 ml of simulated gastric juice. Viable cell counts were carried out at initial time (0h), 3 and 4 h of exposure to simulated gastric juice, in order to simulate intestinal transit tolerance. The BLS29 cells that survived to pH 2.0 (acid-exposed) were inoculated in simulated intestinal juice as well as a control consisting in a suspension of non-acid-exposed BLS29 cells.

Hemolysis

Lactobacilli strains were cultured on blood agar plates and incubated for 48 hours at 30 ºC (Maragkoudakis et al., 2006; Meira et al., 2012). Strains that did not change the medium (γ-hemolysis) or that produced a clear green halo around the colony (α-hemolysis) were classified as non-hemolytic, while strains that showed a lytic zone around the colony were classified as hemolytic (β-hemolysis).

Table 1. 16S rDNA gene and 16S-23S ITS sequence identification of lactic acid bacteria isolated from starter-free pork sausage produced in Southern Brazil.

Isolate	Sequence identity	Sequence length	% similarity	Corresponding GenBank strain accession number
BLS02	*Lactobacillus plantarum*[2]	438	100	AB083123
BLS03	*Lactobacillus plantarum*[2]	441	100	AB362387
BLS04	*Lactobacillus plantarum*[1]	720	100	KF472174
BLS05	*Enterococcus durans*[1]	847	100	KF317897
BLS06	*Enterococcus hirae*[1]	1148	100	AB841310
BLS07	*Enterococcus faecium*[1]	658	100	KF012874
BLS08	*Enterococcus faecium*[1]	1145	100	JN792505
BLS09	*Lactobacillus plantarum*[1]	1161	100	KF472174
BLS10	*Lactobacillus plantarum*[2]	471	100	AB362387
BLS11	*Enterococcus faecium*[1]	1148	100	EU717954
BLS12	*Enterococcus faecium*[1]	858	99	HQ293078
BLS13	*Enterococcus faecium*[1]	1166	100	KF318400
BLS14	*Enterococcus faecium*[1]	1162	100	KF149621
BLS15	*Enterococcus faecium*[1]	988	99	KF318400
BLS16	*Enterococcus faecium*[1]	1149	99	KF149621
BLS17	*Lactobacillus plantarum*[2]	467	100	AB102857
BLS19	*Lactobacillus plantarum*[2]	481	100	AB083123
BLS20	*Lactobacillus plantarum*[2]	464	100	AB102857
BLS21	*Lactobacillus plantarum*[1]	1153	100	KF472174
BLS23	*Lactobacillus plantarum*[2]	760	100	JN587506
BLS26	*Lactobacillus plantarum*[2]	817	100	HE646365
BLS27	*Lactobacillus brevis*[1]	1126	100	KF307784
BLS29	*Lactobacillus plantarum*[1]	1363	100	KF472174
BLS30	*Lactobacillus plantarum*[2]	466	100	AB102857
BLS31	*Lactobacillus plantarum*[2]	481	100	AB083119
BLS32	*Lactobacillus plantarum*[2]	468	100	AB102857

[1]Identification based on the 16S rDNA sequence data. [2] Identification based on the ITS sequence data.

RESULTS

Isolation and characterization of microorganisms

Twenty-six (26) isolates of LAB were isolated from pork sausage starter-free manufactured in a meat processing unit in the western part of the State of Santa Catarina, Southern Brazil. On the basis of 16S rRNA gene and ITS region sequencing data, isolates were identified as belonging to the genus *Lactobacillus* and *Enterococcus* (Table 1). *L. plantarum* was the most frequently isolated microorganisms. The accession numbers were identified and corresponded to the sequences of Genbank, where the highest identified scores after submitting the sequence query to the BLAST tool were obtained.

Exopolysaccharide, CO_2 and hemolysis production

All strains produced exopolysaccharides on MRS plates supplemented with 5% of sucrose. None of the *Lactobacillus* strains were positive for hemolytic reaction and only BLS27 *L. brevis* producing CO_2.

Acidification capacity, salt tolerance and growth at different temperatures

All the isolated strains were tested for their ability of acidification, tolerance to curing salts and capacity of growing at different temperatures (Table 2). This was done in order to select a fermentative strain with the potential to be used in the preparation of sausage with functional culture.

Among the *L. plantarum* strains, BLS29 was chosen and tested for its probiotic potential, based on the high acidification of MRS broth; BLS29 grow well in the presence of curing salts and grow at temperature of 15°C, what is usually practiced during sausages ripening.

Antimicrobial activity

All lactobacilli isolated from pork sausages were tested

Table 2. Ability of acidification, tolerance to curing salts and growth at different temperatures of lactic bacteria isolated from fermented pork sausage produced in Southern Brazil.

Strain	Salts tolerance			Growth at different temperatures				pH
	NaCl 3% + $NaNO_2$ 200 ppm	5% NaCl	8% NaCl	4ºC	15ºC	37ºC	45ºC	
BLS02	+	+	+	-	+	+	-	3.89±0.03[a]
BLS03	+	+	+	-	+	+	-	3.87±0.00[a]
BLS04	+	++	+	-	+	+	-	3.92±0.02[a]
BLS05	+	+	-	-	+	+	-	4.54±0.05[b]
BLS06	+	+	-	-	+	+	-	4.67±0.01[b]
BLS07	+	++	-	-	+	+	+	4.56±0.00[b]
BLS08	+	++	-	-	+	+	+	4.53±0.01[b]
BLS09	+	++	+	-	+	+	-	3.90±0.00[a]
BLS10	+	++	+	-	+	+	-	3.96±0.00[a]
BLS11	+	++	-	-	+	+	+	4.58±0.00[b]
BLS12	+	+	-	-	+	+	+	4.58±0.00[b]
BLS13	+	+	-	-	+	+	+	4.58±0.00[b]
BLS14	+	+	-	-	+	+	+	4.59±0.01[b]
BLS15	+	++	-	-	+	+	+	4.59±0.01[b]
BLS16	+	++	-	-	+	+	+	4.61±0.01[b]
BLS17	+	++	+	-	+	+	-	4.00±0.01[c]
BLS19	++	+	++	-	+	+	-	4.00±0.01[c]
BLS20	+	++	+	-	+	+	-	3.90±0.01[a]
BLS21	+	++	+	-	+	+	-	3.83±0.06[a]
BLS23	+	+	+	-	+	+	-	3.81±0.00[a]
BLS26	+	+	+	-	+	+	-	3.90±0.01[a]
BLS27	+	+	+	-	+	+	-	4.61±0.01[b]
BLS29	++	++	++	-	+	+	-	3.89±0.01[a]
BLS30	+	++	+	-	+	+	-	3.91±0.07[a]
BLS31	+	+	+	-	+	+	-	3.90±0.01[a]
BLS32	+	+	+	-	+	+	-	3.82±0.01[a]

-, No growth; +, Growth; ++ Strong growth. a,b,c Different letters indicate significant difference (P<0,05) between pH values by Tukey test.

for inhibitory potential against *Salmonella enterica* serovar Enteritidis SE86, *Salmonella* Typhimurium (ATCC 14078), *S. aureus* (ATCC 14458), *E. coli* O157:H7 (ATCC 43888), *B. cereus* (ATCC 33010) and *L. monocytogenes* (ATCC 6477) (Table 3), and demonstrated to be able to inhibit all pathogens.

Tolerance to simulated gastric juice and intestinal juice

The BLS29 strain showed good potential to be used as a probiotic for the production of pork sausages, because demonstrated ability to produce exopolysaccharides, but not CO_2, was tolerant to cure salts, was able to acidify MRS broth, was able to grow at different temperatures and demonstrated the ability for inhibiting food pathogens. Based on these characteristics, BLS29 was chosen for further experiments and was tested for the resistance to simulated gastric juice and intestinal juices (Tables 4 and 5).

BLS29 was incubated at 37°C, and then subjected to simulate gastric conditions for up to four hours. An initial reduction of 3 log was observed, however about 5 log of BLS29 remained viable. Both preparations of *L. plantarum* BL29 exposed to the gastric juice at pH ranging from 4.0 to 2 as the control non-acid-exposed were able to survive and could also tolerate the exposure to the bile salts for four hours, demonstrating tolerance to the passage through the intestinal tract, emphasizing that there were no significant difference in the viable cells counts, demonstrating its potential as a probiotic microorganism.

DISCUSSION

In spontaneous fermentation, it is common to find several

Table 3. Antimicrobial activity of *Lactobacillus* bacteria isolated from pork sausages in Southern Brazil against foodborne pathogens.

Strains	*S.* Enteritidis SE86	*S.* Typhimurium ATCC 14028	*S. aureus ATCC 14458*	*E. coli O157:H7 ATCC 43888*	*B. cereus ATCC 33010*	*L. monocytogenes ATCC 6477*
L. plantarum BLS2	54.95^{aA}	51.20^{abABC}	48.45^{bA}	55.80^{aACE}	32.55^{cACD}	54.60^{aACE}
L. plantarum BLS3	53.00^{aA}	49.25^{Babc}	59.05^{cC}	53.00^{cC}	44.05^{dB}	58.75^{cAC}
L. plantarum BLS4	44.50^{aB}	47.4^{aABC}	39.00^{abBD}	45.15^{aBCD}	33.35^{bACD}	45.80^{aBDE}
L. plantarum BLS9	56.65^{aA}	43.70^{abAB}	49.15^{abA}	46.05^{abABCD}	36.30^{bACD}	55.95^{aAC}
L. plantarum BLS10	51.30^{aAB}	49.35^{aAB}	52.10^{aA}	50.10^{aABC}	42.50^{aBC}	42.75^{aBD}
L. plantarum BLS17	44.40^{aB}	42.25^{aABC}	51.75^{bA}	43.65^{aBCD}	34.00^{cABCD}	49.20^{bABE}
L. plantarum BLS19	43.05^{aB}	38.00^{bABC}	40.05^{abA}	48.40^{cABCD}	34.65^{bACD}	44.55^{acBDE}
L. plantarum BLS20	42.00^{aBC}	39.60^{abAB}	38.05^{bdBDE}	55.30^{cACE}	35.15^{dACD}	51.55^{cABCE}
L. plantarum BLS21	51.70^{aAB}	37.85^{bB}	37.05^{bdBDE}	52.90^{aABC}	35.80^{bACD}	48.60^{aBDE}
L. plantarum BLS23	48.30^{acAB}	44.95^{acB}	33.70^{bBDE}	43.65^{acBCD}	34.05^{bACD}	52.40^{adBDE}
L. plantarum BLS26	43.50^{aBC}	37.75^{bB}	43.45^{aAB}	39.15^{abBD}	34.85^{bcACD}	40.55^{abBD}
L. brevis BLS27	45.85^{abBC}	44.65^{abB}	41.85^{abAB}	56.40^{cBD}	37.10^{bACD}	55.60^{cBD}
L. plantarum BLS29	57.10^{acA}	57.55^{acAC}	60.80^{acC}	64.00^{aAE}	37.05^{bABCD}	53.20^{cACE}
L. plantarum BLS30	44.85^{aB}	41.00^{aAB}	37.70^{bDE}	47.90^{aABCD}	34.80^{abACD}	45.30^{aBDE}
L. plantarum BLS31	49.50^{aAB}	42.15^{abAB}	39.70^{bBD}	47.00^{abABCD}	30.50^{cACD}	45.40^{abBDE}
L. plantarum BLS32	51.50^{aAB}	48.15^{aABC}	35.10^{bBDE}	47.60^{aABCD}	38.70^{bABC}	49.55^{aABE}

^{aA}Different letters indicate significant difference (P < 0,05) between halos diameter of growth inhibition measured in mm on the agar surface.

Table 4. *Lactobacillus plantarum* BLS29 isolated from a pork sausage survival at different pH. simulating gastric conditions.

Strain	Time (h)	pH			
		2	2.5	3	4
Lactobacillus plantarum BLS29	0	8.23 ± 0.17^{aA}	8.48 ± 0.08^{aA}	8.23 ± 0.17^{aA}	8.23 ± 0.17^{aA}
	3	5.44 ± 0.13^{bB}	6.28 ± 0.25^{bB}	8.71 ± 0.13^{aA}	8.31 ± 0.0^{aA}

^{aA}Different letters in the same column or same line differ significantly (P < 0.05) by Tukey test between the different pH values.

Table 5. *L. plantarum* BLS29 strain tolerance nonacid-exposed and acid- exposed (BLS29ae) in simulated intestinal juice. NC (negative control), PC (intestinal juice containing pancreatin), PC+SB (intestinal juice containing pancreatin and bile salts).

Strain	Time (h)	Log_{10} (CFU/ml^{-1})		
		NC	PC	PC+SB
BLS29	0	8.78 ± 0.09^{a}	7.99 ± 0.14^{a}	8.21 ± 0.05^{b}
BLS29ae	0	8.03 ± 0.25^{b}	8.18 ± 0.07^{a}	8.07 ± 0.01^{b}
BLS29	4	7.92 ± 0.11^{b}	8.17 ± 0.10^{a}	8.5 ± 0.08^{a}
BLS29ae	4	8.02 ± 0.06^{b}	8.27 ± 0.11^{a}	8.49 ± 0.03^{a}

^{a}Different letters in the same column differ significantly (P < 0.05) by Tukey test.

species of lactobacilli like *L. sakei* and *L. curvatus* (Hugas et al., 1993), staphylococci, micrococci, and enterococci, however in the present study we reported the prevalence of *L. plantarum* and *E. faecium* in a pork sausage. *L. plantarum* are homofermentative and facultatively heterofermentative microorganisms widely distributed in nature (fermented vegetables, meats, dairy products and intestines), and possesses an expressive metabolic versatility, what is an important characteristic for potential probiotics intended to be used in foods. Depending on the glycolytic pathway, *L. plantarum* can produce different amounts of lactic acid, being that in the homo-fermentative route, more than 90% of substrate is converted in lactic acid under anaerobic conditions. On the other hand, the heterofermentative pathway produces, in addition of lactic acid, acetic acid and carbon dioxide, both undesirable by-products in sausage. Nevertheless, it was demonstrated that the salt concentrations (6-8% NaCl) found in dry sausages may alter the pattern of fermentation, leading to the production

of less acetic acid and more acid latic under aerobic conditions, showing the plasticity of using this kind of microorganism for meat fermentation (Bobillo and Marshall, 1991).

The flavor of sausages can be attributed to *Lactobacillus,* enterococci, and other LAB that have several metabolic pathways, such as proteolytic, lipolytic and esterolytic (Sarantinopoulos et al., 2001). As examples of this, the flavor both of the Mediterranean cheeses and the fermented sausages have been attributed to LAB (Hugas et al., 1993). *Enterococci* are generally associated with the fermentation of meat products, being detected in low numbers as $10^2 – 10^5$ CFU g^{-1} at the final products (Samelis et al., 1998; Franz et al., 2003). These bacteria are especially found in handmade products of Southern Europe, and can contaminate meat at the time of slaughter.

In our study, only bacteria belonging to the genus *Lactobacillus* were tested, since, in spite of being characterized as probiotics, enterococci may possess virulence factors that precludes its use as a food starter (Franz et al., 2003). Although some *Enterococci* strains have been characterized as functional microorganisms, some of these bacteria may have plasmids, including some that may be related to resistance factors to antibiotic and pathogenicity. Therefore, is not recommended to use this type of microorganism in food as starter (Franz et al., 2003). In the production of sausages, it is important that LAB be homofermentative to prevent the production of voids caused by the production of carbon dioxide, which would result in a displeasing appearance (Terra et al., 2004;Ordoñez Pereda, 2005). Although BLS29 to be facultatively homofermentative, the bacteria did not produced gas during tests, because it is isolated from sausage with sensory characteristics acceptable to the population. Heterolactic bacteria can produce acetic acid, which in high quantities can produce astringent taste, as is the case *L. brevis* BLS27.

Production of exopolysaccharides by the isolates investigated in this study were considered a positive characteristic for the production of sausages, because it can contribute to the organoleptic properties, such as flavor and texture of the final fermented food, since the exopolysaccharides can improve thickening and emulsifying properties of the product (Ballús et al., 2010). Furthermore, production of exopolysaccharides may contribute to the adhesion of probiotics to the intestine, contributing to the colonization of this surface (Sanders and Klaenhammer, 2001).

Although bacteriocins produced by LAB are generally inefficient to inhibit Gram-negative bacteria, our results demonstrate that *S*. Enteritidis and *S*. Typhimiurium were inhibited by the LAB isolated. Similar results were demonstrated by Hu et al. (2013), who described a bacteriocin called plantaricin 163, produced by *L. plantarum that* showed broad inhibitory activity against foodborne pathogens including *S.* Typhimurium*, E. coli,* and also *L. monocytogenes,* and *S. aureus.* The authors indicated that this bacterium has potential to be used as a biopreservative in the food industry. Gong et al. (2010) have reported the action of plantaricin MG against *L. monocytogenes* and *S.* Typhimurium. Lee et al. (2014) reported that *L. plantarum* HY7712 demonstrated good results against the impairment of NK-Cells activity caused by y-irradiation in mice. These studies showed the benefit action of *L. plantarum* in functional food, highlighting the possible use of this functional starter.

In order to provide beneficial effect to the hosts, microorganisms used as probiotics must reach and colonize the intestine, and for that they must survive the gastric acidic fluid and bile salts. These abilities were demonstrated by BLS29. It is remarkable that BLS29 demonstrated a population increase in the presence of pancreatin with or without bile salts and similar results were obtained by Pennacchia et al. (2004) studying *Lactobacillus* strains from fermented sausages, in Italy.

Some factors may negatively impact on the development of functional cultures in meat environment, as the concentration of curing salts, low pH and water activity (De Vuyst et al., 2008), so it is appropriate to use strains adapted to these conditions (Hugas et al., 1993; Pennacchia et al., 2006). Therefore, the BLS29 strain was chosen for follow-up study, since the strain survived well to this kind of conditions, i.e. growing at temperatures prevailing during maturation of fermented meat products and developed well in the presence of curing salts.

Besides the Good Manufacturing Practices (GMP) that should be adopted in the production of meat products in order to prevent contamination, a quick acidification carried out by LAB and the production of bacteriocins may contributes to the inhibition of food pathogens such as *Salmonella*, *E. coli, S. aureus* and *L. monocytogenes.*

The flavor of fermented foods depends on several factors, mainly by the quantity and quality of the source, amount and type of ingredients (meat, salt, and spices), temperature, processing time, smoking, and type of starter culture used (Leroy et al., 2006). The involvement of proteolytic enzymes also have their contribution to the liberation of peptides that together with free fatty acids, resulting in the action of lipases, lead to volatile compounds, giving the characteristic odor and flavor characterized of each fermented sausage (Leroy et al., 2006). These enzymes may originate in parts by the raw material itself, and most often by the starter culture fermentation. With the exception of the post- mortem glycolysis, the catabolism of carbohydrates is carried mostly by LAB which produces compounds such as lactic acid, acetic acid, ethanol, acetoin, and other complex compounds which contribute for the flavor of sausages (Viallon et al., 1996; Leroy et al., 2006). The fast acid production observed in BLS29 is an important factor that contributes not only to the development of pleasant sensory characteristics, but also to the microbiological

safety of the product.

In conclusion, this study demonstrated that BLS29 *L. plantarum* presents probiotic potential, and can be used in the production of pork sausages. However, in order to confirm its functional and sensory properties more studies are necessary, mainly those that apply this microorganism in the production of pork meat sausages.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Arihara K (2006). Strategies for designing novel functional meat products. Meat Sci. 74: 219-229. http://dx.doi.org/10.1016/j.meatsci.2006.04.028

Ayad EHE, Verheul A, Wouters JTM, Smit G (2000). Application of wild starter cultures for flavour development in pilot plant cheese making. Int. Dairy J. 10: 169-179. http://dx.doi.org/10.1016/S0958-6946(00)00041-8

Ballús CA, Klajn VM, Cunha MF, Oliveira ML, Fiorentini AM (2010). Aspectos científicos e tecnológicos do emprego de culturas probióticas na elaboração de produtos lácteos fermentados: revisão. B. CEPPA 28: 85-96. http://dx.doi.org/10.5380/cep.v28i1.17900

Bobillo M, Marshall VM (1991) Effect of salt and culture aeration on lactate and acetate production by Lactobacillus plantarum. Food Microbiol. 8:153-160. http://dx.doi.org/10.1016/0740-0020(91)90008-P

Brazil (2000). Ministério da Agricultura, Pecuária e do Abastecimento . Secretaria de Defesa Agropecuária. Instrução Normativa no. 22 de 31 de julho de 2000. Aprova o Regulamentos Técnicos de Identidade de Qualidade de Salames. Diário Oficial da União, Brasília, p. 15-28, 03 de agosto de 2000.

Dalla Santa OR (2008). Avaliação da qualidade de salames artesanais e seleção de culturas starter para a produção de salame tipo italiano. Thesis, Federal University Paraná).

De Almeida Júnior WLG, Ferrari IS, de Souza JV, Barbosa AL, da Costa MM, Menezes DF, Dias FS (2015). Principal criteria for selection of lactic acid bacteria for potential use as probiotic in foods. Afr. J. Microbiol. Res. 9: 671-686.

De Vuyst L (2000). Technology aspects related to the application of functional starter cultures. Food Technol. Biotechnol. 38:105-112.

De Vuyst L, Falony G, Leroy F (2008). Probiotics in fermented sausages. Meat Sci. 80: 75-78. http://dx.doi.org/10.1016/j.meatsci.2008.05.038

El-Ghaish S, Ahmadova A, HadjiSfaxi I, Mecherfi KEE, Bazukyan I, Choiset Y, Rabesona H, Sitohy M, Popov YG, Kuliev AA, Mozzi F, Chobert J-M, Haertl T (2011). Potential use of lactic acid bacteria for reduction of allergenicity and for longer conservation of fermented foods. Trends Food Sci. Technol. 22: 509-516. http://dx.doi.org/10.1016/j.tifs.2011.05.003

Erkkilä S, Suihko ML, Eerola S, Pestäjä E, Mattila-Shandholm T (2001). Dry sausage fermented by Lactobacillus rhamnosus strains. Int. J. Food Microbiol. 64: 205-210. http://dx.doi.org/10.1016/S0168-1605(00)00457-8

Fiorentini AM, Sawitzki MC, Bertol TM, Cunha Júnior A, Sant'Anna ES (2010). Influence of a native strain of Staphylococcus xylosus on the microbiological, physicochemical and sensorial characteristics on milano salami type. Braz. Arch. Biol. Technol. 53: 961-974. http://dx.doi.org/10.1590/S1516-89132010000400027

Food and Agriculture Organization of United Nations; World Health Organization – FAO/WHO (2001). Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation, Córdoba, Argentina. ftp://ftp.fao.org/es/esn/food/probioreport_en.pdf. Accessed 12 April 2014.

Franz CMAP, Stiles ME, Schleifer KL, Holzapfel WH (2003). Enterococci in foods – a conundrum for food safety. Int. J. Food Microbiol. 88: 105-122. http://dx.doi.org/10.1016/S0168-1605(03)00174-0

Gong H-S, Meng X-C, Wang H (2010). Mode of action of plantaricin MG, a bacteriocin active against Salmonella typhimurium. J. Basic Microbiol. 50:S37-S45. http://dx.doi.org/10.1002/jobm.201000130

Guetouache M, Guessas B (2015). Characterization and identification of lactic acid bacteria isolated from traditional cheese (Klila) prepared from cow's milk. Afr. J. Microbiol. Res. 9: 71-77.

Gurtler V, Stanisich VA (1996). New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology 142: 3-16. http://dx.doi.org/10.1099/13500872-142-1-3

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

Hammes WP, Hertel C (1998). New developments in meat starter cultures. Meat Sci. 49:125-138. http://dx.doi.org/10.1016/S0309-1740(98)90043-2

Hu M, Zhao H, Zhang C, Yu J, Lu Z (2013). Purification and characterization of plantaricin 163, a novel bacteriocin produced by Lactobacillus plantarum 163 isolated from traditional chinese fermented vegetables. J. Agric. Food Chem. 61: 11676-11682. http://dx.doi.org/10.1021/jf403370y

Hugas M, Garriga M, Aimerich T, Monfort JM (1993). Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18: 107-113. http://dx.doi.org/10.1016/0168-1605(93)90215-3

Incze K (1998). Dry fermented sausage. Meat Sci. 49: 169-177. http://dx.doi.org/10.1016/S0309-1740(98)90046-8

Lane DJ (1991). 16S/23S rRNA sequencing. In: E Stackebrandt, MN Goodfellow (Eds.). Nucleic Acid Techniques in Bacterial Systematics, Wiley, Chichester. pp. 115-147.

Lee H, Ahn YT, Park SH, Park DY, Jin YW, Kim SC, Sung SH, Huh C-S, Kim DH (2014). Lactobacillus plantarum HY7712 protects against the impairment of NK-cell activity caused by whole-body γ-irradiation in mice. J. Microbiol. Biothecnol. 24: 127-131. http://dx.doi.org/10.4014/jmb.1307.07001

Leroy F, Verluyten J, De Vuyst L (2006). Functional meat starter cultures for improved sausage fermentation. Int. J. Food Microbiol. 106: 270-285. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.06.027

Macedo REF, Pflanzer OS, Gomes CL (2012). Probiotic Meat Products. In: Rigobelo IC, (Org.) Probiotic in animals. http://www.intechopen.com/books/probiotic-in-animals http://dx.doi.org/10.5772/50057

Maldonado A, Ruiz-Barba JL, Floriano B, Jime'nez-Dı'az R (2002). The locus responsible for production of plantaricin S, a class IIb bacteriocin produced by Lactobacillus plantarum LPCO10, is widely distributed among wild-type Lact. plantarum strains isolated from olive fermentations. Int. J. Food Microbiol. 77: 117-124. http://dx.doi.org/10.1016/S0168-1605(02)00049-1

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. Int. Dairy J. 16: 189-199. http://dx.doi.org/10.1016/j.idairyj.2005.02.009

Martins AKS, Martins FS, Gomes DA, Elian DAS, Vieira AT, Teixeira MM, Cara DC, Nardi RMD, Nicoli JR (2010). Evaluation of in vitro antagonism and of in vivo immune modulation and protection against

pathogenic experimental challenge of two probiotic strains of Bifidobacterium animalis var. lactis. Arch. Microbiol. 192: 995-1003. http://dx.doi.org/10.1007/s00203-010-0626-0

Meira SMM, Helfer VE, Velho RV, Lopes FC, Brandelli A, (2012). Probiotic potential of Lactobacillus spp. isolated from Brazilian regional ovine cheese. J. Dairy Res. 79: 119-127. http://dx.doi.org/10.1017/S0022029911000884

Metchnikoff E (1907). Lactic acid as inhibiting intestinal putrefaction. In: The prolongation of life: Optimistic studies. W. Heinemann, London. pp.161-183.

Muthukurasami P, Holley RA (2006). Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated Lactobacillus reuteri. Int. J. Food Microbiol 111: 164-169.

http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.036

Ordo-ez Pereda JA (2005). Tecnologia de Alimentos – Alimentos de origem animal. Vol. 2. Tradução: Murad F. Artmed, Porto Alegre.

Pennacchia C, Ercolini D, Blaiotta G, Pepe O, Mauriello G, Villani F (2004). Selection of Lactobacillus strains from fermented sausages for their potential use as probiotics. Meat Sci. 67: 309-317. http://dx.doi.org/10.1016/j.meatsci.2003.11.003

Pennacchia C, Vaughan EE, Villani F (2006). Potential probiotic Lactobacillus strains from fermented sausages: Further investigations on their probiotic properties. Meat Sci. 73:90-101. http://dx.doi.org/10.1016/j.meatsci.2005.10.019

Raigorodsky B (2011). Embutidos: da sobrevivência à gastronomia. Senac, São Paulo.

Samelis J, Metaxopoulos J, Vlassi M, Pappa A (1998). Stability and safety of traditional Greek salami – a microbiological ecology study. Int. J. Food Microbiol. 44:69-82. http://dx.doi.org/10.1016/S0168-1605(98)00124-X

Sanders ME, Klaenhammer TR (2001). The scientific basis of Lactobacillus acidophilus NCFM functionality as a probiotic. J. Dairy Sci. 84:319-331. http://dx.doi.org/10.3168/jds.S0022-0302(01)74481-5

Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G, Tsakalidou E (2001). Biochemical properties of enterococci relevant to their technological performance. Int. Dairy J. 11:621-647. http://dx.doi.org/10.1016/S0958-6946(01)00087-5

Soccol CR, Vandenberghe LP De S, Spier MR, Medeiros ABP, Yamaguishi CT, Lindner JDD, Pandey A, Thomaz-Soccol V (2010). The potential of probiotics: a Review. Food Technol. Biotechnol. 48:413-434.

Terra, N, Terra, AB de M, Terra L de M (2004). Defeitos nos produtos cárneos: origens e soluções. Varela, São Paulo.

Tondo EC, Ritter AC (2012). Salmonella and Salmonellosis in Southern Brazil: A review of the last decade. In: Monte AS, De Santos PE (eds). Salmonella: Classification, Genetics and Disease Outbreaks; New York, NY, USA. pp. 175-191.

Viallon C, Berdague´ JL, Montel MC, Talon R, Martin JF, Kondjoyan N, Denoyer C (1996). The effect of stage of ripening and packaging on volatile content and flavour of dry sausage. Food Res. Int. 29:667-674. http://dx.doi.org/10.1016/S0963-9969(96)00061-0

Yoon HS, Ju JH, Lee JE, Park HJ, Lee JM, Shin HK, Holzapfel W, Park KY, Do MS (2013). The probiotic Lactobacillus rhamnosus BFE5264 and Lactobacillus plantarum NR74 promote cholesterol efflux and suppress inflammation in THP-1 cells. J. Sci. Food Agric. 93:781-787. http://dx.doi.org/10.1002/jsfa.5797