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Safety of a low-fat fermented sausage produced with *Enterococcus faecium* CRL 183 and *Lactobacillus acidophilus* CRL1014 probiotic strains

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Abstract

This work is aimed at studying the effect of using lactic acid bacteria with lipid-lowering properties (*Enterococcus faecium* CRL183 and *Lactobacillus acidophilus* CRL1014) on the physicochemical characteristics and safety of fermented sausages with fat and curing salt reduction. The probiotic cultures were submitted to preliminary tests for evaluating their resistance to curing salts and their capacity to produce antimicrobial substances. The sausage quality was evaluated by analyses at the beginning of the processing and during the ripening and storage periods. The *E. faecium* CRL 183 exhibited no log reduction in the population of viable cells (10^9 CFU mL) while the *L. acidophilus* CRL 1014 showed a reduction in comparison with the control treatment (10^8 CFU/mL). All formulations presented appropriate physicochemical and microbiological characteristics to the standards established. The formulations F2-F7 (with fat reduction) showed an increase in oleic acid and decrease in saturated acid content, compared to control sausage. The microbiological safety and technological properties it was not compromise.

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Introduction

Currently, the food industry is faced with a dilemma: the search for safety foods that are minimally processed and free of food additives at the same time. This market trend has encouraged the use of alternative forms of food preservation. Fermentation has been used as a preservation technique for millennia, and is considered an effective way to increase the shelf life of food and beverages by a combined action of antimicrobial metabolites produced during the process (Ross *et al*., 2002).

Microorganisms able to confer health benefits to humans, known as probiotics, are traditionally added to dairy products, including yogurt, fermented milk and dairy desserts. Total or partial replacement of traditional starter cultures for probiotic can contribute to food product safety and offer sensory, technological and nutritional advantages, representing an attractive alternative for the food industry (Pidcock *et al*., 2002; Muthukumarasamy and Holley, 2007).

In the meat industry, the use of probiotics is more promising in fermented products such as sausage, which are usually processed and consumed without heating (Työppönen *et al*., 2003; Ammor and Mayo 2007). Therefore, selecting an appropriate probiotic is crucial to get the desired beneficial effects. Considering the high prevalence of cardiovascular disease and the importance of lipid parameters in the etiology of this pathology, many researchers have been devoted to identifying strains with lipid-lowering potential. Rossi *et al.* (1994) studied 18 bacterial strains regarding their *in vitro* cholesterol removal ability, and the best results were obtained with *Lactobacillus acidophilus* CRL1014 and *Enterococcus faecium* CRL183. Other studies indicate that the strain of *Enterococcus faecium* CRL 183 is able to modulate the intestinal microbiota (Bedani, 2008; Cavallini *et al*., 2011) and reduce the risk of developing colon (Sivieri *et al.*, 2008) and breast cancer (Kinouchi, 2006), and intensity of inflammatory bowel disease (Celiberto, 2014). Nowadays, such strains and their beneficial properties were evaluated only in soy-based products (Rossi *et al*., 2000; Rossi *et al*., 2003; Rossi *et al*., 2008; Cavallini *et al*., 2009). The aim of the present work was to produce a fermented sausage with fat and nitrite reduction using two probiotic bacteria (*Enterococcus faecium* CRL 183 and *Lactobacillus acidophilus* CRL1014) with lipid-lowering properties in order to

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enhance the safety and nutritional properties of the product.

Materials and Methods

Bacterial strains

The traditional cultures (*Pediococcus pentosaceus* and *Staphylococcus xylosus*) were purchased from Christian Hansen, Brazil. The *Enterococcus faecium* CRL183 and *Lactobacillus acidophilus* CRL1014 probiotic cultures were obtained from the Centro de Referencia para Lactobacilos – CERELA (San Miguel de Tucumán, Argentina). The pathogenic cultures (*Listeria monocytogenes* IAL 628, *Salmonella enterica* subsp. *enterica* ser. *typhimurium* IAL 2431 and. *Escherichia coli* IAL 339) used as indicators were obtained from the Adolfo Lutz Institute – Sao Paulo, Brazil.

Sensitivity of probiotic cultures to sodium chloride (NaCl) and sodium nitrite ($NaNO_2$)

The probiotic cultures' resistance to sodium chloride (NaCl) (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) and sodium nitrite ($NaNO_2$) (80, 100, 120, 150 and 200 ppm) added to an MRS agar medium *(L. acidophilus)* or M17 agar *(E. faecium)* were assessed. After the reactivation, 1 mL probiotics culture (7 log CFU/mL) was seeded in a specific medium that was supplemented either with different concentrations of curing salts or not (control), and then incubated at 37°C/48 hours. After incubation the colonies were enumerated and the results were expressed as colony-forming units per milliliter (CFU/mL) and compared with those of the control treatment (Arihara and Itoh, 2000).

Bacteriocin assay

The production of bacteriocins by probiotic cultures (*E.faecium* CRL 183 and *L.acidophilus* CRL1014) was studied by the spot-on-lawn technique, using *Listeria monocytogenes* IAL 628, *Salmonella enterica* subsp. *enterica* ser. *typhimurium* IAL 2431 and *Escherichia coli* IAL 339 as indicating microorganisms (Lewus *et al*., 1991). The production of bacteriocins was indicated by the presence of inhibition halo around the colony.

Fermented sausage formulation and processing

Three batches of fermented sausages were processed on different days. The fermented sausages were obtained in seven formulations (10 kg for each formulation in each batch), according to the Italian salami manufacturing procedure, and as proposed by Severini *et al.* (2003), Koutsopoulos *et al.* (2008) and Macedo *et al.* (2008) with a few modifications.

The formulation 1 (F1) was processed with traditional cultures (*Pediococcus pentosaceus* and *Staphylococcus xylosus*) and without fat (20% of pork fat) and curing salt reduction (0.015% nitrite and 0.005% nitrate). The formulation 2 (F2) with traditional cultures and without curing salt reduction. The formulation 3 (F3) with traditional cultures and with curing salt reduction (0.007% nitrite and 0.003% nitrate). The formulations 4 and 5 (F4 and F5) with probiotic culture (*E. faecium* CRL183) and without and with curing salt reduction, respectively. The formulations 6 and 7 (F6 and F7) with probiotic culture (*L. acidophilus* CRL1014) and without and with curing salt reduction, respectively. F2 to F7 was used 8% of pork fat and 2% of extra virgin olive oil.

The basic low-fat sausage mixture was prepared with the following ingredients and additives: 61.5% pork meat, 28.5% cow meat, 8.0% pork fat, 2.0% olive oil, 2.5% sodium chloride, 0.5% sodium ascorbate, 0.5% sucrose, 0.7% lactose, 0.05% garlic powder, and 0.13% white pepper. The probiotic and commercial starter cultures were added in a sufficient amount to reach at least 8 log CFU/g. The probiotic cultures were propagated in M17 broth (Himedia, India) for 24 hours at 37°C (*Enterococcus faecium* CRL183) or an MRS medium (Man Rogosa Sharpe, Accumedia, USA for 72h at 37°C (*Lactobacillus acidophilus* CRL1014).

The meat material was ground in a 5 to 8 mm thick stainless disc, and then mixed with the other ingredients in a meat mixer for 5 minutes. Frozen pork fat was manually cut and added to the mixture. Afterwards, the starter probiotic and traditional cultures were incorporated, and the meat mixture was embedded in cellulose sausage casings that were 50 mm in diameter. Pieces of about 15cm in length were maintained in fermentation (7 days) and ripening (23 days), totaling 30 days in chamber (Table 1) (Koutsopoulos *et al.*, 2008; Macedo *et al.*, 2008). After the fermentation and ripening period, the sausages were vacuum-packed and stored under refrigeration (4°C) for a period of 90 days, totaling 120 days of study.

Physicochemical analyses

All Physicochemical analyses were carried out in triplicate for each batch at different sampling times.

pH and water activity measurement

pH was measured in homogenate prepared by blending 20 g of sausage with 80 ml of distilled water for 30 s. Readings were taken with a pHmetro Qualxtron (model 8010) that was calibrated by

Table 1. The conditions of each formulation and of fermentation and ripening

Time (Days)	Temperature (ºC)	Relative humidity (%)
Fermentation		
1	25	89
2	24	89
3	23	88
5	22	88
6	21	87
7	20	86
Ripening	18	80
8-30		
Storage	15	75
31-120		
	4	-

Source: Koutsopoulos, Koutsimanis and Bloukas (2008); Macedo, Pflanzer Jr, Terra, and Freitas (2008)

using buffers 7.0 and 4.0. (Liaros *et al.*, 2009). The water activity was determined in previously crushed samples using an AquaLab Water Activity Meter (model CX-2).

Lipid oxidation determination

The 2-thiobarbituric acid (TBA) test was used to determine the oxidative rancidity extent (Koutsopoulos *et al.*, 2008). Readings were obtained on an Ultrospec™ 1100 pro (Amersham Biosciences Limited) at 532 nm.

Fatty acid profile determination

The fatty acids (FA) quantification was preceded by the extraction of the lipid fraction (Folch, 1957), and the fatty acid methyl esters (FAMEs) were obtained according to Hartman and Lago (1973) adapted to microscale (Menezes *et al.*, 2013). Analyses of FAMEs were carried out on a Varian GC gas chromatograph (model 430 GC, Varian Chromatograph Systems, Walnut Creek, CA, USA), equipped with a CP 8412 auto injector. The Galaxie software was used for the quantification and identification of peaks. The injections were performed on a 100-m fused silica capillary column (ID = 0.25 mm), coated with 0.2 micrometers of polyethylene glycol (SP-2560, Supelco, USA), using helium as the carrier gas at an isobaric pressure of 37 psi, linear velocity of 20 cm/s, make-up gas: helium at 29 mL/min, split ratio of 1:50, volume injected: 1.0 µL. The injector temperature was set at 250°C and the detector temperature at 280°C. The oven temperature was initially held at 140°C for 5 min, set to increase to 240°C at a rate of 4°C/min, and then held isothermally for 30 min. The qualitative FA composition of the samples was determined by comparing the retention times of the peaks produced after injecting the methylated samples with those of the respective standards of FA. The quantitative composition was obtained by area normalization and expressed as mass percentage, according to the AOCS Official Method Ce 1-62. All samples were analyzed in duplicate and the reported values are the average of the two runs (Silva *et al.*, 2011).

Microbiological analysis

Microbiological analyses were performed at baseline, at the end of fermentation and ripening steps, and at intervals of 30 days during the storage time, totalizing 120 days. For each period of analysis, 25 g of sausage (without casing) was removed under aseptic conditions and homogenized for 30min with 225 mL peptone water using a Stomacher (Nova Etica, Ethik Techonlogy, Brazil). The homogenate was serially diluted and used to determine microbiological safety and viable cell population.

Microbiological safety

The microbiological quality of fermented sausages was evaluated by studying: *Staphylococcus aureus* - enumeration on Baird Parker agar (Accumedia, USA), at 37°C/48h and confirmation by the coagulase test (Silva *et al.*, 2001); total coliforms and *E.coli* (CFU/g) - cultured on Petrifilm™ *E.coli*/Coliform count plate method at 37ºC/48h 48h (Silva *et al.*, 2001); *Salmonella* spp. (CFU/g) - pre-enrichment in buffered peptone water (BPW); selective enrichment in Tetrathionate broth (TT - Accumedia, USA) and Rappaport Vassiliadis broth (RV - Accumedia, USA); detection in Hektoen enteric agar (HE - Accumedia, USA) and agar Xylose Lysine Desoxycholate (XLD - Accumedia, USA) (Downes and Ito 2002). *Listeria monocytogenes* and *Clostridium botulinum* were analyzed at the Science Center and Quality Food (SCQF) in the Food Technology Institute, Campinas - SP. Three replicates were carried out for each microorganism group/specie.

Viable cell counts

The population of microorganisms of the genus *Enterococcus* spp. was performed by plating on a KF *Streptococcus* agar selective medium (37ºC/48h,

Table 2. Viable cell population of *Enterococcus faecium* CRL183 and *Lactobacillus acidophilus* CRL1014 exposed to different concentrations of NaCl and $NaNO_2$ (Mean values ± standard error)

Curing salt concentration		Viable cells (log CFU/mL) *Enterococcus faecium* CRL 183	Viable cells (log CFU/mL) *Lactobacillus acidophilus* CRL 1014
NaCl (%)	0	9.44 ± 0.08^{b}	9.41 ± 0.07^{a}
	1	9.77 ± 0.02^{a}	8.11 ± 0.01^{b}
	1.5	9.73 ± 0.02^{a}	8.11 ± 0.04^{b}
	2	9.66 ± 0.03^{a}	8.15 ± 0.03^{b}
	2.5	9.52 ± 0.05^{b}	8.10 ± 0.09^{b}
	3	9.39 ± 0.07^{b}	8.14 ± 0.06^{b}
$NaNO_2$ (ppm)	0	$9.41 \pm 0.05^{b,c}$	9.43 ± 0.03^{a}
	80	9.61 ± 0.07^{a}	$8.92 \pm 0.01^{b,c}$
	100	$9.53 \pm 0.03^{a,b}$	8.93 ± 0.02^{b}
	120	$9.43 \pm 0.03^{b,c}$	$8.89 \pm 0.01^{b,c,d}$
	150	$9.41 \pm 0.10^{b,c}$	8.96 ± 0.01^{b}
	200	9.34 ± 0.06^{c}	8.85 ± 0.01^{d}

Means with the same letter in the same column do not differ from the Tukey test (P<0.05).

Himedia, India) with confirmation in agar bile esculin azide (Accumedia, USA) for all colonies with different morphologies. *Lactobacillus* spp. was enumerated using MRS agar (37ºC/72h, anaerobic, Accumedia, USA).

Statistical analysis

The means were obtained for carrying out 3 replicates and the experiment was conducted in 3 batches. The quantitative results were reported as mean ± SE. The results were tested by the one-way analysis of variance (ANOVA), and individual means were compared by Tukey's post test (P<0.05). The concentration of curing salt was analyzed as an independent variable and the starter culture was considered as a dependent variable of the salt concentration. All analyses were carried out using the statistical software Bioestat 5.0.

Results and Discussion

Resistance to NaCl and curing salts

The use of starter cultures that are resistant to curing salts is fundamental to obtaining fermented probiotics sausages, since the addition of NaCl and nitrite are necessary to ensure the microbiological safety of the product (Papamanoli *et al.*, 2003). The sensitivity to curing salts analysis revealed that *Enterococcus faecium* CRL183 kept the population of viable cells at a range of 9 log CFU/mL for all evaluated NaCl and nitrite concentrations with no difference from the control treatment (P<0.05) (Table 2). The total *Lactobacillus acidophilus* CRL1014 viable cells population was reduced in the presence of different NaCl and nitrite concentrations, differing from the control treatment (P<0.05). The reduction in the population of *Lactobacillus acidophilus* CRL 1014 was similar for all NaCl concentrations (- 1.3 log CFU/g), while the greatest curing salt reduction was observed with the addition of sodium nitrite 200 ppm (- 0.5 log CFU/g).

The ability to resist to the curing salts is a strain-specific effect. The work conducted by Sameshima *et al*. (1998) concluded that among the 202 *Lactobacillus* species evaluated, *L. paracasei* ssp. paracasei, *L. rhamnosus* and *L. acidophilus* were resistant to curing salts. Macedo (2005) evaluated the *Lactobacillus casei* (LC 01), *Lactobacillus paracasei* ssp. *paracasei* (ATCC 10746/CCT 0566) and *Lactobacillus casei* ssp. *rhamnosus* (ATCC 7469/6645 CCT) strains resistance to curing salts. The results showed that all strains were resistant to the tested concentrations of NaCl (1% to 3%) and nitrite (80 ppm to 200 ppm), with no reduction in microbial population in comparison with the control treatment. Despite the reduction observed in its viability, the probiotic bacteria evaluated in the present study can be used as starter cultures in fermented meat products because the viable cell population exceeds the minimum recommended requisite for beneficial effects attributed to probiotics (8 log CFU/ ingested daily portion) (Hill *et al*., 2014).

Bacteriocin production

Probiotic cultures (*Enterococcus faecium* CRL183 and *Lactobacillus acidophilus* CRL1014) did not produce antimicrobial substances, specifically against the microorganisms strains used as indicators (*Listeria monocytogenes* IAL 628, *Salmonella enterica* ssp. *enterica* serovar *typhimurium* IAL 2431, and *Escherichia coli* IAL 339) because it was not detected an inhibition halo in the spot-on-lawn test. As the initial test was negative for the production of antimicrobial substances, it was not necessary

Table 3. Mean values (± standard error) for water activity, pH and TBARS (mg/kg) during the study period

Time/Attributes	Formulations						
	F1	F2	F3	F4	F5	F6	F7
T0							
pH	5.96 ± 0.36^{aA}	5.99 ± 0.05^{aA}	6.11 ± 0.07^{aA}	6.02 ± 0.19^{aA}	6.08 ± 0.13^{aA}	5.59 ± 0.21^{aA}	5.87 ± 0.08^{aA}
Wa	0.984 ± 0.010^{aA}	1.000 ± 0.015^{aA}	1.000 ± 0.013^{aA}	$0.998^{aA}\pm0.001^{aA}$	0.981 ± 0.022^{aA}	0.997 ± 0.002^{aA}	0.981 ± 0.016^{aA}
TBARS	2.30 ± 0.27^{aF}	1.12 ± 0.10^{cD}	1.42 ± 0.37^{bcD}	1.48 ± 0.18^{bcD}	1.55 ± 0.27^{bD}	1.24 ± 0.17^{bcE}	1.24 ± 0.18^{bcD}
T7							
pH	5.11 ± 0.20^{aB}	4.97 ± 0.08^{aBC}	5.15 ± 0.10^{aBC}	5.03 ± 0.09^{aB}	$5.15^{aCD}\pm0.12$	4.99 ± 0.18^{aB}	5.14 ± 0.05^{aBC}
Wa	0.978 ± 0.012^{aA}	0.996 ± 0.007^{aA}	0.998 ± 0.014^{aA}	0.971 ± 0.001^{aA}	$0.993^{aA}\pm0.006$	0.992 ± 0.002^{aA}	0.982 ± 0.003^{aAB}
TBARS	3.61 ± 0.24^{aE}	2.35 ± 0.42^{bcC}	2.41 ± 0.26^{bcC}	2.40 ± 0.19^{bcC}	$2.09^{cC}\pm0.08$	2.63 ± 0.20^{bD}	2.35 ± 0.05^{bcC}
T30							
pH	5.21 ± 0.28^{aB}	5.19 ± 0.22^{aB}	5.26 ± 0.14^{aB}	4.98 ± 0.16^{aB}	$5.25^{aBCD}\pm0.07$	5.11 ± 0.03^{aB}	5.04 ± 0.20^{aC}
Wa	0.916 ± 0.026^{aAB}	0.921 ± 0.008^{aB}	0.915 ± 0.017^{aB}	0.912 ± 0.027^{aB}	$0.910^{aB}\pm0.023$	0.893 ± 0.038^{aB}	0.916 ± 0.053^{aBC}
TBARS	5.27 ± 0.22^{aD}	4.85 ± 0.10^{abB}	4.42 ± 0.18^{bcB}	4.44 ± 0.27^{bcB}	$4.16^{cB}\pm0.28$	4.22 ± 0.36^{cC}	4.19 ± 0.17^{cB}
T60							
pH	5.14 ± 0.23^{bB}	5.03 ± 0.11^{bB}	5.05 ± 0.15^{bC}	4.88 ± 0.16^{bB}	$5.53^{aB}\pm0.33$	5.12 ± 0.01^{bB}	5.02 ± 0.14^{bC}
Wa	0.852 ± 0.042^{aBC}	0.863 ± 0.012^{aC}	0.870 ± 0.050^{aBC}	0.908 ± 0.009^{aB}	$0.844^{aC}\pm0.032$	0.843 ± 0.028^{aBC}	0.851 ± 0.062^{aC}
TBARS	6.27 ± 0.20^{aC}	5.67 ± 0.25^{bA}	5.41 ± 0.21^{bcA}	5.49 ± 0.38^{bcA}	$5.15^{cA}\pm0.19$	5.51 ± 0.32^{bcA}	5.49 ± 0.23^{bcA}
T90							
pH	5.13 ± 0.30^{bB}	5.09 ± 0.05^{bB}	5.18 ± 0.04^{bBC}	4.98 ± 0.01^{aA}	$4.98^{bB}\pm0.14$	5.02 ± 0.10^{bB}	5.12 ± 0.05^{bBC}
Wa	0.820 ± 0.054^{aC}	0.832 ± 0.046^{aC}	0.835 ± 0.001^{aC}	0.869 ± 0.005^{aB}	$0.851^{aCD}\pm0.016$	0.810 ± 0.011^{aCD}	0.859 ± 0.002^{aCD}
TBARS	8.53 ± 0.26^{aB}	5.03 ± 0.31^{cdB}	5.37 ± 0.26^{bcA}	5.52 ± 0.24^{bcA}	$4.78^{dA}\pm0.48$	5.58 ± 0.37^{bcA}	5.69 ± 0.25^{bA}
T120							
pH	5.46 ± 0.56^{aAB}	4.47 ± 0.71^{bC}	5.17 ± 0.11^{abBC}	5.02 ± 0.18^{abB}	5.39 ± 0.27^{aBC}	4.97 ± 0.18^{abB}	5.48 ± 0.43^{aB}
Wa	0.810 ± 0.079^{aC}	0.828 ± 0.067^{aC}	0.769 ± 0.051^{aD}	0.828 ± 0.042^{aC}	0.821 ± 0.066^{aC}	0.753 ± 0.072^{aD}	0.820 ± 0.052^{aD}
TBARS	9.44 ± 0.30^{aA}	4.61 ± 0.24^{bB}	4.65 ± 0.28^{bB}	4.39 ± 0.33^{bB}	3.77 ± 0.33^{cB}	4.83 ± 0.08^{bB}	4.45 ± 0.38^{bB}

F1 - traditional cultures without curing fat and curing salt reduction (0.015% nitrite and 0.005% nitrate); F2 - traditional cultures without curing salt reduction; F3 – traditional cultures with curing salt reduction (0.007% nitrite and 0.003% nitrate); F4 and F5 - probiotic culture (*E. faecium* CRL183) without and with curing salt reduction, respectively; F6 and F7 - probiotic culture (*L. acidophilus* CRL1014) without and with curing salt reduction, respectively. T0 = initial time; T7 = end of the fermentation period; T30 = end of the ripening period; T60, T90 and T120 = storage time at 4° C. Analysis of treatments: means with the same lowercase letters in the same line, at the same time interval, do not differ from the Tukey test ($P<0.05$). Analysis of time: means with capital letters for the same formulation at different times, do not differ from the Tukey test ($P<0.05$).

the continuation of the bacteriocins production confirmation analysis (sensitivity to protease and bacteriophages lytic action).

The conditions used in this test (medium free of sucrose or other fermentable sugar - TSAYE - and incubating anaerobically) enabled the exclusion of inhibiting pathogens due to the presence of organic acids and hydrogen peroxide (Lewus *et al*., 1991). Thus, the probiotic cultures could exert the desired protective effect by the production of organic acids and/or hydrogen peroxide, or even through the production of bacteriocins against other genera/species of microorganisms which were not evaluated in this study.

Physicochemical analyses

pH and Water activity measurements

Table 3 presents the results of pH and water activity during the different stages of the present study. The sausage's final pH can vary from 5.5 to 4.6, depending on the acidification rate of the starter cultures and the presence of yeast, as Derbaryomyces hansenii, which is capable to raise the product's pH (Ammor and Mayo 2007).

In this study, the pH of sausages ready for consumption (T30) ranged from 4.98 to 5.26, however, no statistical difference between the formulations was observed at the end of the fermentation (T7) and ripening (T30) periods, indicating that the use of probiotics did not affect the fermentation process. When comparing the pH values at the end of storage time (T120) with those at the end of the fermentation period (T7), formulations F2 and F7 had the lowest and highest means, respectively. An eventual pH increase can be attributed to basic compounds derived from protein degradation, buffering substances and the decrease of electrolyte (Fernandez *et al*., 1997; Fonseca, 1999).

Water activity represents the amount of available water for chemical and enzymatic reactions, development of microorganisms and toxin production (Jay, 1994). In this way, the reduction of water activity inhibits the proliferation of spoilage and pathogenic microorganisms (Siqueira, 1995). According to the Brazilian legislation (MAPA, 2000), sausages ready for consumption should present a maximum water activity of 0.92. By the end of the ripening period, all formulations with the exception of F2 and F7 exhibited water activity values lower than 0.92, being in accordance with current legislation. The decrease in this parameter remained during the storage period, and after 30 days of storage at refrigeration temperature (T60), all formulations

Table 4. Mean values (± standard error) obtained in the fatty acid profile (g/100g) for sausages obtained by different treatments and processing steps

Fatty Acid	Treatments						
	F1	F2	F3	F4	F5	F6	F7
T0							
C14:0 Myristic Acid	1.47±0.02^{bB}	1.55±0.00^{aC}	1.40±0.02^{cB}	1.31^{dB}±0.01	1.45±0.01^{bC}	1.56±0.00^{aB}	1.37^{cA}±0.00
C16:0 Palmitic Acid	25.86±0.06^{aA}	24.54±0.02^{bC}	23.66±0.14^{dC}	23.45^{dC}±0.05	24.82±0.02^{bBC}	24.84±0.01^{bB}	24.39^{cA}±0.01
C16:1 Palmitoleic acid	2.55±0.01^{cB}	2.67±0.00^{aA}	1.97±0.01^{fD}	2.43^{eC}±0.00	2.55±0.00^{cB}	2.64±0.00^{bB}	2.46^{dA}±0.00
C18:0 Stearic Acid	13.04±0.01^{bC}	12.54±0.01^{eC}	14.36±0.04^{aA}	12.16^{fC}±0.01	12.87±0.00^{cdB}	12.92±0.01^{cC}	12.82^{dD}±0.00
C18:1n9c Oleic Acid	47.96±0.06^{dA}	46.44±0.01^{eD}	48.57±0.11^{abA}	48.49^{bcB}±0.03	48.35±0.02^{cA}	46.40±0.01^{eD}	48.14^{dC}±0.01
C18:2n6c Linoleic Acid	8.27±0.01^{gD}	11.47±0.00^{aA}	9.29±0.02^{eB}	11.39^{bA}±0.02	9.21±0.01^{fC}	10.95±0.00^{cA}	10.06^{dA}±0.01
C18:3n3 Alpha-Linolenic Acid	0.83±0.00^{aA}	0.69±0.00^{cB}	0.76±0.01^{bAB}	0.77^{bA}±0.00	0.75±0.00^{bA}	0.70±0.01^{cC}	0.75^{bA}±0.00
T30							
C14:0 Myristic Acid	1.48±0.00^{cB}	1.61±0.01^{aB}	1.37±0.01^{dB}	1.45^{cA}±0.01	1.57±0.01^{bA}	1.32±0.01^{eD}	1.32±0.01^{eC}
C16:0 Palmitic Acid	25.64±0.00^{aB}	25.45±0.01^{bB}	24.49±0.05^{deB}	24.39^{eAB}±0.08	25.11±0.01^{cA}	24.40±0.02^{eC}	24.61±0.01^{dA}
C16:1 Palmitoleic acid	2.51±0.02^{cC}	2.77±0.08^{aA}	2.51±0.00^{cB}	2.56^{bcA}±0.00	2.64±0.01^{bA}	2.50±0.00^{cC}	2.28±0.00^{dB}
C18:0 Stearic Acid	13.19±0.01^{bB}	12.82±0.02^{dB}	13.04±0.02^{cC}	12.79^{dB}±0.02	12.82±0.01^{dB}	12.99±0.01^{cB}	13.28±0.01^{aC}
C18:1n9c Oleic Acid	46.64±0.02^{fC}	47.59±0.07^{eB}	48.58±0.03^{bA}	48.79^{aA}±0.05	48.18±0.00^{dA}	48.80±0.01^{aB}	48.41±0.00^{cA}
C18:2n6c Linoleic Acid	9.75±0.00^{aB}	9.09±0.01^{dA}	9.27±0.01^{cB}	9.28^{bcD}±0.01	8.98±0.02^{eD}	9.23±0.00^{cB}	9.32±0.01^{bC}
C18:3n3 Alpha-Linolenic Acid	0.80±0.01^{dB}	0.67±0.00^{fB}	0.75±0.00^{cdAB}	0.74^{dBbA}±0.00	0.70±0.00^{eB}	0.76±0.00^{bcB}	0.77±0.01^{bA}
T120							
C14:0 Myristic Acid	1.60±0.00^{aA}	1.44±0.01^{bD}	1.39±0.00^{cB}	1.43^{bA}±0.01	1.44±0.0^{bC}	1.46^{bC}±0.01	1.36±0.00^{cA}
C16:0 Palmitic Acid	25.89±0.03^{aA}	24.30±0.04^{dD}	24.58±0.04^{cB}	24.26^{dB}±0.01	24.87±0.13^{bABC}	23.51^{eD}±0.00	24.45±0.03^{cdB}
C16:1 Palmitoleic acid	2.26±0.00^{dD}	2.45±0.02^{abcB}	2.40±0.02^{cC}	2.49^{abB}±0.02	2.39±0.03^{cC}	2.52^{aC}±0.00	2.45±0.02^{bcA}
C18:0 Stearic Acid	14.74±0.00^{aA}	13.25±0.01^{dA}	13.53±0.01^{bcB}	13.26^{dA}±0.00	12.15±0.03^{fC}	12.79^{eD}±0.00	13.51±0.02^{cA}
C18:1n9c Oleic Acid	44.29±0.04^{eD}	48.36±0.07^{bA}	47.40±0.05^{dB}	48.37^{bC}±0.02	47.22±0.09^{dB}	50.41^{aA}±0.00	48.04±0.01^{cD}
C18:2n6c Linoleic Acid	10.40±0.00^{bA}	9.47±0.00^{dB}	9.94±0.01^{cA}	9.45^{dB}±0.00	11.19±0.04^{aA}	8.52^{eC}±0.00	9.45±0.02^{dB}
C18:3n3 Alpha-Linolenic Acid	0.83±0.01^{aA}	0.73±0.00^{dA}	0.77±0.01^{cA}	0.74^{dB}±0.01	0.75±0.00^{dA}	0.79^{bA}±0.00	0.75±0.00^{cdA}

F1 - traditional cultures without curing fat and curing salts reduction (nitrite and nitrate 0.015% 0.005%); F2 - traditional cultures without curing salts reduction; F3 – traditional cultures with curing salts reduction (nitrite and nitrate 0.007% 0.003%); F4 and F5 - probiotic culture (*E. faecium* CRL183) without and with curing salts reduction. respectively; F6 and F7 - probiotic culture (*L. acidophilus* CRL1014) without and with curing salts reduction. respectively. T0 = initial time; T30 = end of the ripening period; T120 = storage time at 4°C. Analysis of treatments: means with the same lowercase letters in the same line. in the same time interval. do not differ by Tukey test (P<0.05). Analysis of time: means with capital letters for the same formulation at different times. do not differ by Tukey test (P<0.05).

were in compliance with the legislation requirements.

Lipid oxidation determination

The lipid oxidation results are shown in Table 3. The malondialdehyde (MDA) is the major secondary by-product of lipid oxidation that reacts with 2-thiobarbituric acid (TBA). However, other compounds present in the product, such as proteins and nitrite, can also react with TBA, thus interfering with the obtained results (Silva *et al.*, 1999).

Some strains of lactic acid bacteria are able to inhibit the oxidation of lipids, although the mechanism involved in this effect is still unknown (Inoue *et al.*, 1998). The results demonstrated that fermented sausages with *Enterococcus faecium* CRL 183 showed the lowest oxidation means at the end of storage time (T120), indicating that the probiotic can have a protective effect on the formation of undesirable compounds in the product. The results were most evident for the formulation with nitrite reduction (F5), suggesting that this additive concentration could also affect the obtained results.

It is observed that, from T90, there was a reduction in the lipid oxidation rate in all formulations, except for formulation F1, and it is supposed that the pH and water activity (Aw) are responsible for it, which made the system inappropriate for oxidation increase (Karel and Young 1981). Additionally, this decrease can be attributed to the reaction by malonaldehyde with proteins during the storage period, although malonaldehyde is a secondary by-product of the oxidation of polyunsaturated FA (Melton, 1993 cited by Marangoni, 2007).

FA profile determination

Table 4 reports the fatty acid composition of the lipid fractions extracted from the sausages obtained by the different formulations in different processing steps. The compositions were normalized by only taking into account the identified FA. In the case of traditional formulation (F1), most of the lipid fraction is provided by the pork fat added as an ingredient to the formulation, besides the low levels that are naturally present in pork meat and beef. The main FA found in this formulation at T0 were oleic (48.0%), palmitic (25.9%), and linoleic acids (8.3%), in addition to myristic, palmitoleic and alpha-linolenic acids, which together represent less than 5.0% of total FA. According to the Codex Alimentarius (CODEX, 1999) and the composition presented by Gunstone and Harwood (2007), pork fat should contain 35-55% oleic acid, 20-30% palmitic acid, 4-12% linoleic acid and up to 8% of myristic, palmitoleic and alpha-linolenic acids together. Thus, the fatty acid composition of F1 at T0 was agreement with that found in literature.

During the production stages, F1 presented some significant changes in fatty acid profile, however

Table 5. Means of viability of probiotic microorganisms and of population potentially pathogenic microorganisms - microbiological safety, during the trial period

		Enterococcus spp. (log UFC/g)	*Lactobacillus* spp. (log UFC/g)	Positive coagulase staphylococci (log UFC/g)	Coliforms 45°C/ *E.coli* (log UFC/g)	*Salmonella* spp.	*Listeria Monocytogenes*	*C.botulinum*
T0	F1	-	-	4.97±0.59^{aA}	3.52±0.16^{aA}	Absent	Absent	Absent
	F2	-	-	4.35±0.10^{aA}	3.32±0.16^{aA}	Absent	Absent	Absent
	F3	-	-	4.30±0.71^{aA}	3.50±0.11^{aA}	Absent	Absent	Absent
	F4	6.82±0.82^{aC}	-	4.25±0.77^{aA}	3.36±0.11^{aA}	Absent	Absent	Absent
	F5	6.96±1.04^{aB}	-	4.19±0.11^{aA}	3.53±0.24^{aA}	Absent	Absent	Absent
	F6	-	6.78±0.04^{bB}	4.38±0.09^{aA}	3.59±0.10^{aA}	Absent	Absent	Absent
	F7	-	7.60±0.17^{aA}	3.77±0.61^{bA}	3.53±0.05^{aA}	Absent	Absent	Absent
T7	F1	-	-	3.81±0.44^{aB}	2.18±0.00^{aB}	Absent	Absent	Absent
	F2	-	-	3.74±0.07^{aB}	1.86±0.53^{aB}	Absent	Absent	Absent
	F3	-	-	3.73±0.02^{aA}	2.24±0.09^{aB}	Absent	Absent	Absent
	F4	8.09±0.76^{aAB}	-	4.25±0.77^{aA}	2.27±0.05^{aB}	Absent	Absent	Absent
	F5	7.40±0.53^{aAB}	-	3.42±0.54^{aAB}	2.35±0.07^{aB}	Absent	Absent	Absent
	F6	-	8.37±0.54^{aA}	3.76±0.05^{aB}	2.27±0.01^{aB}	Absent	Absent	Absent
	F7	-	7.63±0.76^{aA}	3.34±0.40^{aA}	2.07±0.16^{aB}	Absent	Absent	Absent
T30	F1	-	-	3.59±0.12^{aB}	< 1	Absent	Absent	Absent
	F2	-	-	3.68±0.19^{aB}	< 1	Absent	Absent	Absent
	F3	-	-	3.70±0.01^{aA}	< 1	Absent	Absent	Absent
	F4	8.54±0.50^{aA}	-	3.69±0.06^{aA}	< 1	Absent	Absent	Absent
	F5	7.98±0.08^{aA}	-	3.45±0.49^{aAB}	< 1	Absent	Absent	Absent
	F6	-	8.52±0.36^{aA}	3.70±0.02^{aB}	< 1	Absent	Absent	Absent
	F7	-	8.45±0.19^{aA}	3.64±0.21^{aA}	< 1	Absent	Absent	Absent
T60	F1	-	-	3.56±0.11^{aB}	< 1	Absent	Absent	Absent
	F2	-	-	3.69±0.32^{aBC}	< 1	Absent	Absent	Absent
	F3	-	-	3.69±0.07^{aA}	< 1	Absent	Absent	Absent
	F4	7.44±0.15^{aABC}	-	3.68±0.03^{aA}	< 1	Absent	Absent	Absent
	F5	8.36±0.54^{aAB}	-	3.01±0.70^{aAB}	< 1	Absent	Absent	Absent
	F6	-	8.36±0.54^{aA}	3.60±0.30^{aB}	< 1	Absent	Absent	Absent
	F7	-	7.83±0.47^{aA}	3.59±0.48^{aA}	< 1	Absent	Absent	Absent
T90	F1	-	-	3.35±0.08^{aB}	< 1	Absent	Absent	Absent
	F2	-	-	3.57±0.33^{aBC}	< 1	Absent	Absent	Absent
	F3	-	-	3.66±0.08^{aA}	< 1	Absent	Absent	Absent
	F4	7.40±0.19^{aABC}	-	3.59±0.20^{aA}	< 1	Absent	Absent	Absent
	F5	7.63±0.20^{aAB}	-	3.29±0.03^{aAB}	< 1	Absent	Absent	Absent
	F6	-	8.15±0.29^{aA}	3.69±0.12^{aB}	< 1	Absent	Absent	Absent
	F7	-	7.72±0.89^{aA}	3.62±0.02^{aA}	< 1	Absent	Absent	Absent
T120	F1	-	-	3.23±0.07^{aB}	< 1	Absent	Absent	Absent
	F2	-	-	3.17±0.03^{aC}	< 1	Absent	Absent	Absent
	F3	-	-	3.46±0.51^{aA}	< 1	Absent	Absent	Absent
	F4	7.15±0.03^{aABC}	-	3.06±0.26^{aA}	< 1	Absent	Absent	Absent
	F5	7.18±0.27^{aAB}	-	2.61±0.01^{bB}	< 1	Absent	Absent	Absent
	F6	-	7.73±0.09^{aA}	3.66±0.12^{aB}	< 1	Absent	Absent	Absent
	F7	-	7.35±0.43^{aA}	3.63±0.31^{aA}	< 1	Absent	Absent	Absent

F1 - traditional cultures without curing fat and curing salt reduction (0.015% nitrite and 0.005% nitrate); F2 - traditional cultures without curing salt reduction; F3 – traditional cultures with curing salt reduction (0.007% nitrite and 0.003% nitrate); F4 and F5 - probiotic culture (*E. faecium* CRL183) without and with curing salt reduction. respectively; F6 and F7 - probiotic culture (*L. acidophilus* CRL1014) without and with curing salt reduction. respectively. T0 = initial time; T7 = end of the fermentation period; T30 = end of the ripening period; T60. T90 and T120 = storage time at 4°C. Means followed by the same lower case letters in a line and capital letters on the column do not differ from Tukey test (P<0.05).

being unimportant. Generally speaking, at the end of the storage period (T120), the saturated fatty acid content increased from 40.4% to 42.2% in detriment of the unsaturated fatty acid content, which decreased from 59.6% to 57.8%. This slight modification can be related to more severe oxidative processes in F1 if compared to other formulations, as presented in Table 3, given that the chain reactions of oxidative rancidity occur preferentially in unsaturated FA (Dransfield, 2008).

The formulations F2 - F7 provided a 60% reduction in animal fat content if compared to F1 (control formulation), and part of this reduction was due to the reduced amount of fat added to the formulation, and partly by the partial replacement of pork fat for olive oil. The Codex Alimentarius (CODEX, 1999) stipulates that olive oil should contain levels of 55 to 83% oleic acid, its most abundant fatty acid, followed by linoleic (3.5 to 21%) and palmitic acids (7.5 to 20%). The pork fat and olive oil have, therefore, the same principal FA, but in different proportions. The addition of olive oil to the formulations resulted in a significant increase in oleic acid content in most cases, especially after 120 days of production, reaching an increase of 44.3% in F1 and 50.4% in F6. A decrease in the saturated fatty acid content was also observed, which is interesting from a nutritional point of view, since myristic and palmitic acids are the main hypercholesterolemic FA (Hayes *et al.*, 2001). Muguerza *et al.* (2003) also verified that a 25% reduction in fat content resulted in changes in the fatty acid profile, which was revealed by an increase in the unsaturated FA (15.22 to 23.96%) and a reduction in the saturated FA (37.83 to 32.81%).

Formulations F2 and F3 were produced using the same traditional cultures and, in addition, F3 had a 50% reduction in the amount of added curing salts. Similarly, F4 and F5 were produced with the addition of the probiotic *E. faecium* CRL 183 strain,

with and without curing salt reduction, respectively. Comparably, F7 and F6 used the probiotic *L. acidophilus* CRL 1014 strain, with and without curing salt reduction, respectively. By comparing the levels of saturated FA of these formulations, considering that each pair was added with the same microorganism (F2/F3, F4/F5, and F6/F7), significant differences can be noted as a function of time or formulation, but that does not indicate a possible influence of curing salt levels on the sausages. These observations can indicate that the curing salt reduction did not result in significant changes in the fatty acid composition of the product, which could emphasize the advantageous aspect of this reduction on nutritional perspectives. It is important to mention, however, that other results are needed to confirm this hypothesis.

Viable cells count

According to the National Health Surveillance Agency's (ANVISA) recommendations, probiotic microorganisms must be present in the range of 8.0 to 9.0 log CFU for a daily intake of the product. This recommendation is in agreement with international guidelines for probiotics foods (Hills *et al*., 2014). For formulations F4 and F5, the population of *Enterococcus faecium* CRL183 (*Enterococcus* spp.) exhibited an increase of two and one logarithmic cycle, respectively, at the end of the ripening period (T30: 8.54±0.50 and 7.98±0.08 log CFU/g) (Table 5). In the storage phase, regardless of which formulation, there was a reduction in the microorganism's population (T120 = F4: 7.15±0.03 log CFU/g and F5: 7.18±0.27 log CFU/g). The culture *Lactobacillus acidophilus* CRL 1014 (*Lactobacillus* spp.) showed an increase of two logarithmic cycles in the population of viable cells at the end of the ripening period (T30) for formulation F6 (8.52±0.36 log UFC/g), and of one cycle for F7 (8.45±0.19 log CFU/g). After 90 days of storage (T120), both formulations exhibited a reduction in the population of *Lactobacillus* spp. It should be noted that the probiotic population showed no difference in all formulations after the fermentation period (T7).

The obtained results confirmed those that had been achieved in the preliminary tests of curing salt resistance, thus indicating that the probiotic starter cultures are able to survive and multiply in the meat matrix. Despite the numerical reduction in the population of probiotic microorganisms, a daily intake to 10 g of sausage (F4, F5, F6 and F7) would be enough to comply with ANVISA's and international guidelines recommendations and ensure its possible beneficial health effects.

Macedo *et al.* (2008) tested three cultures of Lactobacillus spp. in combination with commercial starter cultures for producing probiotic salami. At the end of the ripening period (T25), the population of *L. paracasei, L. rhamnosus* and *L. casei* was 9.50 x 10^7, 5.50 x 10^7 and 3.45 x 10^7 CFU/g, respectively. Comam *et al*. (2012) have found that the probiotic cultures *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 developed in Italian and Swiss salami, respectively, exhibiting a viable cell population of 108CFU/g at the end of the storage period. Similar results were published by Rubio *et al.* (2013a), who have found that *L. rhamnosus* GG was able to dominate the natural microbiota of meat in fermented Spanish salami, reaching levels of 10^8CFU g at the end of ripening and storage periods. In another study, Rubio *et al.* (2014) evaluated six strains of *Lactobacillus* spp. (*L. casei* CTC1677, *L. casei* CTC1678, *L. rhamnosus* CTC1679, *L. plantarum* 299v, *L. rhamnosus* GG, *L. casei* Shirota) as starter cultures in fermented salamis with sodium and fat reduction, and only *L. rhamnosus* CTC1679 was able to multiply and reach levels of 10^8CFU/g at the end of the ripening period.

Table 5 shows the results for contaminants in meat sausage at different stages of the study. There was a reduction in all microorganism groups studied throughout the experimental period, and *Salmonella* spp., *Listeria monocytogenes* and *Clostridium botulinum* were absent in all formulations from the beginning of the process (T0). Fermentation was essential for reducing the population of microorganisms because, at the end of this step (T7), all formulations exhibited a decrease in populations of coliforms at 45°C/*E.coli*. After the ripening stage (T30), it was not detected the presence of coliforms at 45ºC/E.coli (Petrifilm™ *E.coli*/Coliform count plate by 3M - results expressed as less than one CFU/g).

The population of coagulase-positive staphylococci was also reduced during the storage period, and only formulations F3, F4 and F7 showed no significant difference in analysis between the time periods. It should be noted that, among potentially probiotic formulations, F5 exhibited the greatest reduction in this group of microorganisms at the end of the 120 days of storage (1.58 log CFU/g). The results obtained are in accordance with the Brazilian National Health Surveillance Agency (ANVISA, 2001), which requires a maximum of 5.103CFU/g (3.7 log CFU/g) for coagulase-positive staphylococci, 103CFU/g (3.0 log CFU/g) coliform 45°C and absence of *Salmonella* ssp. in 25 grams of fermented meat products.

Conclusion

The results clearly show that it was possible to obtain a potentially probiotic fermented meat product with enhanced nutritional properties using bacteria that have hypolipidemic properties as starter culture. The reduction in curing salt content and replacement of traditional cultures by probiotic ones neither compromised the sausages' microbiological safety, nor their physicochemical characteristics. Among the formulations, F5 (fermented by *E. faecium* CRL 183, with fat and curing salt reduction) was considered the most promising for presenting the greatest reduction in the population of coagulase-positive staphylococci and lower lipid oxidation at the end of the storage period. Future studies are needed to confirm the potential health effect of this product.

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