MICROBIOLOGICAL AND PHYSICOCHEMICAL ASPECTS OF "SALPICÃO", A TRADITIONAL DRY SAUSAGE PRODUCED IN THE NORTHEAST OF PORTUGAL

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Abstract

Counts of total viable mesophilic bacteria (TVC), lactic acid bacteria (LAB), Microccocaceae, Enterobacteriaceae, Salmonella spp. and Listeria monocytogenes, in traditional Portuguese dry sausages from two industrial producers, were compared in batter and final product. During the production process, the TVC increased significantly, most likely due to the multiplication of fermentative flora. Enterobacteriaceae decreased from batter to final product while the S. aureus increased. Great variability was verified in detection of L. monocytogenes both between batches and industrial producers.

Keywords: *dry-fermented sausage, salpicão, safety, microbial quality*

1. INTRODUCTION

Portugal, as the other Mediterranean countries, has a long tradition in dry fermented sausage-making. In Portugal, there are many different types of dry sausages, with distinctive organoleptic and sensory characteristics. Trás-os-Montes in the north of Portugal, is a region known by its great variety and high-quality traditional meat products. One of the most appreciated by Portuguese consumers is "salpicão", a dry fermented sausage ready-to-eat (RTE) that reaches the highest prices in the market. This traditional product is manufactured by small production units following spontaneous fermentation. For its production, loin pork meat cut in large pieces are mixed with a specific combination of ingredients, such as salt, laurel, garlic, red/white wine or water according to the traditions and know-how of the regional producers. The mixture is stored at 4ºC for 2-6 days, and it is then stuffed into pork large intestine casings. Sausages are then hung vertically in smoking and drying chambers for 4-5 weeks. The variations in both ingredients and production process are responsible for the distinct quality and sensory characteristics found in the products of each manufacturer.

Most of the research to date, related with food safety traditional sausages, has been performed on dry-fermented sausages from Spain, Italy, France and Greece, and only a few scientific studies investigate Portuguese sausages. They have reported that, on certain occasions, these sausages may harbour foodborne pathogens. (Ferreira et al, 2009; Elías and Carrascosa, 2010).

Thus, this study aimed to investigate the presence of total viable mesophilic bacteria (TVC), *Enterobacteriaceae*, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* from raw ingredients to final product as well to study the evolution of fermentative flora during maturation of "salpicão" produced in two different traditional producers.

2. MATERIAL AND METHODS

2.1. Samples

A total of 3 batches of "salpicão" were studied on two different producers. Three sausages from each batch were collected in diced meat (raw meat), raw meat mixed with condiments (batter), after maceration and before filling, after smoking and final product. All samples were randomly collected using sterile equipment and stored aseptically in appropriate collection bags. The time of processing steps during ripening process was different for each batch and producer (Table1).

Table 1. Time of sampling of "salpicão" for each batch and producer.

Batch	Sampling	Time of sampling	
		Producer 1	**Producer 2**
A	Diced meat	day 0	day 0
	Batter	day 0	day 1
	Before filling	day 3	day 6
	After smoking	day 8	day 27
	Final product	day 13	day 32
B	Diced meat	day 0	day 0
	Batter	day 0	day 0
	Before filling	day 2	day 3
	After smoking	day 7	day 21
	Final product	day 17	day 28
C	Diced meat	day 0	day 0
	Batter	day 0	day 1
	Before filling	day 6	day 10
	After smoking	day 13	day 33
	Final product	day 15	day 35

2.2. *Physicochemical analysis*

The pH was measured by introducing a pH meter HI8424 (Hanna Instruments) into the centre of the samples, the water activity (a_w) was measured using a Rotronic HygroPalm AW1. All chemical determinations were made in triplicate for each sample.

2.3. Microbiological analysis

For the microbiological analysis, except for *L. monocytogenes*, 25 g of the test sample was taken aseptically, diluted in 225 mL of sterile buffered peptone water (BPW, VWR Chemicals Prolabo) and homogenized for 2 min (Stomacher 400, Seward). After serial decimal dilutions in BPW, appropriate dilution samples (1 or 0.1 mL) were poured or spread on petrifilms or agar plates.

Total viable counts (TVC) were determined on Petrifilm™ Aerobic Count, incubated at 30ºC for 72 h. *Enterobacteriaceae* were enumerated on Petrifilm™ *Enterobacteriaceae* Count, incubated ate 37ºC for 24 h. *E. coli* was determined on Petrifilm™ Select E. coli (SEC), incubated at 42ºC for 24 h. *Staphylococcus aureus* was enumerated on Petrifilm™ Staph Express Count, incubated at 37 ºC for 24 h, and confirmed with Petrifilm™ Staph Express Disk.

LAB count was performed on MRS agar (Liofilchem) overlaid with 5 mL of agar 0.8 %, incubated at 30 ºC for 48-72h. *Microccocaceae* were counted on Baird-Parker agar (VWR Chemicals Prolabo), incubated at 37 ºC for 48 h. LAB and *Microccocaceae* counts were performed in batter and final product of two samples from batch B and C.

Salmonella spp. detection was performed using the *Salmonella* 1-2 Test® (Biocontrol) AOAC official methods according to the manufacturer's instructions. All the positive samples were confirmed and *Salmonella* enumerated by MPN method. 25 g portion of sample were added to 225 mL of BPW and three subsequent dilutions were also prepared in BPW. The BPW aliquots were pre-enriched at 37 ºC for 24 h. One-mL volume of each pre-enrichment tube was sub-cultured to 9 mL of Rappaport-Vassiliadis Broth (Biopark Diagnostics). After incubation at 42 ºC for 18 h, aliquots of the broth were streaked onto Xylose-Lysine-Tergitol 4 Agar plates (Oxoid).

For the microbiological analysis of *Listeria monocytogenes*, 25 g of sample was homogenized for 2 min in 225 mL of Half Fraser Base CM0895 (Oxoid). The enumeration was performed according to the procedure adapted from ISO 11290-2:1998/Amd. 1:2004(E). After incubation of the initial suspension for 1 h at 20 ºC, 0.1 mL were surface-inoculated on Oxoid Chromogenic Listeria Agar (OCLA, Oxoid), in duplicate, and incubated at 37 ºC for 24 h. The samples with no growth were analysed for detection of *Listeria monocytogenes* according to the procedure adapted from ISO 11290-1:1996/Amd.1:2004(E). The initial suspension was supplemented with SR 166 (Oxoid), incubated at 30ºC for 24 h and streaked on OCLA (incubated at 37 ºC for 24 h). If no growth was detected, 0.1 mL of the same initial supplemented suspension was transferred into 10 ml Fraser Broth supplemented with SR 166 (Oxoid), incubated at 37 °C for 48 h and streaked onto OCLA (incubated at 37 ºC for 24 h). The presumptive colonies of *Listeria* spp. were confirmed using API® Listeria (BioMérieux) biochemical strips according to the manufacturer's instructions.

2.4. Statistical analysis

All the analysis was performed in triplicate and results are expressed as mean values and standard deviation. The data was analysed using SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Shapiro–Wilks test ($n < 50$) and Levene's test, respectively. For each producer, to test significant differences between batches on the physicochemical and microbiological data, a one-way ANOVA was applied. If a significant effect was found, the means were compared using Tukey's multiple comparison test. For each physicochemical and microbiological parameter significant differences between producers were also assessed. All statistical tests were performed at a 5 % significance level.

3. RESULTS AND DISCUSSION

Regarding the manufacture process between both producers, pronounced variations in time were evident during the different processing steps, not only between producers but also between batches (Table 1). Producer 2 had the longest production process, between 28 and 35 days, as much as the double of producer 1. The period of smoking in producer 2 was not only longer (18 to 23 days), but the approach was also different. This is, whereas in the producer 1 the smoking was carried out without cessations during 5-6 days, in the producer 2 it was a discontinuous process: the "salpicão" was removed several times from the smoking chamber to an ambient temperature room, and vice versa. In general, the differences in the duration of the production phases between producers and batches were observed mainly after the batter step.

3.1. Physicochemical analysis

The physicochemical characteristics, namely pH and aw, of samples collected at different stages of ripening for both producers is displayed in Table 2.

Table 2. Values of pH and aw of "salpicão" analysed at different stages of development.

	Producer 1			P_1	Producer 2			P_2	P_{ind}
	A	B	C		A	B	C		
pH									
Diced meat	5.63 ± 0.21	5.73 ± 0.20	5.64 ± 0.20	ns	5.51 ± 0.09^{a}	5.55 ± 0.06^{ab}	6.30 ± 0.53^{b}	0.036	ns
Batter	6.04 ± 0.31	6.05 ± 0.30	5.97 ± 0.22	ns	5.46 ± 0.01^{a}	5.57 ± 0.16^{a}	6.32 ± 0.20^{b}	0.001	ns
Filling	5.83 ± 0.25	6.03 ± 0.06	5.74 ± 0.24	ns	5.33 ± 0.05	5.74 ± 0.29	5.76 ± 0.10	n.s.	0.040
Smoking	6.12 ± 0.08	6.17 ± 0.12	6.06 ± 0.05	ns	5.12 ± 0.09^{a}	5.31 ± 0.10^{ab}	5.44 ± 0.06^{b}	0.009	<0.001
Final product	6.12 ± 0.08	6.09 ± 0.07	6.18 ± 0.05	ns	4.93 ± 0.10^{a}	5.39 ± 0.12^{b}	5.58 ± 0.07^{b}	0.001	<0.001
aw									
Diced meat	0.968 ± 0.003^{a}	0.969 ± 0.006^{a}	0.984 ± 0.004^{b}	0.013	0.984 ± 0.004	0.986 ± 0.005	0.984 ± 0.003	n.s.	0.003
Batter	0.944 ± 0.004	0.952 ± 0.005	0.954 ± 0.009	ns	0.968 ± 0.008^{a}	0.990 ± 0.004^{b}	0.980 ± 0.009^{ab}	0.029	<0.001
Filling	0.935 ± 0.003^{a}	0.953 ± 0.006^{b}	0.955 ± 0.005^{b}	0.004	0.965 ± 0.003^{a}	0.966 ± 0.007^{a}	0.986 ± 0.002^{b}	0.002	<0.001
Smoking	0.927 ± 0.009^{a}	0.951 ± 0.003^{b}	0.946 ± 0.003^{b}	0.004	0.929 ± 0.000	0.928 ± 0.006	0.923 ± 0.004	n.s.	0.003
Final product	0.914 ± 0.007^{a}	0.922 ± 0.009^{a}	0.942 ± 0.004^{b}	0.007	0.941 ± 0.004	0.920 ± 0.003	0.910 ± 0.023	n.s.	ns

P_1 values between batches for producer 1 at $p<0.05$ (values with different superscript letters are significantly different)

P_2 values between batches for producer 2 at $p<0.05$ (values with different superscript letters are significantly different)

P_{ind} values between producers at $p<0.05$

ns No significant difference

Regarding the pH values, during the production process of "salpicão" of producer 2 significant differences were observed between batches, except at the stage of filling; and the pH evolution followed the typical trend of fermented sausages, decreasing during ripening. The pH of the final product ranged between 4.9 and 5.6 and these values are comparable to those recorded in other studies on Iberian dry sausages (Ferreira et al., 2009; Linares et al., 2013; Casquete et al., 2012; Elías and Carrascosa, 2010; Ferreira et al., 2007; Garcia Fontán et al., 2007). In contrast, for producer 1 the pH of samples increased during the process, presenting the final product a pH of about 6.1. However, no significant differences were found in pH between batches. The rise of pH towards the end of the process may be attributed to the proteolytic microbiota (Elías and Carrascosa, 2010). Significant differences were observed in the last three stages of ripening between producers. Differences between industries for the same product have already been reported (Casquete et al., 2012; Elías and Carrascosa, 2010; Ferreira et al., 2009).

Water activity of samples decreased, as expected, during the processing of sausages and ranged from values of 0.97 – 0.98 in diced raw meat up to 0.91 – 0.94 in the final product. In almost all stages of production, significant differences between producers were observed, except in the final product. This suggests that the additional duration of the production process did not further influence the final value of aw. Great variability of aw has been reported in traditional dry sausages, namely 0.91 – 0.92 in sausages produced in the North East of Italy (Comi et al., 2005), 0.87 – 0.98 in Botillo Spanish sausage (García Fontán et al., 2007), 0.80 – 0.83 in traditional Iberian "salchichón" and "chorizo" (Casquete et al., 2012) and 0.82 in Portuguese "Paio do Alentejo" (Elías and Carrascosa, 2010).

3.2. Microbiological analysis

Concerning the fermentative flora, lactic acid bacteria (LAB) and *Micrococcaceae*, the results obtained for batter and final product of both producers are presented in Figure 1.

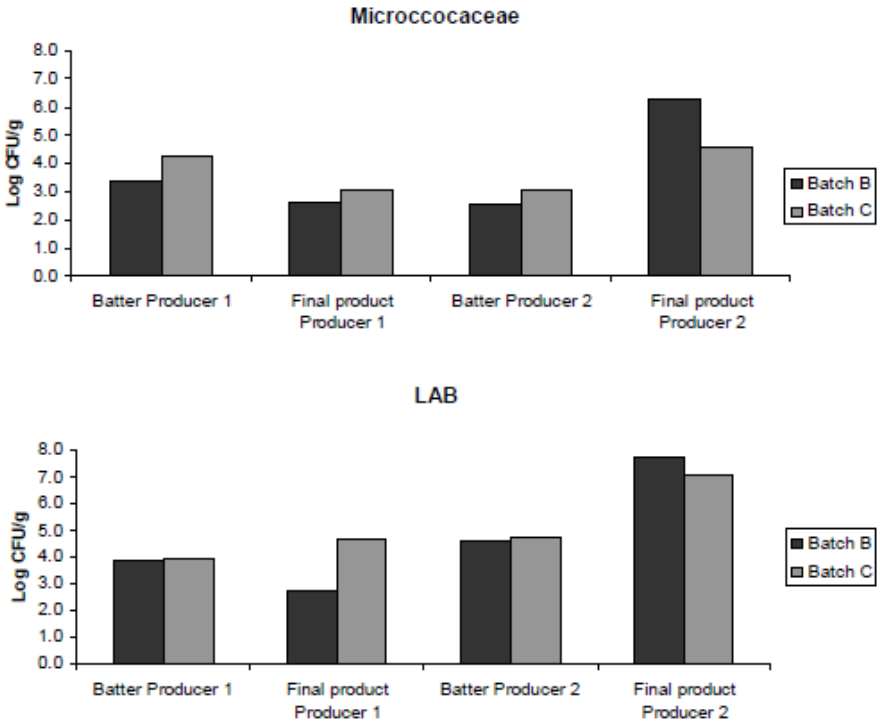

Figure 1. Levels of lactic acid bacteria (LAB) and *Microccocaceae* in batter and final product for batches B and C of both producers.

The behaviour of fermentative flora was different in both producers. In general for producer 1, LAB and *Microccocaceae* were present in equal proportions and their development was not boosted during production process. Furthermore, in batch B both populations dropped from around 3.5 log CFU/g in batter to 2.6 log CFU/g in final product. In contrast, for producer 2, an increase of fermentative flora was observed during production, with LAB reaching the highest values. The levels of these bacteria in final product were around 7 log CFU/g and *Microccocaceae* ranged from 4.5 to 6 log CFU/g. These values are in agreement with previous studies on fermented dry sausages (Elías and Carrascosa, 2010; Ferreira et al., 2009).

These differences in composition and quantity of endogenous fermentative flora can partially explain the variations of pH between producers. LAB are responsible for acid production and consequently drop of pH, contributing therefore to assure the safety of the product. Additionally, the proteolytic activity of the microorganisms involved in the fermentation can be responsible for the increase of pH. However, more studies on ecology and identification of microorganisms during meat fermentation would be necessary.

The microbiological analysis of total viable mesophilic bacteria (TVC) and *Enterobacteriaceae* revealed significant differences between batches and producers (Table 3).

Table 3. Hygiene indicators (log CFU/g) in "salpicão" analysed at different stages of production.

	Producer 1			P_1	Producer 2			P_2	P_{ind}
	Batch A	Batch B	Batch C		Batch A	Batch B	Batch C		
TVC									
Diced meat	4.14 ± 1.15	4.86 ± 0.29	5.72 ± 0.23	ns	5.09 ± 0.37	5.07 ± 0.37	4.44 ± 0.53	ns	ns
Batter	5.12 ± 0.61^{ab}	4.75 ± 0.16^{a}	5.93 ± 0.35^{b}	0.035	5.98 ± 0.16^{a}	5.09 ± 0.23^{b}	5.49 ± 0.10^{b}	0.002	ns
Filling	5.42 ± 0.12^{a}	4.69 ± 0.15^{b}	7.57 ± 0.25^{c}	0.000	5.87 ± 0.03^{a}	5.20 ± 0.18^{b}	6.33 ± 0.19^{c}	0.000	ns
Smoking	2.82 ± 0.34	3.56 ± 0.03	4.00 ± 0.81	ns	7.76 ± 0.10^{a}	8.32 ± 0.10^{b}	7.46 ± 0.10^{c}	0.000	0.000
Final product	4.55 ± 2.08	4.02 ± 1.43	4.38 ± 1.50	ns	7.99 ± 0.09^{a}	8.04 ± 0.23^{a}	7.28 ± 0.31^{b}	0.011	0.000
Enterobacteriaceae									
Diced meat	1.87 ± 0.91	3.09 ± 0.56	3.11 ± 0.19	ns	3.77 ± 0.20^{a}	2.33 ± 0.31^{ab}	2.13 ± 1.00^{b}	0.034	ns
Batter	3.53 ± 0.52^{a}	2.07 ± 0.17^{b}	3.47 ± 0.49^{a}	0.009	3.78 ± 0.12^{a}	1.70 ± 0.04^{b}	1.65 ± 0.10^{b}	0.000	ns
Filling	3.72 ± 0.14^{a}	2.14 ± 0.16^{b}	3.68 ± 0.09^{a}	0.000	3.42 ± 0.12^{a}	2.70 ± 0.19^{b}	2.57 ± 0.26^{b}	0.004	ns
Smoking	nd	nd	nd	---	< 1	3.69 ± 0.43	< 1	---	---
Final product	nd	1.39 ± 2.42	nd	---	1.16 ± 0.15	< 1	< 1	---	---

P_1 values between batches for producer 1 at $p<0.05$ (values with different superscript letters are significantly different)

P_2 values between batches for producer 2 at $p<0.05$ (values with different superscript letters are significantly different)

P_{ind} values between producers at $p<0.05$

ns no significant difference

nd not detected;

< 1 mean counts were lower than one

For both producers, the raw meat presented comparable levels of aerobic mesophilic flora, and during the maceration of the meat with the condiments up to the filling step (2 - 4 days), the levels of TVC slightly increased. However, in producer 1, smoking had a positive effect on the reduction of mesophilic flora (from values of 4.7 – 7.6 log CFU/g to 2.8 – 4.0 log CFU/g) and the final product presented a relatively-low microbial load (4 – 4.5 CFU/g). Nevertheless, in producer 2, the counts of TVC increased significantly from raw meat (4.4 – 5.1 log CFU/g) until the final product (7.3 – 8.0 log CFU/g). Although the levels of TVC in the final product of producer 2 may seem high, they are in accordance with the ones reported in similar products (Casquete et al., 2012; García Fontán et al., 2007; Ferreira et al., 2009).

The levels of *Enterobacteriaceae* revealed no significant differences between producers, but showed significant differences between batches. This can be correlated with the initial level of contamination of raw meat. The presence of *Enterobacteriaceae* in raw meat can have origin on animal tissues, since these microorganisms are natural inhabitants of the gastrointestinal tract of animals, or in the factory environment, material or equipment or even also from manipulation practices (García Fontán et al., 2007; Ferreira et al., 2007). The casings used (natural pork intestine) for filling the sausages, can also contribute to the product contamination. The initial contamination of raw material presented identical profile for both producers and ranged between 1.9 and 3.8 log CFU/g. Identical values were found in raw meat used for Italian sausages production (Comi et al., 2005). In general, in the first three stages of processing, the levels increased slightly, which can be related with conditions favourable to the growth of these microorganisms, namely the concentration of nutrients, pH or a_w (Comi et al., 2005; García Fontán et al., 2007). Smoking had a decreasing effect on the *Enterobacteriaceae* population for both producers, although for producer 1 there was a greater reduction, until non-detectable levels. For producer 1, the presence of *Enterobacteriaceae* in final product of batch B can be related with post-

processing contamination due to deficient hygienic practices, since there was only one sample from three that presented enterobacterias. However, in the case of producer 2, the smoking conditions applied during processing appeared to be insufficient to eliminate *Enterobacteriaceae* in final product, and, as a consequence, the presence of these microrganisms in all batches was observed. The reduction/ elimination of *Enterobacteriaceae* during the production process of dry sausages has been reported by several authors (Elías and Carrascosa, 2010; Casquete et al., 2012; Ferreira et al., 2009) as well as the increase of these microorganisms (Comi et al., 2005; Ferreira et al., 2009).

The results of the foodborne pathogens *Listeria monocytogenes* and *Staphylococcus aureus* in "salpicão" samples are presented in Table 4.

Table 4. Pathogenic microorganisms (log CFU/g) in "salpicão" analysed at different stages of production.

	Producer 1			Producer 2		
	Batch A	**Batch B**	**Batch C**	**Batch A**	**Batch B**	**Batch C**
S. aureus						
Diced meat	1.55 ± 0.13	1.53 ± 0.47	2.50 ± 0.17	1.30 ± 0.30	<1	< 1
Batter	1.43 ± 1.25	2.46 ± 0.15	< 1	nd	2.00 ± 1.73	1.43 ± 1.25
Filling	2.36 ± 0.39	2.75 ± 0.08	1.33 ± 1.15	nd	1.10 ± 1.91	2.67 ± 0.19
Smoking	< 1	1.40 ± 0.17	< 1	3.49 ± 0.20	1.53 ± 1.33	2.33 ± 0.35
Final product	2.76 ± 1.81	< 1	nd	2.10 ± 1.83	nd	2.42 ± 0.39
L. monocytogenes						
Diced meat	-	-	+	1.13 ± 0.98	+	< 1
Batter	< 1	1.13 ± 0.98	2.02 ± 0.28	+	+	+
Filling	+	+	2.02 ± 0.28	+	+	+
Smoking	-	-	-	-	-	-
Final product	-	-	-	-	-	-

< 1 mean counts were lower than one

nd not detected

\+ present

\- absent

L. monocytogenes results were different between batches and producers, mainly in the first three stages of the ripening process. In diced meat of producer 1, *L. monocytogenes* was only detected in batch C, while for producer 2 it was present in all batches and quantified in two of them. In batter and filling samples, it was detected in all batches of both producers, and higher values were present in "salpicão" of producer 1. Although *L. monocytogenes* was detected in raw material and after maceration in all batches and for both producers, it was not recovered after smoking. The non-detection/reduction of this foodborne pathogenic during the ripening process of dry fermented sausages has been reported by other authors (Casquete et al., 2012; Lindqvist and Lindblad, 2009; Hajmeer et al., 2011; Linares et al., 2013, Ferreira et al., 2009). Conditions resulting from fermented meats generally inhibit the growth of *L. monocytogenes* due to a combination of several factors such as aw, pH, smoke, spices and fermentative flora and general hygienic measures (Ferreira et al., 2007).

S. aureus was present in samples of both producers. For producer 1, the initial contamination was slightly higher, yet smoking was more efficient in reducing the microbial load than for producer 2. In

fact, smoking was found to be effective against some bacterial pathogens in sausages, due to the high temperatures employed (Hajmeer et al., 2011). However, for producer 2, the longer smoking period (between 18 and 23 days) apparently did not contribute positively to the inhibition of this pathogen. Indeed, as this process was not performed in a continuous way (smoking periods intercalated several times with room temperature), it appeared to influence the amount of *S. aureus* in the final product. This microorganism is one the most common causes of poisoning transmitted by food, particularly by consumption of contaminated meat and milk products, and normally indicate an inadequate or excessive manipulation of food products. According to the Food Safety Authority of Ireland Guidelines (2014), 50% of the batches analyzed would be considered satisfactory (lower than 20 CFU/g), and the remaining batches would be in the borderline (counts of *S. aureus* between 20 and 10^4 CFU/g). According to those guidelines, a batch's hygiene is considered unsatisfactory when the *Enterobacteriaceae* average is above 10^4 CFU/g, which was not found in any of the "salpicão" batches tested.

Salmonella spp. was not detected in any producer and in any of the sausage samples after processing or during ripening. Similar results have already been reported in the traditional dry sausage "salpicão" (Ferreira et al., 2007; Ferreira et al. 2009).

4. CONCLUSIONS

As "salpicão" is a meat product that can be consumed without cooking, it is imperious to carry out more investigations to identify the risk factors that determine the presence of foodborne pathogens in this product. Despite the variations observed during manufacture in both producers, no significant differences were observed in the water activity of the final products.

Even though *L. monocytogenes* and *Salmonella* spp. were not detected in the final products, the microbiological safety of "salpicão" still cannot be assured if the raw meat was highly contaminated and the manufacture practices were not optimized and standardized. In the same way, the current levels of TVC and *Enterobacteriaceae* in producer 2 and the presence of *S. aureus* in the final product of both producers, although not at high levels, hint that there is a need to further control the microbiological quality of the product as well as to reinforce the good practices of hygiene and manufacture in the production of these traditional sausages.

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