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SURVIVAL OF *LISTERIA MONOCYTOGENES* IN LOW ACID ITALIAN SAUSAGE PRODUCED UNDER BRAZILIAN CONDITIONS

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ABSTRACT

Dry sausages have been considered ready-to-eat products with low risk of causing listeriosis due to the hurdles created during the manufacturing process such as low pH and a_w, high salt concentration and presence of lactic acid bacteria (LAB). However, several studies have detected survival of *Listeria monocytogenes* in these products and also shown that process parameters, LAB and *L. monocytogenes* strains directly influence the results. In this work, survival of the pathogen in sausages prepared with three different formulations (one standard formulation, one formulation added of *Lactobacillus plantarum* and one added of 2% sodium lactate), using the manufacturing process usually employed in Brazil, was evaluated. Naturally contaminated sausages presented a small increase in the counts of *L. monocytogenes* in the first days of the process, followed by a gradual decrease until the end of the process. In experimentally contaminated samples containing *L. plantarum*, the reduction of counts of *L. monocytogenes* during processing was considerable, but there wasn´t significant differences between the treatments.

Key words: *Listeria monocytogenes*, *Lactobacillus plantarum*, Fermented dry sausage, Survival, Brazilian salami

INTRODUCTION

Salamis are dry fermented sausages manufactured with pork, pork fat, salt, ripening adjuvants and spices. This kind of sausage is considered a ready-to-eat product, because it does not require any thermal treatment befora comsumption (5,10, 18,22). The manufacturing process varies according to the production region and regional consumer preferences (1,8,25,28).

Fermented meat products have been considered safe products (23) . During fermentation the acid lactic bacteria (LAB) uptake of the oxygen in the raw matter, decreasing the redox potential and turning nitrite into a more effective tool to prevent the growth of aerobic spoilage and pathogenic bacteria. In addition, low pH causes a decrease in the protein water holding capacity, accelerating sausage dehydration and leading to low a_w and high NaCl concentration in the final product (30,31).

Listeria monocytogenes has been considered the most important foodborne pathogen due to the high death rate in risk groups (29). During fermentation and drying, the count of *L.monocytogenes* in sausages tends to decrease because of a set of hurdles created in the manufacturing process (8,30). However, *L. monocytogenes* is often isolated from fermented meat products, due to the capability to survive to the adverse conditions of this type of product (2,3,12,33).

Due to the lack of studies on the behavior of *L. monocytogenes* in Brazilian fermented meat products, the aim of this study was to evaluate the survival of *L. monocytogenes* in low acid Italian sausages produced under Brazilian conditions and in the presence of intentionally added inhibitory compounds.

MATERIAL AND METHODS

Raw meat, spices and ingredients were obtained in a pork slaughter and industrial plant in Videira-SC, Brazil. The commercial strain of *Lactobacillus plantarum* (Holbac 100™) was supplied by Danisco Brasil LTDA.

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Preparation of the inoculum

The *L. monocytogenes* strain ATCC 7644 (OXOID C3970L) was resuspended in Brain Heart Infusion – BHI (MERCK 1.10493) and incubated overnight at 36ºC. The culture was transferred to Trypticase Soy Agar – TSA (OXOID CM 131) and kept under refrigeration. For usem, the cultures were transfered to BHI broth, incubated overnight at 36ºC, and then submitted ot decimal dilutions in 0.85% sterile saline. The diluted culture containing 10^4 UFC mL^{-1} was kept under refrigeration until the moment of use.

Preparation of sausages

Three different formulations of sausages were prepared: one standard formulation with no inhibitor additive, one formulation with *Lactobacillus plantarum* and one with 2% sodium lactate. Each formulation was performed in two batches: one control batch and one added of *Listeria monocytogenes* ATCC 7644 (Table 1). Assays were performed in duplicates.

Table 1. Codification of sausage batches prepared to evaluate survival of *Listeria monocytogenes*.

Formulation	Batches	Batch description
A – Standard	A1	Raw mixture standard
	A2	Raw mixture standard + *L. monocytogenes*
B – *Lactobacillus plantarum*	B1	Raw mixture + *L. plantarum*
	B2	Raw mixture + *L. plantarum* + *L. monocytogenes*
C – Sodium Lactate	C1	Raw mixture + Sodium Lactate
	C2	Raw mixture + Sodium Lactate + *L. monocytogenes*

The six batches (A1, A2, B1, B2, C1, C2) were prepared with the same raw components: pork meat (74.9%), pork fat (16.0%), salt (2.8%), curing salt (sodium nitrate and nitrite) (0.3%), sodium eritorbate (0.04%), monosodium glutamate (0.25%), maltodextrin (0.5%), dried milk (4.0%), glucono delta-lactone (GDL) (1.2%) and *Staphylococcus carnosus* (BACTOFERM SB 61 – Chr. Hansen) (0.025%).

The dry sausages were prepared in two steps. In the first step, a mixture was prepared in a cutter (MADO Granrant®) with pork (15%), salt (10% of the entire content), frozen pork fat, ground with the half graining desired. In the second step, *L. monocytogenes* inoculum (A2, B2, C2 batches), cultures, spices and additives were added and the cutting continued until the particles were about 5 mm in diameter. Then, the minced mixture was stuffed (stuffer Heinrich Frey Maschinenbau GmmH – Henry 20®) in collagen casings (43 mm), making pieces of 350-400 g. Sausages were hung in a climate-controlled chamber for fermentation and drying.

Batches B1 and B2 were added of 0.025% Holbac 100® (DANISCO) dissolved in 50mL of distilled water. Batches C1 and C2 were added of 2.0% sodium lactate (Purasal - PURAC).

Technological parameters

Fermentation and drying were performed in a climate-controlled chamber (Reich®). For ripening, the chamber temperature and relative humidity were 22-24ºC and 94-98%, respectively, during 48 h. The conditions for fermentation and drying up to 28 days are shown in Table 2.

Table 2. Technological parameters for ripening, fermentation and drying of sausages

Day	Temperature (Cº)	Relative Umidity (RU%)
1, 2	22-24	94-98
3	20-22	92-96
4	18-20	90-94
5	16-18	88-92
6	12-14	85-90
7 - 28	12-14	82-87

Microbiological analysis

Samples were taken from each batch after 0, 7, 14, 21 and 28 days of production. Portions of 25 g were homogenized with 225 mL of Buffered Peptone Water (OXOID CM 509) using a stomacher (model 400, Seward Medical, England). Further decimal dilutions were prepared with Buffered Peptone Water (OXOID CM 509). The counts of *L. monocytogenes* were determined by the most probable number dilution technique (MPN), (3 tubes each at - 1,0 g, 0,1 g, 0,01 g, 0,001 g e 0,0001 g inocula), using Modified *Listeria* Enrichment Broth (ACUMEDIA 7409A) for pre-enrichment (24 h at 30ºC), Fraser broth (ACUMEDIA 7502A) for a second enrichment (48 h at 35ºC) and *Listeria* selective agar (ALOA – BIOLIFE 404605) (24-48 h at 35ºC) for isolation (32). Three to five typical colonies of *L. monocytogenes* were submitted to catalase test, motility by microscopy (32), CAMP test (Tryptic Soy Agar - OXOID CM 131 added of sheep blood - NEWPROV), acid production from L(+)-rhamnose (Phenol-red Broth Base - MERCK 1.10987, L(+)-rhamnose - MERCK 1.04736) and *Listeria O Antisera Types 1,4* (DIFCO 223021). LAB were counted in MRS Agar (OXOID CM 361) incubated att 30ºC for 48 to 72 h in microaerophilic conditions (26). Typical colonies were confirmed by catalase reaction.

Chemical analsysis

Sausage samples were ground and homogeneized in adequate miller. pH was measured inserting the spear electrode of a pH meter (model 410 - 060547, ORION) into the samples. Aw was measured calculating water and salt percentages according to Krispien, Rödel and Leinster (15). Moisture was determined by gravimetry at 105ºC until constant weight and chloride was determined by a mercurometric method (4).

Statistical analysis

Three repetitions of each batch were carried through. The results of *Listeria monocytogenes* were expressed as log NMP g^{-1} and the counts of LAB were expressed as log$CFUg^{-1}$. The data of pH, Aw, NMP of *Listeria monocytogenes* and LAB counts were submitted to analysis of variance (ANOVA) using Statistica software version 6.0. Tukey´s Test was applied when the detected difference among the values was significant with 5% of significance ($p<0.05$).

RESULTS AND DISCUSSION

pH and a_w

The differences in pH and Aw for the three formulations (Table 3) were not significant ($P>0.05$). The decrease of pH until the 14th day, a rise in the 21st day and then a stabilization up to the 28th day in all formulations were considered normal in the Brazilian salamis manufacturing process. The decrease is a consequence of the the activity of naturally occurring LAB and the subsequent rise is caused by proteolysis and lipolysis, probably performed by yeasts, also responsible for the development of the maturation characteristic flavor (19,29,34).

Aw reduction during ripening in all formulations was similar, but from the 14th day on, the Aw decrease in formulations C1 and C2 was more intense than in the other formulations. This fact can be explained by the humectant effect of the sodium lactate, which increases the water retention capacity causing the reduction of Aw (24).

LAB Counts

Counts of LAB (Table 3) ranged from 6.0 to 7.0 log $CFUg^{-1}$, with no differences between the batches ($p>0.05$), except of batch A1. Except for batches B1 and B2, the LAB detected in the other batches originated from the raw material from the production site.

Survival of* L. monocytogenes *in naturally contaminated samples

As shown in Fig. 1, *L. monocytogenes* counts in naturally contamined sausages (batches A1, B1 and C1) were very low, as already reported by Peccio (20) and Silva (27). The *L. monocytogenes* curves for batches A1 e B1 were different from those of the other batches. In batch B1, the initial count was higher than in batches A1 and C1, and the difference between the beginning and the end of the process was 2.42 log. For this batch, a constant decrease until the 14th day was observed, followed by a small increase at the 21st day and a rapid decrease at the 28th day. In batch A1, the difference in counts between the beginning and the end of the process was only 0.04 log. In this batch, the counts increased until the 7th day, and decreased afterwards, with a very small increase at day 28. Campani et al. (6) presented similar results when comparing the effect of two *L. plantarum* strains, one bacteriocin-producing and another non-bacteriocin-producing, on the survival of *L. monocytogenes* during the manufacture of Italian sausage. The growth of *L. monocytogenes* in batch A1 in the first week may be a response to the gradual acidity increase and lack of hurdles, which is expected in strains adapted to environmental stress (21,35).

In batch C1, there ere no *L. monocytogenes* counts, certainly because raw material contamination was under the detection limit of the analytical method.

Table 3. LAB counts, pH and Aw values during the ripening and maturation of sausages.

Batch	Day 0			Day 7			Day 14			Day 21			Day 28		
	LAB	pH	A_w	*LAB*	pH	A_w	*LAB*	pH	A_w	*LAB*	pH	A_w	*LAB*	pH	A_w
A1	5.00	5.61	0.958	5.97	4.99	0.938	6.88	4.93	0.916	6.35	5.08	0.895	5.80	5.16	0.892
A2	5.48	5.74	0.959	6.43	5.03	0.941	6.80	4.82	0.917	6.37	5.12	0.901	6.40	5.12	0.897
B1	6.21	5.62	0.958	6.87	5.08	0.939	7.34	4.89	0.917	7.05	5.03	0.898	6.95	5.17	0.895
B2	6.17	5.74	0.958	6.85	5.07	0.940	7.24	4.91	0.917	7.03	5.06	0.899	6.78	5.19	0.896
C1	6.00	5.60	0.959	6.74	5.06	0.937	6.60	4.76	0.915	6.40	5.07	0.885	6.00	5.19	0.883
C2	6.26	5.36	0.959	6.98	5.08	0.937	6.93	4.84	0.915	6.59	5.15	0.885	6.43	5.10	0.883

A1: Batch A1 – standard formulation; A2: Batch A2 – standard formulation inoculated with *L. monocytogenes*; B1: Batch B1 – standard formulation inoculated with *L. plantarum*; B2: Batch B2 – standard formulation inoculated with *L. plantarum* and *L. monocytogenes*; C1: Bacth C1 – standard formulation added of 2% sodium lactate; C2: Batch C2 – standard formulation added of 2% sodium lactate and inoculated with *L. monocytogenes*.

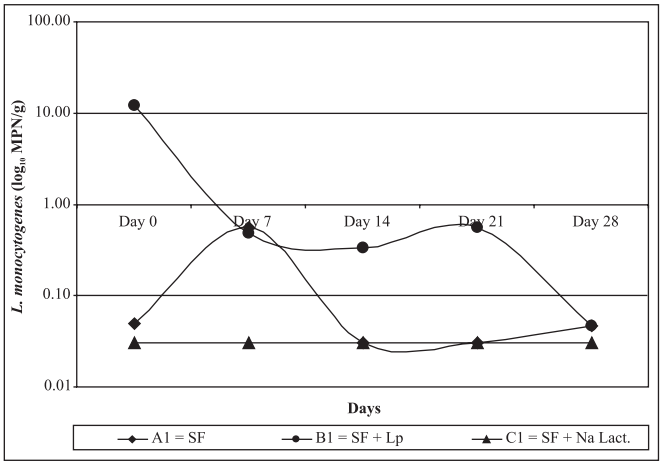

Figure 1. Survival of *L. monocytogenes* in naturally contaminated Italian sausages during fermentation and maturation time. A1 = SF: Batch A1 – standard formulation; B1 = SF + Lp: Batch B1 – standard formulation inoculated with *L. plantarum*; C1 = SF + Na Lact: Bacth C1 – standard formulation added of 2% sodium lactate.

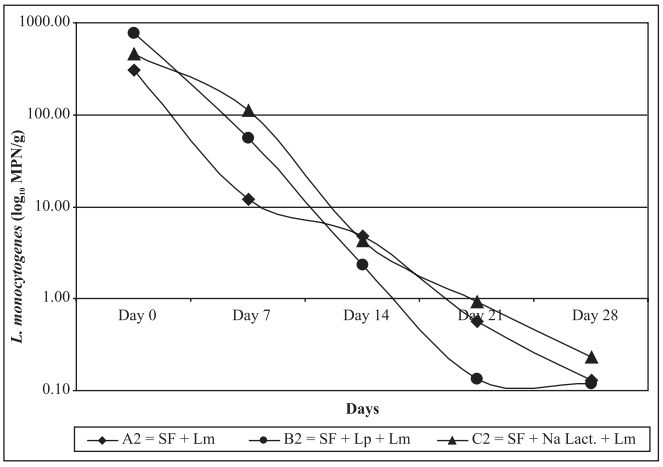

Figure 2. Survival of L. monocytogenes in experimentally contaminated Italian sausages during fermentation and maturation time. A2 = SF + Lm: Batch A2 – standard formulation inoculated with L. monocytogenes; B2 = SF + Lp + Lm: Batch B2 – standard formulation inoculated with L. plantarum and L. monocytogenes; C2 = SF + Na Lact. + Lm: Batch C2 – standard formulation added of 2% sodium lactate and inoculated with L. monocytogenes.

Survival of* L. monocytogenes *in experimentally contaminated samples

The behavior of *L. monocytogenes* in experimentally contaminated sausages (batches A2, B2 and C2) is presented in Fig. 2. All batches presented a continual decreasing in the count curves with no significant differences between them ($p>0.05$). The difference between the initial and the final *L. monocytogenes* counts for batches A2, B2 and C2 were 2.57 log, 3.81 log and 3.3 log, respectively.

Comparing the two batches of standard formulation (A1 naturally contaminated and A2 experimentally contaminated), differences in the two *L. monocytogenes* count curves were noted. Batch A1, which presented a lower initial count, which increased in the first 7 days and decreased gradually afterwards. On the other hand, batch A2 with higher initial count, presented a constant decrease during the maturation time. This is probably due to the typical trait of the different strains present in each batch. While batch A2 was manufactured with a control strain (ATCC 7644) isolated from humans, batch A1 contained one or more native *L. monocytogenes* strains from raw meat or processing plants.

Batch B2, manufactured with a *L. plantarum* strain, presented a *L.monocytogenes* growth curve that decreased until the 14th day, when the performance was better than in the other batches. At day 21, the *L. monocytogenes* counts were lower than in batches A2 and C2. Batch B2 presented the best performance if compared to batches A2 and C2, once it had the highest *L. monocytogenes* initial count and the lowest count at the end of the experiments.

The survival of *L. monocytogenes* in batch C2 was similar to batches A2 and B2, showing a 3.3 log count reduction during the process.

Several studies have evaluated the efficiency of sausage manufacturing process in controling *L. monocytogenes*, and their results differ considerably (7,8,17,18,25,29,31). These differences range between the efficacy of the process in decreasing and increasing *L. monocytogenes* populations and are related to the parameters used in each case (17) and the features of the strains in each experiment (29).

There are hurdles, during the manufacturing process, which could act synergically creating an inhibitory environment for the pathogens (16). In the fermentation stage, the decrease of pH represents an important hurdle to the growth and survival of *Listeriae* (29). However, in studies where LAB were not used, Chikthimmah *et al.* (7) and Glass & Doyle (11) reported a growth of Listeriae at this stage. In drying and maturation stages, even at low pH, there was a decrease of A_w and an increase of salt concentration (29). In addition, the presence of bacteriocin-producing and non-bacteriocin-producing LAB cultures is another important hurdle (6).

There are many important reasons to explain *L. monocytogenes* survival during the manufacturing process and at the final product: its ability in becoming acid-resistant (3,22) and, according to several studies, the effect of initial population size on the *L. monocytogenes* survival under stressful

conditions (13,14,17,18). This ability is related to its pathogenicity and it's usually found in strains isolated from fermented food or from meat processing facilities (35).

Under the fermentation and maturation conditions employed in this work, the decrease of *L. monocytogenes* counts in Italian sausage is less intense than that reported for other European studies (8,18,31), where an expressive decreasing was observed at the beginning of the process. This is probably due to the lowe pH and higher water activity achieved in these processes. When occuring in the first days the increase of *L. monocytogenes* is higher than that observed in other studies (6).

Although no significant differences were detected among the three artificially contaminated batches ($p > 0.05$), batch B2 (inoculated with *L. plantarum*) presented a slightly different reduction in counts of *L. monocytogenes* when compared to the standard batch (A2). The curve of the C2 treatment (added of sodium lactate) was very similar to the standard curve. Therefore, the use of bioprotective cultures such as *Lactobacillus plantarum* is highly recommended in commercial production of Italian sausages. However, the use of sodium lactate must be better evaluated, mainly when used with other inhibitory substances.

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RESUMO

Sobrevivência de *Listeria monocytogenes* em salame tipo italiano de baixa acidez, produzido sob condições brasileiras de fabricação

Salames têm sido considerados produtos prontos para o consumo com baixo risco de provocar listeriose devido aos obstáculos criados no processo de fabricação e suas características de pH e atividade água baixos, alta concentração de sal e presença de bactérias lácticas. Entretanto, a sobrevivência de *Listeria monocytogenes* nesta classe de produtos é verificada e estudos de processo visando à redução da contaminação por este patógeno, têm demonstrado que particularidades como variação dos parâmetros de processo, cepas de bactérias lácticas e de *L. monocytogenes* influenciam diretamente os resultados. Neste estudo três formulações foram avaliadas (uma padrão, uma com inoculação da cultura *Lactobacillus plantarum* e outra com adição 2% de lactato de sódio) empregando parâmetros de processo comumente praticados no Brasil. Os salames naturalmente contaminados apresentaram discreto aumento da população de *L. monocytogenes* no início do processo, seguidos por redução até o final da maturação. Os salames artificialmente contaminados tiveram redução considerável da contagem de *L. monocytogenes* não havendo diferenças significativas entre os tratamentos.

Palavras chave: *Listeria monocytogenes*, *Lactobacillus plantarum*, embutido seco fermentado, sobrevivência, salame brasileiro.

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