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Effect of *Lactobacillus plantarum* starter cultures on the behavior of *Listeria monocytogenes* during sausage maturation

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Abstract

The objective of this study was to evaluate the effect of a bacteriocinogenic starter culture against *L. monocytogenes* in artificially contaminated sausages. During the maturation of sausages the following procedures were carried out: physical-chemical analysis, lactic acid bacteria counts, and enumeration of *L. monocytogenes*. In the treatment inoculated only with a pool of *L. monocytogenes*, the multiplication of the pathogen was verified during the 19 days of maturation and the increase in the pathogen population was 2.01 log CFU.g^{-1}. In the treatments inoculated with starter cultures of bacteriocinogenic and bacteriocin-negative *L. plantarum*, we observed a 1.7 log CFU.g^{-1} reduction in levels of *L. monocytogenes* during the sausages maturation. However, no significant differences ($P > 0.01$) were observed between the treatments inoculated with starter cultures. The pH values of the control treatment remained almost constant at 6.07 during the maturation, differing significantly ($P < 0.01$) from treatments inoculated with starter cultures.

Introduction

The production and consumption of raw fermented sausage in southern Brazil is significant. Even in light of this, an identity and quality standard for this type of product was created (Brazil, 2000), allowing a typical feature, keeping the colonial designation. However, the inexistence of minimum standards for pH and water activity maximum in the legislation, may favor pathogen development making the safety of this product doubtful. Studies have shown that the genus *Listeria* is commonly found in fermented raw meat products, which can make this type of product a problem for public health (Gianfranceschi *et al*., 2006; Degenhardt; Sant'anna, 2007).

L. monocytogenes is a Gram-positive non-sporulating food pathogen. It is especially dangerous for very young (children), old, pregnant and immunocompromized persons. Despite the various hurdles in the dry sausage manufacturing process, it is able to survive the commercial manufacturing process (Työppönen *et al*., 2003). During the fermentation and drying of sausages, the numbers of *L. monocytogenes* tend to decrease substantially. However, the survival of the micro-organism may occur in fermented products even under unfavorable conditions such as low pH, low water activity (a_w), and elevated sodium chloride levels (Johnson *et al*., 1988).

It is thus not surprising that in more recent years the focus has shifted towards the selection of starter cultures with the ability to produce bacteriocins. Bacteriocins produced by lactic acid bacteria (LAB) are antibacterial peptides or proteins that kill or inhibit the growth of other Gram-positive bacteria. The application of bacteriocin-producing LAB in the meat industry therefore offers a method of natural food preservation (Leroy *et al*., 2006). Considering that the survival of *L. monocytogenes* may constitute a potential risk to consumer health, the present study aimed to assess the antimicrobial activity of a bacteriocin-producing *L. plantarum* strain against *L. monocytogenes*, in comparison with the bacteriocin-negative variant during the fermentation and drying of colonial sausages.

Materials and Methods

Preparation of inocula

The cultures of lactic acid bacteria (LAB) used as a starter culture in the preparation of sausages were: a culture of *Lactobacillus plantarum* bacteriocin-producing (Bac$^+$), previously evaluated for anti-listerial activity (Zanette *et al*., 2011) and a culture of *L. plantarum* non-bacteriocin producing (Bac$^-$) that was used as a negative control for the formation of

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bacteriocin. The cultures were isolated from naturally fermented sausage and presented technological characteristics making them suitable for use as starters (Dalla Santa *et al.*, 2012).

The LAB cultures were preserved at -20°C in Man Rogosa Sharpe broth (Difco) with 20% (v/v) glycerol. Prior to the experiment, *L. plantarum* strains were cultured twice in 10 ml MRS broth at 30°C for 18–24 h. A loop-full was streaked onto MRS agar and incubated at 30°C for 24 h. One isolated colony was transferred to 100 ml of MRS broth and incubated at 30°C for 24h. The cells were harvested by centrifugation (10,844 g for 10 min), washed twice, and resuspended in 0.85% (w/v) NaCl solution. Each culture was added to give a final concentration in the meat mixture of 6 log CFU/g.

Four different strains of *L. monocytogenes* (three isolated from fermented sausage and one ATCC 7644) were used in this study. Cultures were stored under refrigeration in TSA-YE medium. When needed, each strain was transferred to 10 ml of tryptone soya broth containing 0.6% of yeast extract (TSB-YE) and incubated at 37°C for 24h. A loop-full was streaked onto TSA-YE and incubated at 37°C for 24 h. One isolated colony was transferred to 100 ml of TSB-YE and incubated at 37°C/24 h. Each culture was diluted in 0.85% (w/v) NaCl solution to give a final concentration in the meat mixture of 4 log CFU/g. Immediately prior inoculation into the meat mixture, the four inocula were mixed.

Preparation and inoculation of colonial sausage

The formulation used was a colonial sausage type and had the following composition: pork meat (85%), pork fat (15%), salt (2.8%), curing salt (0.6%), glucose (0.5%), dextrose (0.5%), sodium eritorbate (0.25%), black pepper (0.1%), garlic (0.05%) and nutmeg (0.02%). The products were prepared in a pilot plant according to commercial processing methods. The pork meat and fat were minced with a 10 mm grinding disc (C.A.F®). The meat mixture was taken to the mixer (Beccaro®, model MB-25I) and the ingredients were added and mixed for 5 minutes. The mixture was divided and six treatments were prepared according to Table 1. Processing was repeated three times with each treatment.

For each trial, the batter was first prepared with the negative control (non-inoculated), followed by the positive control (inoculated with the Bac^- starter) and then the test samples (inoculated with Bac^+ starter) to ovoid carryover of the bacteriocin-producing strain into the batter intended to contain the Bac^- culture. The minced mixture of each treatment was stuffed (Jamar EJVI-15) into collagen casings (45 mm), making pieces of 350-400 g. Sausages were hung in a climate-controlled chamber (Instalafrio®) for fermentation and drying.

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

Treatment	Batch description
A	Raw mixture standard
B	Raw mixture standard + *L. monocytogenes*
C	Raw mixture standard + *L. plantarum* (Bac^-)
D	Raw mixture standard + *L.plantarum* (Bac^-) + *L. monocytogenes*
E	Raw mixture standard + *L. plantarum* (Bac^+)
F	Raw mixture standard + *L. plantarum* (Bac^+) + *L. monocytogenes*

On the first day the temperature was maintained at 25°C with a relative humidity of 95%. The values of temperature and relative humidity were gradually reduced in accordance with the following conditions: 24°C/93% (2nd day), 23°C/90% (3rd day), 22°C/85% (4th day), 21°C/80% (5th day), 20°C/75% (6th day) and 18°C/75% (7th day). After 7 days the weather conditions were maintained at a constant until the 19th day of ripening. Two samples were taken from each treatment after 0, 3, 5, 7, 13, 16 and 19 days of production to perform microbial counts and physico-chemical analysis.

pH measurement

The pH values of colonial sausages were measured using a digital potentiometer (Marte, MB-10). Fifty grams of sample was homogenized in 10 ml of distilled water for two minutes and the reading was taken after five minutes of stabilization.

Water activity (a_w)

Water activity was measured using AQUALAB LITE® equipment by direct measurements on previously ground samples.

Microbiological analysis

LAB were counted in MRS Agar (DIFCO) incubated at 30ºC for 48 to 72 h in microaerophilic conditions. The detection and enumeration of *L. monocytogenes* was carried out according to ISO 11290-1 and 11290-2 methods, respectively (Anonymous, 1998, 1996). For the identification of *Listeria* spp., typical colonies in Agar Listeria Ottavani and Agosti (ALOA) and Palcam agar plates were submitted to Gram staining, catalase production, motility testing at 25°C, hemolysis on blood sheep agar and fermentation of rhamnose and

xylose (Anonymous, 1998, 1996).

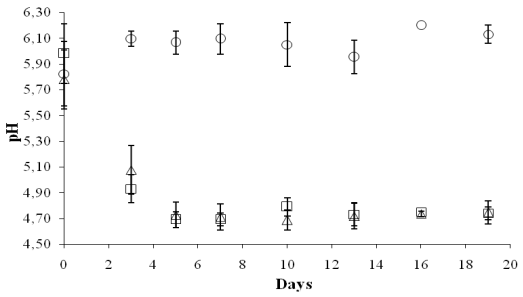
Statistical analysis

Three repetitions of each treatment were carried through. The counts of LAB and *L. monocytogenes* were expressed as log CFU g^{-1}. The data of pH, Aw, LAB and *L. monocytogenes* counts were submitted to analysis of variance (ANOVA) using SigmaStat for Windows® Version 3.0.1. Tukey´s Test was applied when the detected difference among the values was significant with a 1% level of significance ($p<0.01$).

Results and Discussion

pH and water activity

The pH values over the course of sausage manufacturing are illustrated in Figure 1.



Treatment identification: ○ – A; □ – C ; Δ- E. Values are the means of three experiments, ±standard deviation (error bars)

Figure 1. pH development during sausage maturation

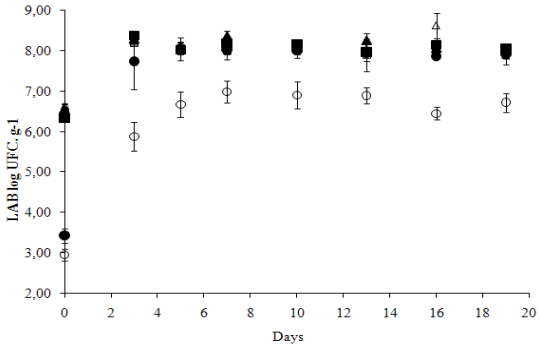
The initial pH values (day 0) of the treatments did not differ (P> 0.01) between them, with values close to 5.7. After 5 days of fermentation, the pH of the treatments C and D (inoculated with *L. plantarum* Bac^-) decreased from 5.98 to 4.69. In treatments E and F (inoculated with *L. plantarum* Bac^+), the pH decreased from 5.78 to 4.71. The pH values of the treatments were maintained until the 19th day of processing, with no significant difference (P> 0.01) between treatments, indicating that the production of lactic acid by cultures was similar.

A rapid pH drop to below 5.3 proved to be important for the inhibition of pathogens. The low pH value is the basis for safety as well as the texture and color of dry sausages (Lücke, 2000; Erkkilä *et al*., 2001; Työppönen *et al*., 2003). In treatments A and B (not inoculated with LAB), pH values were found to increase from 5.82 to 6.13 at the end of processing. The pH values during the 19 days of processing were statistically different (P <0.01) from the treatments inoculated with *L. plantarum* (Bac^- and Bac^+) and without addition of LAB treatment. Campanini *et al*. (1993) also found that pH in salami cultures not inoculated with *L. plantarum* showed no decline during the ripening process, as the values remained between 5.7 and 5.8.

In fermented sausages, a decrease in pH occurs primarily by the accumulation of lactic acid, resulting from the metabolism of carbohydrates added in the meat mixture, which occurs during the fermentation by the action of lactic acid bacteria (Lücke, 2000). Aw reduction during ripening in all treatments was statistically similar (P >0.01) and a linear aw decrease was observed from 0.95 to 0.82 at the end of maturation period.

LAB counts

The evolution of the population of LAB in colonial sausages is shown in Figure 2.



Treatment identification: ○ – A; ●- B; □ C-; ■ – D; Δ- E; ▲- F. Values are the means of three experiments, ±standard deviation (error bars)

Figure 2. LAB counts during sausage maturation

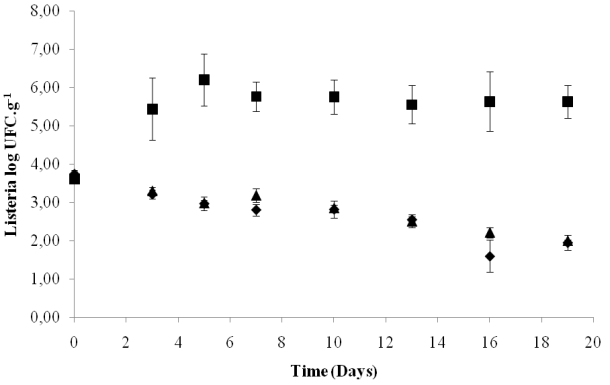
At the beginning of the fermentation, treatments A and B (not inoculated with starter cultures) showed counts of 2.95 and 3.43 log CFU g^{-1}, respectively. According to Coppola *et al.* (2000) and Papamanolli *et al.* (2003), in sausages produced without the addition of starter cultures, the LAB count is less than 4 log CFU.g^{-1} at the beginning of fermentation. Treatments C, D, E and F (inoculated with starter cultures) showed an initial count ranging from 6.33 to 6.64 log CFU g^{-1}, which was significantly (P <0.01) higher than non-inoculated treatments.

After 7 days of maturation, treatment A had a LAB count of approximately log 7 CFU g^{-1}. This population was maintained until the end of the process of maturation and was significantly lower (P <0.01) than other treatments. Treatments B,C, D, E and F had a LAB count of approximately log 8 CFU g^{-1} after 3 days of processing. Values remained constant until the end of processing, with no significant difference (P> 0.01) among treatments. Similar counts of *L. plantarum* in fermented sausage were observed in

studies by Työppönen *et al*. (2003) and Sawitzki *et al.* (2008). These results indicate that cultures of *L. plantarum* Bac$^-$ and *L. plantarum* Bac$^+$ are able to multiply in colonial sausages and compete with microbial contaminants. According to Schillinger and Lücke (1989), lactic acid bacteria originally isolated from meat and meat products are probably the best candidates for improving the microbiological safety of these foods, because they are well adapted to the conditions in meats and should therefore be more competitive than lactic acid bacteria from other sources.

Survival of L. monocytogenes *in experimentally contaminated treatments*

During ripening of colonial sausages in treatment B (inoculated only with the pool of *L. monocytogenes*), growth of the pathogen was observed, as can be seen in Figure 3.



Treatment identification: ●- B; ■ – D; ▲- F. Values are the means of three experiments, ±standard deviation (error bars)

Figure 3. Survival of *L. monocytogenes* in experimentally contaminated sausages during fermentation and maturation time

The initial count was 3.62 log CFU.g^{-1} and on the 5th day of maturing population of *L. monocytogenes* reached the maximum count of 6.21 log CFU.g^{-1}. At the end of the maturation step, the difference between the final and initial population was 5.63 logCFU.g^{-1}, and the increase in the population of *L. monocytogenes* was 2.01 log CFU.g^{-1}. The counts obtained at different times of maturation evaluated in the treatment inoculated only with the pool of *L. monocytogenes* were significantly ($P < 0.01$) higher than those obtained in treatments D and F (inoculated with starter culture).

Several studies have evaluated the efficiency of the sausage manufacturing process in controlling *L. monocytogenes*, but results from these studies vary considerably and are most likely due to the different process parameters used in each study (Nightingale *et al.*, 2006). Degenhardt; Sant'anna (2007) reported a reduction of 2.57 log CFU.g^{-1} of initial levels of *L. monocytogenes* throughout a 28-day fermentation period during the manufacture of Italian sausage produced under Brazilian conditions. Campanini *et al*. (1993) reported growth (ca. 1.0 log CFU/g) of *L. monocytogenes* during the fermentation of Italian salami at 18°C and 78% RH for 7 days. Farber *et al*. (1993) reported a slight increase in *L. monocytogenes* populations during the fermentation and drying of Italian style fermented sausages made without a starter culture.

This study verified a high increase in the population of *L. monocytogenes* (2.01 log) inoculated into sausage colonies compared with previous studies (Farber *et al.*, 1993; Campanini *et al*., 1993). The inoculum of *L. monocytogenes* used in the preparation of sausages was composed of a colonial strain ATCC 7644 and three cultures isolated from fermented sausage. A possible adaptation of these cultures to hurdles in fermented sausages (low pH, low a_w, and high concentration of NaCl) may have contributed to the growth of the pathogen on the product.

In treatments D and F (inoculated with starter culture), a reduction in the population of *L. monocytogenes* during ripening of colonial sausages was observed, which was not seen in the treatment without starter culture (B). The reduction in the population of *L. monocytogenes* for treatment D (*L. plantarum* Bac$^-$) was 1.71 log and 1.79 log for treatment F (*L. plantarum* Bac$^+$), with no statistically significant difference ($P > 0.01$) between them. Similar results obtained in this study were also observed by Campanini *et al.* (1993). The use of starter cultures of *L. plantarum* bacteriocin-producing (Bac$^+$) in sausage artificially contaminated with *L. monocytogenes* did not significantly reduce the pathogen population when compared with cultures that were not bacteriocin-producing (Bac$^-$).

In this study, a higher reduction of the count of *L. monocytogenes* in treatments with added bacteriocin-producing starter cultures than in the negative control (*L. plantarum* Bac$^-$) was expected. *In vitro* results demonstrated the production of the bacteriocin isolated from *L. plantarum* and anti-listerial activity. However, the bacteriocin activity was not detected when the isolate was inoculated in colonial sausage (data not shown).

Studies show that the bacteriocin activity of lactic acid bacteria in a food matrix is less effective when compared with results obtained in vitro (Schillinger; Kaya; Lücke, 1991). Factors that contribute to a reduction of bacteriocin activity in foods are: spontaneous loss of bacteriocin-producing ability, poor solubility and uneven distribution in the

food matrix, binding to food components such as fat particles or protein and pH effects on bacteriocin stability and activity.

Conclusion

The addition of starter cultures of *L. plantarum* in colonial sausages caused a significant reduction of *L. monocytogenes*, regardless of whether or not bacteriocins were produced. The addition of starter cultures in colonial sausages should be considered as the main obstacle to the development of *L. monocytogenes*; its effect may be potentiated by obstacles resulting from the manufacturing process of the product.

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