

## INCORPORATION OF NISIN IN NATURAL CASING FOR THE CONTROL OF SPOILAGE MICROORGANISMS IN VACUUM PACKAGED SAUSAGE

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### ABSTRACT

This study aimed to evaluate the effectiveness of natural casing treatment with nisin and phosphoric acid on control of spoilage microorganisms in vacuum packaged sausages. Ovine casings were dipped in the following baths: 1) 0.1% food grade phosphoric acid; 2) 5.0 mg/L nisin; 3) 0.1% phosphoric acid and 5.0 mg/L nisin; and 4) sterile water (control). The sausages were produced in a pilot plant, stuffed into the pretreated natural casings, vacuum packaged and stored at 4 and 10 °C for 56 days. The experiments were performed according to a full factorial design 2<sup>3</sup>, totalizing 8 treatments that were repeated in 3 blocks. Aerobic plate counts and lactic acid bacteria analysis were conducted at 1, 14, 28, 42 and 56 days of storage. Treatment of casings with phosphoric acid 0.1% alone did not inhibit the growth of lactic acid bacteria and reduced the aerobic plate count by 1 log. The activity of nisin against lactic acid bacteria was enhanced by the addition of phosphoric acid, demonstrating a synergistic effect. Furthermore nisin activity was more evident at lower storage temperature (4 °C). Therefore treatment of the natural casings with nisin and phosphoric acid, combined with low storage temperature, are obstacles that present a potential for controlling the growth of lactic acid bacteria in vacuum packaged sausage.

**Key words:** Nisin, Bacteriocin, Sausage, Natural casing

### INTRODUCTION

In recent years, consumers are demanding for more convenient foods, minimally processed, with a fresh appearance, free of chemical additives, with an extended shelf life, but also safe. To prevent the growth of pathogens and reduce the microbial spoilage, a new alternative is biopreservation, based on the use of microorganisms and/or their metabolic products to inhibit the growth of undesired microorganisms in foods (8, 15).

Bacteriocins are antimicrobial peptides that are produced by gram-positive and gram-negative species of microorganisms. However only bacteriocins produced by food-grade lactic acid bacteria are of particular interest to the food industry as biopreservatives (12). Nisin is a bacteriocin generally recognized as safe (GRAS) by the US Food and Drug Administration and World Health Organization since 1969. It is produced by certain strains of *Lactococcus lactis* subsp. *lactis*, and is commercially available for use in foods (14, 21). Nisin is a heat stable peptide, with 34 aminoacid residues, and

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a molecular weight of 3510 Da. It is classified as a lantibiotic, due to presence of lanthionine and  $\beta$ -methylanthionine residues in the molecule. These unusual residues form covalent bridges between amino acids, resulting in internal rings responsible for the characteristic structural features (8). Nisin is active against gram-positive bacteria, but is not effective against gram-negative, yeast and mold (20).

Nisin has been shown to be effective in controlling spoilage and pathogenic bacteria in many meat products (6, 7, 16, 19, 23, 24, 26, 34, 36). However nisin activity may be affected by many factors, such as concentration, the target microorganisms, interaction with food components, fat content and phosphate type, processing and storage conditions of food (5, 10, 35).

In minimally heat-treated, cured, vacuum-packaged and chilled meat products, such as Frankfurters and Vienna sausages, nisin can be applied to inhibit spoilage by lactic acid bacteria selected by the low storage temperature, presence of nitrite and curing salts, and microaerophilic conditions (10, 13).

As the growth of spoilage microbial population on vacuum packed cured meats occurs essentially on the surface of the products, the effectiveness of an antimicrobial treatment could be enhanced by applying the antimicrobial compound to the surface of the cooked meats (18). This could be achieved by dipping products in an antimicrobial bath (17), applying a spray (9), coating by an edible gel (11), or using a packaging system (20, 33). Moreover surface addition of bacteriocins facilitates distribution through the product and reduces interaction with meat component, improving the antimicrobial activity (2, 28).

In Brazil, nisin was authorized to be applied, by immersion or spraying, on the surface of frankfurters at the end of the thermal processing step at a level of 200 mg/L of Nisaplin® (commercial nisin preparation from Danisco), dissolved in 0.1% food grade solution of phosphoric acid (15).

In this study, a new alternative of applying antimicrobials to sausage surface was evaluated. Nisin, phosphoric acid and the combination of the two were incorporated to sausage

natural casings, at the soaking step, and the effectiveness of these treatments for the control of lactic acid bacteria and total aerobic counts was evaluated during the storage of vacuum package sausages. The influence of storage temperature was also evaluated.

## MATERIALS AND METHODS

### Natural casing preparation and its activity

Ovine natural salted casing was provided by Kienast & Kratschmer (Kraki, Brazil) and stored at 4 °C. Before use, casings were washed and soaked for 2 h in potable water to remove the salt. Subsequently, casings were divided in 4 groups and each of them dipped for 1 h and 40 min in the following baths: 1) 0.1% food grade phosphoric acid (pH 2,3); 2) 5.0 mg/L nisin (pH 4,6); 3) 0.1% phosphoric acid and 5.0 mg/L nisin (pH 2,3); and 4) sterile water (pH 5,4). Nisin was provided by Danisco, Brazil, in the form of the commercial product Nisaplin® (Applin & Barret) that contained 2.5% pure nisin. The concentration of nisin (5.0 mg/L) used in this study corresponds to 200 mg/L Nisaplin®.

Nisin activity in the solutions and in the natural casings was monitored using the agar diffusion assay (30). Molten De Man, Rogosa & Sharpe agar (MRS – Oxoid) was cooled to 45 °C and seeded with an overnight culture of the indicator microorganism, *Lactobacillus sakei* ATCC 15521. After agar solidification, wells (5 mm diameter) were cut using a sterile glass tube and 40  $\mu$ L of each solution were added to each well. Natural casings containing the antimicrobials were cut into circles (9 mm diameter) and also placed on the agar surface. Plates were stored at 4 °C for 1 h to allow the bacteriocin to diffuse into the agar, before incubation at 30 °C for 24 h. Nisin activity was observed as a zone of inhibition of the indicator microorganism around the wells and the casings. Inhibition radius was measured (mm) from the edge of the well or the casing to the edge of the halo.

### Sausage production

The sausages were produced in a pilot plant, according to

the following composition: lean beef, 25.8%; lean pork, 30.7%; lard, 15.0%; spices and additives, 1.0%; sodium chloride, 2.0% and water, 25.5%. Spices and additives were used as a commercial mixture (Sausage Mixture 314, Kienast & Kratschmer, Kraki, Brazil) composed by sodium tripolyphosphate, sodium polyphosphate, sodium erythorbate, nitrite, nitrate, monosodium glutamate, natural spices and flavorings. The raw ground lean beef and fat were obtained in a local market and stored at -18 °C in a plastic bag. Ingredients were mixed (STEPHAN/GEIGER, UMMSK-25/40E) and during operation the temperature of the emulsion was maintained below 16 °C by addition of crushed ice. The emulsion was stuffed into the pre-treated natural casings using a manual stuffer (MADO, MWF 591). The sausages were heat processed at 60 °C for 2 h and then cooked in steam bath for 15 min after the internal temperature reached 74 °C. After cooking, the sausages were maintained in a cooling chamber for 24 h, and then vacuum packed in bags (CRYOVAC Sealed Air Corporation) and stored at 4 °C or 10 °C for 56 days.

### Microbiological analysis

Aerobic plate counts and lactic acid bacteria analysis were conducted at 1, 14, 28, 42 and 56 days of storage. Sausage portions (25 g) were aseptically weighed into a stomacher 400 bag (Seward Medical, London, UK) with 225 mL of 0.1% peptone water and homogenized for 1 min. Serial dilutions were prepared in the same diluent and 1.0 mL was inoculated onto suitable media using the pour plate technique, in duplicates. Aerobic bacteria were cultivated on Plate Count Agar (Oxoid) at 36 ± 1°C for 48 h (29). Lactic acid bacteria were enumerated on De Man Rogosa e Sharpe Agar (MRS, Oxoid), incubated at 32 ± 1°C for 48 h in anaerobic system, Anaerogen, Oxoid (22). Mean values of bacterial counts were converted into log cfu.g<sup>-1</sup>.

### Experimental Design

A 2<sup>3</sup> factorial design (3) was applied to evaluate the effect of the three variables: (1) nisin, (2) phosphoric acid and (3) temperature. As response variables, the aerobic plate counts

and lactic acid bacteria counts were determined during sausage storage. The levels assumed for the variables and the experimental design are presented in Table 1. Treatments were repeated in three blocks, totalizing 24 experiments.

**Table 1.** Experimental design

Treatment	(X <sub>1</sub> ) Nisin (mg/L)	(X <sub>2</sub> ) Phosphoric acid (% w/v)	(X <sub>3</sub> ) Storage temperature (°C)
1	0	0	4
2	0	0.1	4
3	5	0	4
4	5	0.1	4
5	0	0	10
6	0	0.1	10
7	5	0	10
8	5	0.1	10

### Statistical analysis

Results from 2<sup>3</sup> factorial design were processed and analyzed using the software Minitab™ 15 (Minitab, Inc. State College, EUA). The statistical significance was determined by analysis of variance (ANOVA).

## RESULTS AND DISCUSSION

### Agar diffusion assay

Natural casing treated with nisin (5.0 mg/L) presented antimicrobial activity against *Lactobacillus sakei* ATCC 15521, although the inhibition halo (0.9 ± 0.3 mm) was significantly smaller (p < 0.05) when compared to the solution inhibition halo (2.1 ± 0.3 mm). Casings treated with nisin (5.0 mg/L) in phosphoric acid solution (0.1%) also presented an inhibitory activity, with no significant difference (p > 0.05) between solution inhibition zone (2.3 ± 0.5 mm) and casing inhibition zone (3.3 ± 0.6 mm). The treatment of natural casing only with water or phosphoric acid resulted in no activity against the indicator microorganism.

### Effect of nisin, phosphoric acid and temperature on bacterial growth

The effect of nisin, phosphoric acid and temperature on the average counts (log cfu.g<sup>-1</sup>) of total aerobes and lactic acid

bacteria in sausages during 56 days of storage are presented in Table 2. In the first day, none of the variables affected significantly the bacterial growth, indicating that the addition of nisin and phosphoric acid did not result in an immediate antimicrobial activity.

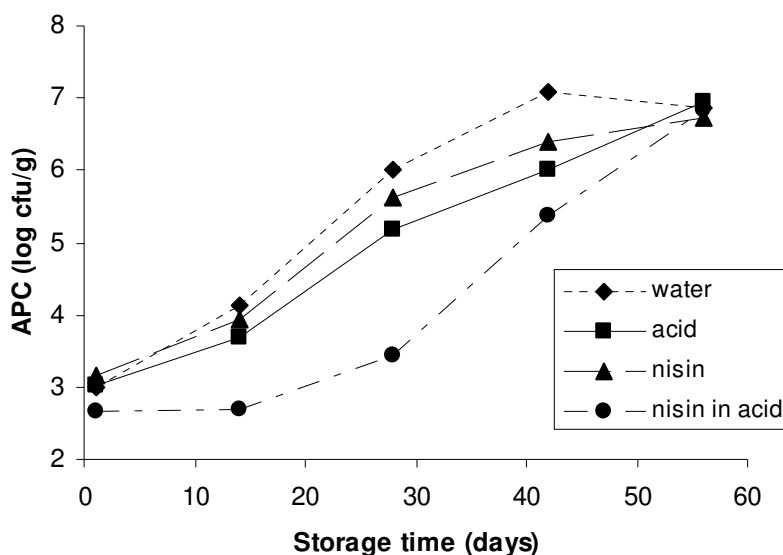
The temperature was the variable that most significantly reduced the bacteria growth in sausages during storage. The highest effect values for this factor were obtained when compared to nisin and phosphoric acid effects (Table 2). At

day 14, total aerobic and lactic acid bacteria counts in sausages stored at 4°C were 3 log cfu·g<sup>-1</sup> lower than in those stored at 10°C. During storage the temperature effect values decreased and at day 56 only lactic acid bacteria counts were significant lower in sausages stored at 4°C than in those stored at 10°C by 0.5 log cfu·g<sup>-1</sup> (Table 2). Figures 1 and 2 present the total aerobic and lactic acid bacteria counts in the sausages during storage at 4°C, respectively. Figure 3 and 4 present the same microorganisms counts in sausages stored at 10°C.

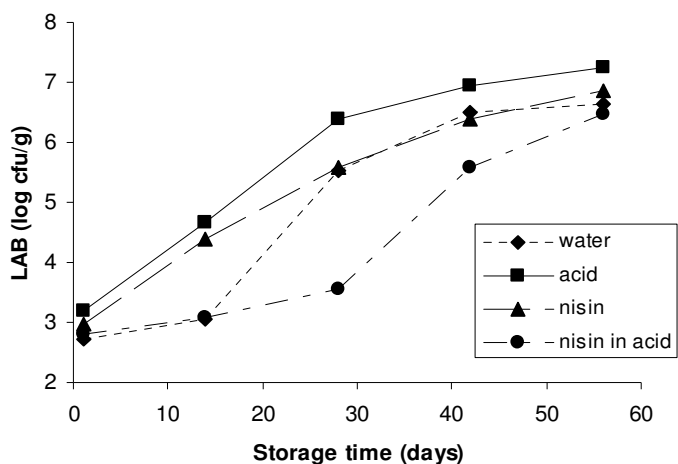
**Table 2.** Effect of nisin ( $X_1$ ), phosphoric acid ( $X_2$ ) and temperature ( $X_3$ ) on total aerobic plate counts (APC) and lactic acid bacteria (LAB) counts in sausages during storage.

Term	1 day		14 day		28 day		42 day		56 day	
	APC	LAB	APC	LAB	APC	LAB	APC	LAB	APC	LAB
$X_1$	-0.11	0.01	-0.60*	-0.31	-0.88*	-0.87*	-0.41	-0.44	0.06	-0.25
$X_2$	-0.25	0.09	-0.50*	-0.18	-0.73*	-0.31	-0.95*	-0.29	-0.31	-0.18
$X_3$	0.12	-0.05	3.00*	2.94*	2.40*	2.24*	1.20*	1.20*	0.30	0.54*
$X_1 \cdot X_2$	-0.16	-0.25	-0.21	-0.83*	-0.18	-0.60*	-0.16	-0.45	-0.09	-0.53*
$X_1 \cdot X_3$	-0.01	0.07	0.00	-0.19	0.18	0.52*	0.26	0.30	0.20	0.03
$X_2 \cdot X_3$	-0.01	-0.05	0.35	-0.32	0.78*	0.28	0.09	-0.11	-0.40*	-0.28
$X_1 \cdot X_2 \cdot X_3$	0.12	0.07	0.18	0.63	0.51	0.85*	-0.17	0.16	-0.12	-0.04

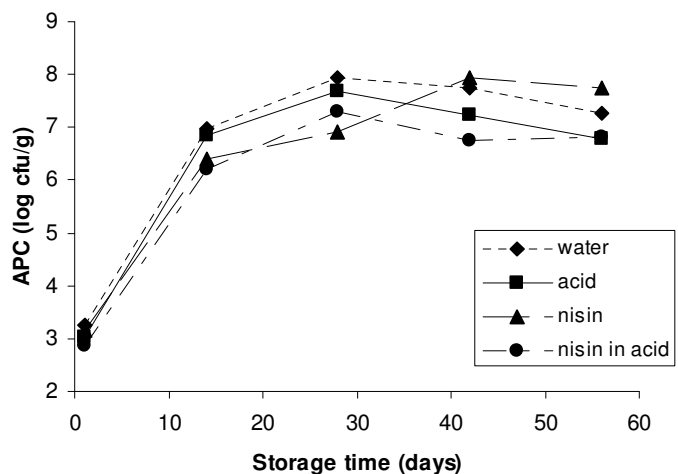
\* Values that are significant ( $p < 0.1$ ).



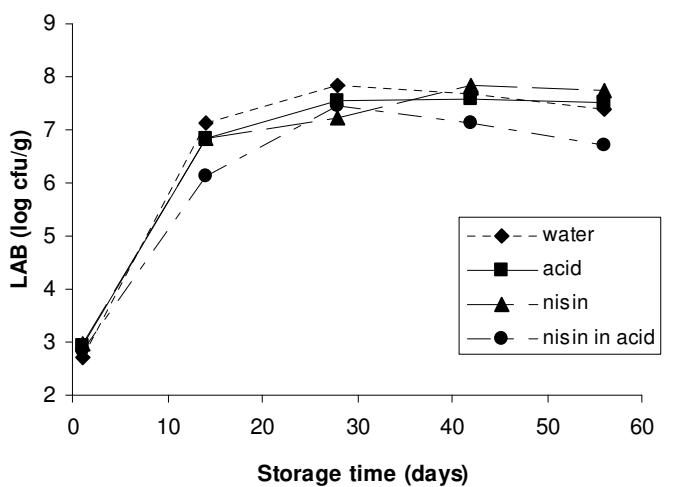
**Figure 1.** Aerobic plate counts in sausages with different treated casings during storage at 4°C.



**Figure 2.** Lactic acid bacteria counts in sausages with different treated casings during storage at 4°C.



**Figure 3.** Aerobic plate counts in sausages with different treated casings, during storage at 10°C.

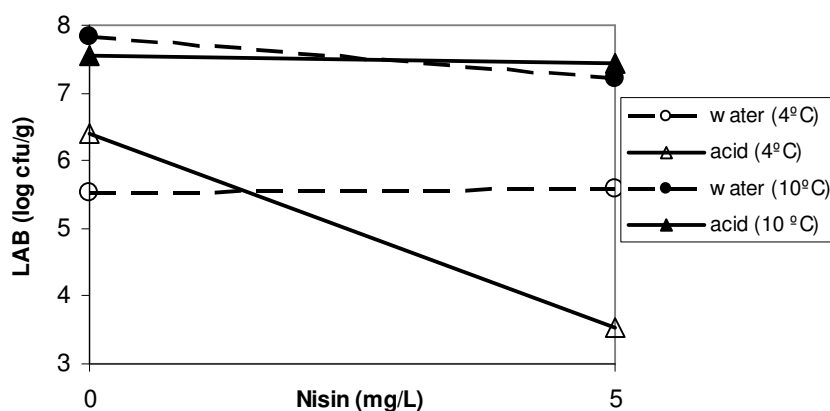


**Figure 4.** Lactic acid bacteria counts in sausages with different treated casings during storage at 10°C.

Phosphoric acid alone reduced significantly total aerobic counts during 42 days of storage. The maximum reduction by 1 log cfu·g<sup>-1</sup> in relation to sausages untreated with the acid was observed at day 28 and 42 for the products stored at 4 °C (Figure 1), and at day 28 for products stored at 10 °C (Figure 3). However no significant reduction was observed in the number of lactic acid bacteria (Figures 2 and 4). This may be explained by the fact that lactic acid bacteria are more resistant to acid conditions caused by the natural casing treated with phosphoric acid solution (pH 2.3). Ariyapipun *et al.* (1) found similar results using lactic acid on the surface of vacuum packaged fresh beef.

The natural casing treatment only with nisin did not significantly reduce lactic acid bacteria counts. It significantly decreased the aerobic plate counts only on days 14 and 28 of storage by 0.6 and 0.9 log respectively (Table 2). The reduction of total aerobic counts in sausages treated with nisin may also be explained by the low pH of nisin solution (pH 4.3), since lactic acid bacteria was not affected. Sausages with casing treated with nisin in phosphoric acid solution presented the

lowest aerobic plate counts during storage at 4 °C (Figure 1). The number of lactic acid bacteria was also lower in sausages treated with both antimicrobials (Figure 2). For these microorganisms, nisin and phosphoric acid showed a significant interaction on days 14, 28 and 56 (Table 2), indicating a synergistic effect. These data are in agreement with other authors, which demonstrated that the acid condition of the solution containing nisin may enhance the antimicrobial effect (16, 27). A significant interaction was observed between the three variables at day 28 for lactic acid bacteria counts (Table 2). For sausages stored at 4 °C, the treatment with nisin in phosphoric acid solution reduced lactic acid count by 3 log cfu·g<sup>-1</sup>, while treatment only with nisin had no inhibitory effect. However, at 10 °C, lactic acid bacteria counts were not influenced by nisin and phosphoric acid (Figure 5). The lower nisin activity at higher storage temperature could explain these results. Raju *et al.* (31) also demonstrated that loss of nisin activity in fish sausage was higher at ambient temperature than at refrigerated storage.



**Figure 5.** Interaction of nisin, phosphoric acid and temperature for lactic acid bacteria counts in sausages at 28<sup>th</sup> day of storage at 4°C and 10 °C.

The results obtained in this study also showed that nisin remained active after the sausage cooking process, confirming the intrinsic thermotolerance, previously demonstrated by other authors (33, 35).

Lactic acid bacteria are frequently associated with spoilage of most heat processed meats (25). As shown in this study, the treatment of casings with nisin in phosphoric acid solution extended the shelf life of sausages stored at 4 °C. The counts of

lactic acid bacteria in non-treated sausages reached  $10^7$  cfu·g<sup>-1</sup> by the 40th day of storage, while in those treated with both antimicrobials, the counts were lower than this, even after 56 days of storage (Figure 2). In spite of the decrease in bacterial growth rate in sausages treated with nisin in acid solution, a decrease in nisin activity over storage period was also verified. The loss of nisin activity in a meat system was also reported by Cutter and Siragusa (9), who suggested that the diminished activity was due an adsorption of nisin onto meat proteins or lipid particles.

Incorporation of nisin to the surface of meat products seems to be more efficient than its direct addition to the meat mass. Many reports have shown that surface application of nisin by spraying, solution bath or packaging systems is an efficient method in controlling spoilage microorganisms. A reduction in total bacterial counts in fresh veal meat in a cellophane packaging containing nisin was observed by Guerra *et al.* (20). Martinez *et al.* (27) verified that spraying a mixture of lactic acid and nisin to beef carcasses can reduce bacterial population by 2 log units. Gill and Holley (17) observed a reduction in the growth of *Lactobacillus* and *Leuconostoc* in bologna sausage when a gel containing nisin and lysozyme was applied. However, contrary to what was observed in this study, Castro (4) did not observe an inhibition of lactic acid bacteria growth or a reduction of total aerobic count with the application of Nisaplin® (200 mg/L) in phosphoric acid solution (0.1%) to the surface of sausages by dipping in a bath. These differences suggest that nisin retention is higher when incorporated into the sausage casing prior to the stuffing step, as compared to its application during the postprocess phase in an antimicrobial bath.

## CONCLUSION

The results obtained in this study demonstrate that nisin (5.0 mg/L) in a phosphoric acid solution (0.1%) can be incorporated to the natural casing prior to the stuffing operation to reduce the aerobic plate count and lactic acid bacteria in vacuum packaged sausages stored at low temperature. It was

also demonstrated that the hurdle technology is necessary to control spoilage bacteria in vacuum packaged sausages. Since natural casings are used in large quantities in the production of sausages (32) such findings could provide the industry with the option of using nisin in acid phosphoric solution to improve the quality and shelf life of these types of product.

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