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Combinations of nisin with salt (NaCl) to control *Listeria monocytogenes* on sheep natural sausage casings stored at 6°C

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This study evaluated the effect of combinations of nisin with salt (NaCl) to control *Listeria monocytogenes* on sheep natural sausage casings. Casings were inoculated with 3.0 x 10^5 cfu/g final inocula of *L. monocytogenes*, stored at 6°C in different solutions of nisin at 0, 100, 150 and 200 µg/g. Each combined with salt at 0, 4, 7 and 12% (w/v). Samples were taken at day 0, 10, 20, 35, 60 and 90 post-inoculation and the number of bacteria present was determined. The bactericidal effect of nisin against *L. monocytogenes* cells was evident where nisin was applied in combination with salts. In all treatments, nisin/salt mixtures induced a bacterial growth inhibitory effect greater than salt alone. These results indicate that nisin and salt synergistically and significantly inhibit the growth of *L. monoctogenes* in sheep natural casings. The use of nisin combined with salt as antibacterial agent will be appropriate for applications on natural sausage casing industries as natural preservatives to control foodborne pathogens. They can be used as growth inhibitors of *L. monocytogenes,* an important foodborne pathogens and spoiling bacterium. The main reason for their appropriateness is their natural origin, which consumers find comforting. These beneficial characteristics could increase casings safety and shelf life.

Key words: Nisin, salt, casings, biopreservative, *Listeria monocytogenes*.

INTRODUCTION

Natural casings from sheep, hog and beef have been used for thousands of years as an edible container for sausage. They are derived from the gastrointestinal tracts of the respective species (Bakker et al., 1999; Madhwaraj et al., 1980; Pearson and Gillett, 1999). Casings are usually preserved by salting, curing and/or drying (Fischer and Schweflinghaus, 1988) to reduce the microbial contamination, but enteric or exogenous microorganisms in the natural casing are inevitable and also the number of microorganisms increases during processing and distribution, especially under unhygienic treatment or high storage temperature (Trigo and Fraqueza, 1998). By their nature, natural casings are contaminated with bacteria (10^4 to 10^7 cfu/g) (Bakker et al., 1999; Byun et al., 2001; Ockerman and Hansen, 2000; Gabbis and Silliker, 1974). Generally, adequately salted casings are considered microbiologically fully acceptable. *Listeria monocytogenes* is an important human food-borne pathogen which causes febrile gastroenteritis in healthy individuals (Piana et al., 2005) and life-threatening invasive infections in susceptible individuals (Mead et al., 2006), such as the young, the old, the pregnant and the immune-compromised, the so called "YOPI" (De Cesare et al., 2006). In Europe it has an incidence of 0.3 cases/year/100,000 population (European Food Safety Authority, 2006), this pathogen has the ability to growth over a wide range of pH values (4.3 - 9.6) (Lou and Yousef, 1999) and can survive under salt concentrations as high as 10% NaCl (McClure et al., 1989). Due to the ubiquitous nature of this pathogen in the slaughterhouse and the meat packaging environments, it is not surprising that the incidence and behavior of this pathogen in meat products are receiving increasing attention.

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Table 1. Explanation of group names, nisin and NaCl Concentrations.

Salt (%)	Nisin (µg/g)			
	0	100	150	200
0	A	B	C	D
4	E	F	G	H
7	I	J	K	L
12	M	N	O	P

Presence and survivability of this pathogen were studied by using fresh and salted beef, sheep and hog casings. *L. monocytogenes* was confirmed in beef and fresh hog casings after 30 days of storage in dry-salted at 15°C (Bockemühl, 2000).

L. monocytogenes was positively identified after the mandatory 30 day preservation period for natural casings at a water activity level of 0.85 or lower (Wijnker et al., 2006). Casings may be used in the manufacture of either fresh sausages, fermented sausages, or cooked/sterilized sausages. Eventually pathogens like *L. monocytogenes* occasionally survive in fermented sausages (Encinas et al., 1999; Levine et al., 2001), which are generally consumed in an uncooked condition. Our research activities focus on the identification of biopreservatives that could improve and partly replace salt/brine methods, which are currently applied, to decrease environmental impact of large quantity of salt used by the casing industry and to improve the casing's technical characteristics. A likely substance to obtain this effect is nisin, the best known and studied bacteriocin produced by lactic acid bacteria (LAB). It is the only bacteriocin that has been approved as a food additive in Europe and it is considered safe as bacteriocin in the United States (Delves-Broughton, 1990; Food and Drug Administration, 1998; Montville et al., 2001). Nisin is effective in controlling a wide range of gram-positive pathogenic bacteria, including *L. monocytogenes* (Ming et al., 1997; Siragusa et al., 1999; Coma et al., 2001). The inhibition of Listeria by nisin has been demonstrated in culture media as well as in different foods such as cottage cheese (Ferreira and Lund, 1996), ricotta-type cheeses (Davies et al., 1997), fresh pork sausages (Scannell et al., 1997), cold-smoked salmon (Nilsson et al., 1997) and Ice cream (Dean and Zottola, 1996). Therefore, the aim of this study was to evaluate nisin applied singly or in combination with salt as bio-conservative solutions, for control of *L. monocytogenes* introduced on sheep natural casings and storage at 6°C.

MATERIALS AND METHODS

L. monocytogenes strain and inoculums preparation

The *L. monocytogenes serovar* 4b CECT 4032 strain was used to artificially contaminate sheep natural casing before treatment,

packaging and storage. Bacteria were grown in 10 ml BHI agar (Difco) for 24 h at 37°C, diluted and counted and adjusted to a final inoculum of approximately 3.0×10^5 cfu/g of sheep natural casings when 0.25 ml of the inoculum was applied to each part of casing, as described below.

Sample preparation

Dry salted Australian sheep casings (AA 20 - 22) were obtained from BOA Company, (Boyauderie d'Atlas Tangier Morocco). These casings were previously cleaned scarped selected and salted according to the Company Standard Operating Procedures. For the experimental treatment, eight hanks were transferred to the microbiology laboratory. Casings were first desalinated for 30 min with flowing lukewarm tap water (temperature 25 ± 2°C). This reduces the residual salt level of dry-salted casings to less than 3 wt% (Lee et al., 1994). Thereafter casings were drained overnight at 3°C to reduce water level. The next day, the casings were divided and weighed of approximately 100 g. Each part was then transferred to the glass recipients for inoculation, treatment, storage and testing, as described below.

Preservation solutions

To preserve casing under controlled conditions, 16 different solutions with specific salt (Salt: 0, 4, 7 and 12%) and nisin concentrations (0, 100, 150 and 200 µg/g) and all possible combinations were prepared with sterilized water. The pH of all solutions was adjusted to 5.8 with HCl (Sigma-Aldrich, Darmstadt, Germany).

Microbiological media and chemicals

PALCAM media and its supplements were purchased from BIOKARD (Beauvais, France). For serial dilutions, TRYPTONE SALT BROTH (BIOKARD) was used. Nisin from *Lactococcus lactis* (subsp lactis) was obtained from Sigma-Aldrich (N5764). Nisin was solubilised in 0.02 M HCl at a concentration of 10 mg/ml with heating (60 - 70°C) to aid solubilisation. The solution was sterilised by filtration through 0.22 µm membrane filters (Millex, Millipore) prior to use and this solution was used throughout.

Product inoculation and treatment with antimicrobials

The desalinated and drained casings were divided into sixteen groups (100 g of each part), placed on glass recipients under a biohazard hood and 0.25 ml of the inoculum was deposited. Inoculated casings were homogenized thoroughly and kept at 6°C for 1 h for inoculum attachment. Thereafter, 100 ml of each antimicrobial solution were added. Each of the groups, were treated separately with different nisin and salt concentrations as showed in Table 1.

Microbiological analysis

Microbiological analysis were realised according to AFNOR V08-055 method, Samples were analyzed for microbiological counts at time-points; 0, 10, 20, 35, 60 and 90 days of storage at 6°C. 10 g of casing for each treatment were transferred into individual sterile plastic bags (Whirl-Paks, Paris, France), mixed with 90 ml of TRYPTONE SALT BROTH (BIOKARD, Beauvais, France) using a Stomacher (Stomacher/Lab Blender 400) for 2 min. Appropriate serial decimal dilutions were made in TRYPTONE SALT BROTH.

The number of *L. monocytogenes* was determined on PALCAM agar (BIOKARD) after incubation at 37°C for 24 h and for further analyses, examination of the TSAYE (BIOKARD) plate with an oblique Henry illumination system, Gram staining, examination for catalase activity, tumbling motility, hemolysis zone on blood agar (BIOKARD), CAMP test were performed according to Harrigan (1998). All treatments were conducted in triplicate. Microbiological counts were expressed as log10 cfu.

Statistical evaluation

Analyse of variance of the data was performed using the ANOVA-MANOVA using statistical software STATISTICA 6.0 (1997 edition). Significant differences (p < 0.05) between mean values of triplicate sample were determined.

RESULTS AND DISCUSSION

No *L. monocytogenes* was detected in the natural casings before inoculation. *L. monocytogenes* populations exceeded 6.3 $\log_{10}$ cfu/g in no treated sample - group A (Figure 1). This demonstrates the ability of the pathogen to undergo abundant growth on sheep natural sausage casings without antimicrobials even when stored under refrigeration and acidified solutions. Nisin (100, 150 and 200 µg/g) used alone had no antilisterial effect before 20 days (p > 0.05). However, at days 20 to 90 nisin reduced the *L. monocytogenes* population in the all nisin treated simples when compared to the control (p < 0.001) (Figure 1). This reduction may be due to acidification of solution of treatment due to lactic acid production by *L. monocytogenes,* which led to a significant acidification (Conner et al., 1986). Nisin is known to act better at pH 5.5 or below (Buncic et al., 1995; De Martinis et al., 1997; Ukuku and Shelef, 1997). The inhibitory action of nisin increased with decreasing pH values .This response could be attributed to acidic damaging effects on target cells concomitant with the higher stability and solubility of nisin (Liu and Hansen, 1990) and with the increase in net positive charge of nisin (Jack et al., 1995). The effect of a combination of nisin (0, 100, 150 and 200 µg/g) with 4% salt to control *L. monocytogenes at* 6°C in casings was studied. At 4% of salt and without nisin, the colonies number of *L. monocytogenes* increased from an initial 5.4 $\log_{10}$ cfu/g to 5.8 $\log_{10}$ cfu/g of casing after 10 days. The bactericidal effect of nisin against *L. monocytogenes* cells was evident in all simple where nisin was applied in combina-tion with 4% (w/v) of salts, regardless of the nisin concentrations, when compared to the control (Figure 2). A decrease was approximately 1 $\log_{10}$ cfu/g at 90 days.

The bactericidal effect of salt at 7% (w/v) against *L. monocytognes* was evident in all groups regardless of the presence of nisin (Figure 3). However, the inhibitory effect was more pronounced in group L, where 200 µg/g of nisin was used (p < 0.001). This inhibition reaches 1.2 $\log_{10}$ at 10 days compared to group I (0 µg/g nisin), J (100 µg/g nisin), K (150 µg/g nisin) where the number of
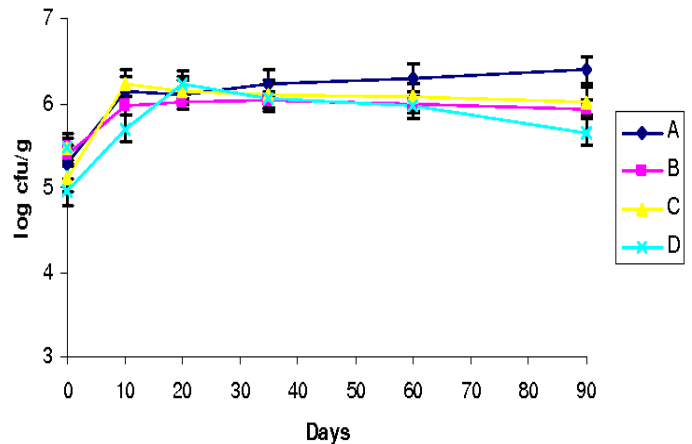

Figure 1. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with (A) 0 µ/g of nisin (B) 100 µg/g of nisin (C) 150 µg/g of nisin and (D) 200 µg/g. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.
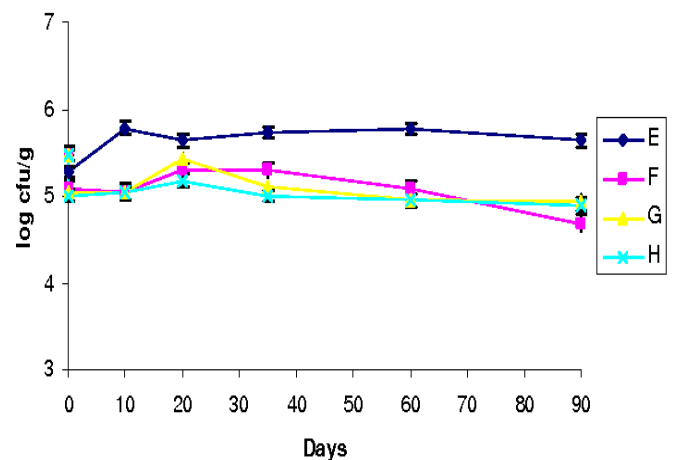

Figure 2. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with 4% of NaCl combined to (E) 0 µg/g of nisin, (F) 100 µg/g of nisin, (G) 150 µg/g and (H) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

colonies formed by *L. monocytogenes* decrease by 0.4 $\log_{10}$ cfu/g (Figure 3).

A considerable decrease of *L. monocytogenes* was shown when casings were treated with 12% of salt alone or in combination with various nisin concentrations. The decrease reached 2.2 $\log_{10}$ in simple L0 µg/g nisin after 90 days, compared with no nisin treated simple with only 0.7 $\log_{10}$ cfu/g decreases (Figure 4).

In all experiments, nisin added to the same salt con-centration induced an inhibitory effect greater than salt alone. Geornaras et al. (2004a) investigated the anti-listerial effects of nisin on commercial bologna and ham. They contaminated the samples with *L. monocytogenes*
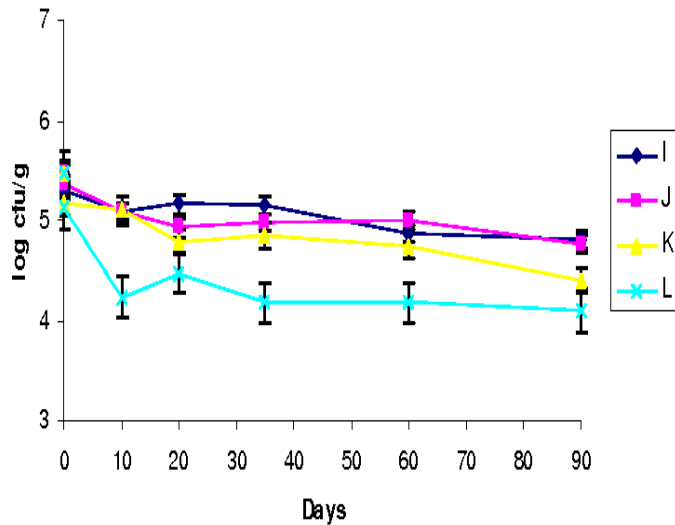
Figure 3. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) isolated from natural sheep casing treated with 7% of NaCl combined to (I) 0 µg/g of nisin, (J) 100 µg/g of nisin, (K) 150 µg/g and (L) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.
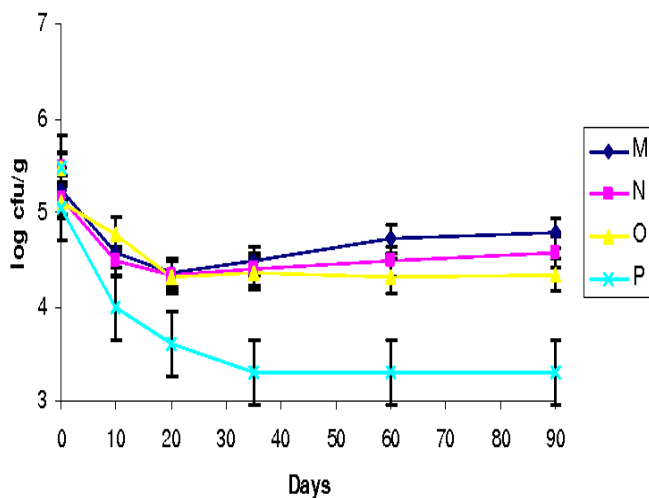

Figure 4. Survival/growth (n = 3) of inoculated *L. monocytogenes* (PALCAM agar) in natural sheep casing treated with 12% of NaCl combined to (M) 0 µg/g of nisin. (N) 100 µg/g of nisin. (O) 150 µg/g and (P) 200 µg/g of nisin. Each point represents the mean of three experiments. Error bars represent standard deviation of the means.

at 3 - 4 log/cm^2 and immersed them in nisin solution (0.5%). They found that nisin reduced the count of *L. monocytogenes* by 2.4 - 2.6 log/cm^2. These researchers performed another study on frankfurter sausages

contaminated with *L. monocytogenes* at 3 - 4 log/cm^2 and dipped in nisin solution (0.5%) and found that nisin reduced the count of *L. monocytogenes* by 2.4 - 3.5 log/cm^2 (Geornaras et al., 2004b). Thus nisin has an antilisterial activity in meat and meat products.

Pawar et al. (2000) found that the growth of *L. monocytogenes* in the treated groups was significantly inhibited compared to the controls. Also, the degree of inhibition increased with increasing concentrations of nisin (Samelis et al., 2005).

Sahl and Bierbaum (1998) proposed that the reaction between nisin and the listerial cell membrane was caused by hydrophobic interaction between the amino acid residues of nisin and the fatty acids of the membrane phospholipids (Henning et al., 1986). It was further suggested that the electrostatic attraction between nisin molecules and the negatively charged phospholipids is involved in the antilisterial effect. Ming and Daeschel (1993) compared the sensitivity to nisin of cells of two strains of *L. monocytogenes* Scott A: one with signi-ficantly decreased phospholipid content compared to the parental strain. They observed low antilisterial activity of nisin in the strain with the low phospholipid content. The nisin resistant cells were found to bind less nisin and release less phospholipid than the sensitive cells when treated with the same concentrations of nisin.

Thomas and Wimpenny (1996) and Parente et al. (1998) found that the presence of NaCl enhances nisin action. The emergence of *L. monocytogenes* as an important food-borne pathogen has led to a resurgence of interest in antimicrobials suitable for its control. At the same time, consumer demand for foods that contain fewer preservatives, are less processed, free from artificial additives and perceived as fresh and more natural food stuffs has increased (Gould, 1992). In the present study, it was found that nisin with various combinations of salt has a great influence as an antilisterial effect in sheep natural casings.

Conclusion

In the present study, we found that the combination of nisin with salt significantly inhibited the growth of *L. monoctogenes* in sheep natural casings. Forthcoming investigations should focus on evaluating antimicrobial activity of nisin against other pathogens founding in natural casings to support a potential application for nisin in natural casing industries as potential biopreservative.

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