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ORIGINAL ARTICLE

Nisin as Biopreservative against *Listeria* spp. in Meat Products

Nisina como bioconservador contra *Listeria* spp. en productos cárnicos

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A B S T R A C T

The antilisterial effect of nisin A and Z in meat and meat products (cooked ham, minced pork meat, deboned chicken breasts, pate, and slightly fermented sausages [espetec]) have been shown. An infective dose of 5 to 10 most probable numbers (MPN)/g to simulate the counts of *Listeria* generally found in meat products was used. Nisin at 4,800 AU/g reduced the numbers of *Listeria innocua* by 7.98 log cycles in cooked ham and by 9 log cycles in pate when stored at 78C for 37 days. In deboned chicken breasts stored at 7ºC for 7 days, 4,800 AU/cm^2 of nisin diminished the *L. innocua* counts in 5.26 log cycles when compared to the control batch. In minced pork meat held at 7ºC for up to 6 days, 1,600 AU/g kept *L. innocua* counts under 3 MPN/g, while the control batch reached 50 CFU/g. In espetec sausages, 648 AU/g diminished the number of *L. innocua* under 50 CFU/g from the fifth day until the end of the process (12 days) while the control batch kept the initial counts (3 x 10^4 CFU/g).

Keywords: Nisin, Meat processed products, *Listeria* spp., Biopreservatives

R E S U M E N

Se ha demostrado el efecto antilisterial de la nisina A y Z en carnes y productos cárnicos (jamón cocido, carne de cerdo picada, pechugas de pollo deshuesadas, paté y salchichas ligeramente fermentadas-espetec). Se utilizó una dosis infecciosa de 5 a 10 números más probables (MPN) / g para simular los recuentos de *Listeria* que se encuentran generalmente en productos cárnicos. La nisina a 4.800 AU / g redujo el número de *Listeria innocua* en 7.98 ciclos logarítmicos en jamón cocido y en 9 ciclos logarítmicos en paté cuando se almacenó a 7°C durante 37 días. En pechugas de pollo deshuesadas almacenadas a 7ºC durante 7 días, 4.800 AU / cm^2 de nisina disminuyeron los recuentos de *L. innocua* en 5.26 ciclos logarítmicos en comparación con el lote de control. En carne de cerdo picada mantenida a 7ºC por hasta 6 días, 1,600 AU / g mantuvieron los conteos de *L. innocua* por debajo de 3 MPN / g, mientras que el lote de control alcanzó 50 UFC / g. En embutidos espetec, 648 AU / g disminuyó el número de *L. innocua* por debajo de 50 UFC / g desde el quinto día hasta el final del proceso (12 días) mientras que el lote control mantuvo los recuentos iniciales (3 x 10^4 UFC / g).

Palabras clave: Nisina, Productos cárnicos procesados, *Listeria* spp., Bioconservantes

INTRODUCTION

The ingestion of products contaminated with *Listeria monocytogenes* is a health threat to a high-risk population such as the immunocompromised, children, pregnant women, and senior citizens. *Listeria* has been involved in 0.7, 1.7, 7.4, and 16.8 cases per million of the foodborne infections in Spain, Canada, the United States, and France, respectively, and an average of 40% was fatal (6, 12, 15, 38). The last outbreak was in the United States; from August to December of 1998, 75 cases of listeriosis distributed in 11 states were detected and 20% of the patients died (10). The percentage of *Listeria* among the food products assayed in several countries was very variable. In pork meat products, the percentage of *Listeria* was 3.1% in carcasses, 7 to 58% in raw meat, 9.6 to 58% in minced meat, 15 to 100% in raw sausages, and 10 to 35% in cooked meat products (36). In general these products contained a low number of *Listeria*, less than 5 CFU/g, although 10^4 to 10^5 CFU/g were found in pate (7, 32, 33). In poultry, the percentage of positive samples for *L. monocytogenes* ranged from 23 to 85%, and the counts were below 10^2 CFU/g (26, 46). The control of *L. monocytogenes* in foods is difficult due to its ability to grow at a wide

range of temperatures (3 to 45ºC), its tolerance to pH (4.8 to 9.6), to high sodium chloride levels (<10%), sodium nitrite (<1,000 ppm), and its resistence to 10 ppm of the majority of disinfectants that are inhibitory to other food pathogens (7, 13, 14, 43). As common control parameters alone will not preclude microbial hazard by *L. monocytogenes* and as consumers demand more natural products, alternative control measures like biopreservation (such as bacteriocinogenic lactic acid bacteria cultures, their bacteriocins, or both), reducing storage temperature to <2ºC, and postprocessing pasteurization have received increasing attention.

Several authors have described the use of bacteriocinproducing starter cultures to inhibit the outgrowth of *L. monocytogenes* in raw meat, German-style fresh sausages, American-style fermented semidry and dry sausages, Italian salami, Spanish dry fermented sausages, frankfurter sausages, and in vacuum-packaged wieners (4, 5, 8, 11, 12, 16, 21–23, 28, 29, 34, 37, 39, 45, 47, 48). Nisin is a bacteriocin produced by a group of Gram-positive bacteria that belongs to *Lactococcus* and *Streptococcus* species.

Nisin is classified as a Type A (I) lantibiotic that is synthesized from mRNA and the translated peptide contains several unusual amino acids due to post-translational modifications. Over the past few decades, nisin has been used widely as a food biopreservative. Since then, many natural and genetically modified variants of nisin have been identified and studied for their unique antimicrobial properties. Nisin A and Z is an FDA approved and GRAS (generally regarded as safe) peptide with recognized potential for eating. Over the past two decades the application of nisin has been extended to industrial and biomedical fields.

Purpose of the study

The aim of this study was to show the antilisterial activity of nisin A and Z produced by *Lactococcus lactis* ATCC 7962 in slightly fermented sausages (espetec), cooked ham, pate, minced pork, and deboned chicken breasts.

MATERIALS AND METHODS

Bacterial cultures and media

Lactococcus lactis ATCC 7962 (nisin A y Z producer) (3, 35) was incubated in MRS broth at 30ºC. *Listeria innocua* CTC1014 (nisin A and Z indicator in the bacteriocin titer assays) (21) was grown in tryptic soy broth with 0.6% yeast extract at 30ºC. *Lactobacillus sake* FVM 148 (nisin Z indicator in the bacteriocin titer assays) (9, 40) and *Pediococcus pentosaceus* FBB63 (nisin Z indicator in the bacteriocin titer assays) (9, 19) were incubated in MRS at 30ºC. Preparation of bacteriocin. *Lactococcus lactis* ATCC 7962 was grown for 18 h at 30ºC in MRS broth. The cells were removed by centrifugation at 5,000 x g for 10 min at 4ºC. The supernatant fluid was collected and the bacteriocins (nisin A and Z) were pre cipitated by adding 300 g/liter of ammonium sulfate. After 30 min incubation at 0ºC, the precipitated proteins were collected by centrifugation at 8,000 x g at 4ºC for 30 min. The precipitates were dissolved in 3 mM sodium phosphate buffer at pH 7.0 and heated at 80ºC for 10 min. The concentrated bacteriocins were stored at -80ºC. A modification of the critical dilution assay (42) was used to determine the bacteriocin titer (21).

Inoculum preparation

Listeria was grown overnight in tryptic soy broth with 0.6% yeast extract at 30ºC. Cells were harvested by centrifugation (5,000 x g for 10 min), resuspended in tryptic soy broth with 0.6% yeast extract and glycerol (50%), and stored at -40ºC. Viable counts were obtained in tryptic soy agar with 0.6% yeast extract. For food application, cells were diluted in 0.85% NaCl when necessary. *L. innocua* CTC1014 was used instead of *L. monocytogenes* CTC1011 in order to avoid dissemination of the pathogen in the pilot plant (21). Previous assays showed that *L. monocytogenes* was more sensitive to nisin A and Z than *L. innocua* (2, 9).

Product manufacture and experimental design: slightly fermented sausages (espetec)

A shoulder/belly proportion of 60 to 40 was ground in a meat grinder by passing through a 6-mm plate (Tegmag, Barcelona, Spain) and mixed with the additives (g/kg): NaCl, 22; KNO_3, 0.15; $NaNO_2$, 0.15; sodium ascorbate, 0.5; sodium citrate, 3; dextrose, 2; milk powder, 20; lactose, 30; casein, 5; black pepper, 2; red cochineal, 0.02; and water, 60 (Sanofi Bio-Industries S.A., Barcelona, Spain). The mixture was vacuum mixed for 3 min in a vacuum blender (Tegmag). The sausages were vacuum stuffed using a continuous stuffing machine (Tecnotrip, Terrassa, Spain) and a 40- to 42-mm-diameter pork intestine casing. The fresh weight of each sausage was 284 g (±27 g). After dipping in a solution of *Penicillium nalgoviensis* (PNT1- NG8, Rhone-Poulenc Texel S.A., Dange Saint Roman, France), they were placed in a controlled room. The conditions during the process were as follows: 16ºC and 72% relative humidity for 4 h to dry the casings, 15ºC, and 85% relative humidity

for 5 days in order to facilitate the growth of the microflora, and 13ºC and 80% relative humidity up to 12 days (18). Three independent sausage treatments were conducted. Batch I, without added bacteriocin; batch II, with 221 AU/g of nisin A and Z; batch III, with 648 AU/g. *Listeria* was added together with the additives at 10^4 CFU/g. Triplicate samples were taken at selected times (0 h, 1 h, and 1, 2, 3, 4, 5, 6, 7, 9, and 12 days of processing).

Cooked ham. Skinless and deboned fresh ham pork (15%) was injected with a brine solution containing per g/kg: NaCl, 18; NaNO2, 0.1; dextrose, 5; carrageenan, 1; and water, 100 by a multineedle Inject Star BI13B (Dordal SA, Barcelona, Spain). The injected fresh ham was equilibrated for 48 h at 4ºC by alternating tumbling (1 h per day) in a stainless steel vacuum massaging drum (Dordal SA). The injected ham was canned and cooked at 70ºC until the internal temperature reached 67ºC (approximately 8 h) (Unimatic oven UM1/1500, Doleschal, Steyr, Germany). After cooling it was sliced. Three independent lots were prepared. Batch IV, without added bacteriocin; batch V, with 1,600 AU/g of nisin A and Z; batch VI, with 4,800 AU/g. The slices were inoculated with Listeria (5 most probable number [MPN]/g) spread over the slice surface. Afterward, appropriate nisin concentrations were also spread over the surface of each slice. The application of Listeria and bacteriocin added a maximum volume of 10 ml/g in order not to change the damp of the slices. The slices were stored at 7ºC for 37 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 4, 10, 18, and 37 days of storage).

Pate

Pork liver (250 g) was ground, brined for 18 h in a solution containing in g/kg: $NaNO_2$ (4.2) and mixed with sodium chloride (7.5), white pepper (1.0), paprika (5.0), onions (20), garlic (5), and liquid whole eggs (50), and homogenized in a cutter at 5 to 8ºC. Milk (250) and emulsified fat (450) at 55ºC were added to the mixture and homogenized with the rest of the ingredients in a three-blade cutter (CM-22, Mainca, Granollers, Spain) to obtain a fine-ground product. The product was stuffed and cooked at 75ºC until the internal temperature reached 728C (approximately 2.5 h in a Unimatic oven, UV 1/1500), cooled down in a refrigerator for 24 h, and sliced. *Listeri*a was spread over the surface at 5 MPN/g. In the appropiate batches, bacteriocin were later applied and spread over the surface. The application of *Listeri*a and nisin added a maximum volume of 10 ml/g in order not to change the damp of the slices. Three independent treatments were done: batch VII, without added bacteriocin; batch VIII, with 1,600 AU/g of nisin A and Z; batch IX, with 4,800 AU/g. The product was stored at 7ºC for 37 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 4, 10, 18, and 37 days of storage).

Minced pork meat

Raw lean pork shoulder and belly fat (50:50) were ground through a 6-mm plate and mixed with *Listeria* at 5 MPN/g for 5 min in a vacuum blender (Tecmag). Afterward, nisin were added in the appropiate batches and the whole mix was vacuum blended during 5 more minutes. The application of *Listeria* and nisin added a maximum volume of 15 ml/g. Four different treatments were prepared: batch X, without added bacteriocin; batch XI, with 1,600 AU/g of nisin A and Z; batch XII, with 3,200 AU/g; and batch XIII, with 4,800 AU/ g. The product was stored at 7ºC for 6 days in sterile plastic boxes. Triplicate samples were taken at selected times (0, 1, 2, 4, 5, and 6 days of storage).

Chicken breasts

Skinless and deboned chicken breasts were selected after slaughtering and three different treatments were conducted: batch XIV, nonadded bacteriocin; batch XV, with 3,200 AU/cm^2 of nisin A and Z; and batch XVI, with 4,800 AU/ cm^2. *Listeria* was spread in all the batches over the surface of the chicken breast with a glass spreader at 10 CFU/cm^2. Nisin were spread afterward in the adequate batches. A maximum volume of 10 ml/g was used for the inoculum of *Listeria* and nisins. The product was stored at 7ºC for 7 days in sterile plastic boxes. Triplicate samples were taken at selected times (0 h, 1 h, and 3 and 7 days of storage).

Physicochemical evaluation

Water activity of the different products assayed was measured with an aw-cryometer (awk-10, Nagy, Ga¨ufelden, Germany). pH was measured with a penetration electrode (Crison Instruments, Barcelona, Spain). The percentage of fat was evaluated by the standard protocol ISO-1443 and total nitrogen content by ISO R-937. The nonprotein nitrogen (NPN) and nitrogen a-amino (N-a-amino) was obtained by standardized protocol no. 24.046 (1). The sodium chloride content was determined by the colorimetric determination of Zall *et al.* (49) adapted to the segmented continuous flow autoanalyzer (Technicon Ltd., Dublin, Ireland). Triplicate samples were taken at

the beginning of the experiments, unless indicated. *Espetec* was evaluated at the end of the manufacturing process.

Microbiological sampling

Triplicates from each treatment were sampled at selected times to determine *Listeria* counts. Ten grams of each sample were mixed (1:10) with dilution medium (0.1% peptone and 0.85% NaCl or *Listeria* enrichment broth (UVM1, Oxoid Division, Unipath Co., Ogdensburg, N.Y.) and placed in a stomacher bag (model 400, Cooke Laboratory Products, Alexandria, Va.) for 1 min. For chicken breasts, an area of 39.3 cm^2 was sampled by the swab method. *Listeria* was enumerated by the spread plate technique in Palcam agar (Merck) at 30ºC for 48 h (44). When the counts of *Listeria* were lower than 10^2 CFU/g or cm^2, the MPN technique using nine tubes (three per dilution, 21, 22, 23) of *Listeria* enrichment broth (UVM1, Oxoid) incubated at 37ºC during 24 h were used. Positive tubes were confirmed by streaking 10 ml in Palcam agar and incubated at 30ºC for 48 h (30). *Listeria* grows as gray–green-colored colonies with a black zone.

Table 1. Physicochemical characteristics of the meat products used[a]
Tabla 1. Características fisicoquímicas de los productos cárnicos empleados[a]

Product	pH	NaCl	a_w	Fat	NMP/N_{total}	*N*-α-amino/N_{total}
Espetec-fermented sausages	6.06 ± 0.01	3.5 ± 0.01	0.87 ± 0.01	41.50 ± 0.32	6.20 ± 0.20	1.86 ± 1.41
Cooked ham	6.36 ± 0.01	2.02 ± 0.06	0.98 ± 0.01	3.48 ± 0.17	8.58 ± 0.20	0.89 ± 0.71
Pâté	6.34 ± 0.00	1.79 ± 0.01	0.97 ± 0.00	20.01 ± 0.16	23.97 ± 0.10	2.79 ± 0.71
Minced meat	5.90 ± 0.01	0.11 ± 0.00	0.99 ± 0.01	13.09 ± 0.13	13.01[b] ± 0.02	1.49[b] ± 0.17
					14.83[c] ± 0.03	1.70[c] ± 0.00
Chicken breast	5.78 ± 0.00	0.19 ± 0.00	1.00 ± 0.00	1.36 ± 0.08	9.97[b] ± 0.1	0.85[c] ± 0.71
					27.80[c] ± 0.06	2.71[c] ± 0.00

[a] Values are the mean of triplicates plus its standard deviation. The values of NaCl, fat, NMP/Ntotal, and N-a-amino/Ntotal are in percentages. [b] Beginning of the storage period. [c] End of the storage period.

RESULTS AND DISCUSSION

Five different types of meat and meat products (espetec, cooked ham, pate, minced pork meat, and chicken breasts) were assayed in order to evaluate the antilisterial effect of bacteriocin. At selected times during storage, samples were taken in triplicate to determine the *L. innocua* CTC1014 counts. In espetec, *L. innocua* CTC1014 grew from 3.2 x 10^4 CFU/g to 2 x 10^5 CFU/g after 4 days of processing and decreased down to the initial counts at the end of the process (12 days) in the control batch without bacteriocin. In batch II inoculated with 221 AU/g of nisin, *Listeria* counts diminished from 3.2 x 10^4 CFU/g to 2.5 x 10^2 CFU/g. In batch III with 648 AU/g of nisin the *Listeria* counts diminished immediately to 100 CFU/g and were reduced to 50 CFU/g after the 5th day. Thus, representing a reduction of 3.61 log CFU/g when compared to the control batch. The physicochemical characteristics of the product were evaluated by its pH, water activity, fat content and the ratio of NPN versus total nitrogen and N-a-amino versus total nitrogen. The product showed a water activity (aw) of 0.875, a final pH of 6.06, and a fat percentage of 41.50% (Table 1).

In cooked ham, an outgrowth of *L. innocua* CTC1014 was observed in the control batch (batch IV) during storage. *Listeria* grew from 5 MPN/g to 1.9 x 10^{10} CFU/g at the end of the 37 days of storage at 7ºC. When 1,600 AU/g of nisin were applied in batch V, the growth of *Listeria* reached 7.9 x 10^7 CFU/g. In batch VI with 4,800 AU/g of bacteriocins, the growth of Listeria at the end of the assay reached 2 x 10^2 CFU/g, thus representing a 7.98 log CFU/ g reduction when compared to the control batch. Physicochemical parameters of the product are in Table 1; a high aw (0.982) and a low percentage of fat (3.48%) were observed. In pate, an outgrowth of *L. innocua* CTC1014 was observed in the control batch (batch VII). *Listeria* grew up to 1.9 x 10^9 CFU/g at the end of the assay (37 days). The *Listeria* population in batch VIII with 1,600 AU/g of bacteriocin only grew to 1 x 10^2 CFU/g at the end of the process. Batch IX with 4,800 AU/g of nisin was able to keep *Listeria* under 3 MPN/g during the 37 days storage at 7ºC (Fig. 3). A high percentage of fat (20.01%) and a high ratio between NPN and total nitrogen content could be observed (23.97%) (Table 1). When minced meat pork was stored for 6 days at 7ºC, *L. innocua* CTC1014 grew from 5 MPN/g to 48 CFU/g in the control batch, but no growth was observed (<3 MPN/ g) when 1,600 AU/g

of nisin were applied (batch XI) (Fig. 4). The pH of the product was slightly acidic (5.9) and the percentatge of fat 13.09% (Table 1). In chicken breasts, *L. innocua* CTC1014 grew up to 6 3 105 CFU/cm2 in the control batch during the 7 days of storage at 7ºC (Fig. 5). The population of *Listeria* was reduced to 2.7 x 10^2 CFU/g when 3,200 AU/cm of nisin were applied (batch XV) and diminished to 3.6 MPN/ cm^2 when 4,800 AU/cm^2 of bacteriocin were applied (batch XVI), thus representing a 5.26 log reduction when compared to the control batch without added bacteriocin. The pH of the product was slightly acidic (5.78) and after 6 days storage of the product at 78C, the percentages of NMP/ Ntotal and the N-a-amino/Ntotal raised by a factor of 2.8 and 3.18, respectively (Table 1).

Initial inocula of *L. innocua* CTC1014 in the different products assayed were standardized about 10 CFU/g in order to reproduce the natural initial contamination of meat products as previously reported (7, 26). In espetec (slightly fermented sausage) the initial inoculum was established as 1 x 10^4 CFU/g because it had previously been reported that *Listeria* survives but not grow in fermented products (21, 22). Temperature abuse (7ºC) has been applied in order to simulate abusive storage conditions. In this study bacteriocin were applied as semipurified food additives as prior *in situ* assays demonstrated that ad dition of *Lactococcus lactis* ATCC 7962 in fermented sausages, hamburgers, and chicken breasts did not exert any positive antilisterial effect when compared to the control batch. It has also been reported that the growth rate of the producer strain and bacteriocin production was highly inhibited at refrigeration temperatures and by salt and pepper (essential ingredients for fermented sausages) (2). The growth of *Listeria* in chicken breasts was high when compared to the growth of *Listeria* in minced pork meat during the 6 to 7 days of storage at 7ºC. Listeria grew from less than 10 MPN/g up to 48 CFU/g in minced pork meat and up to 6 x 10^5 CFU/cm^2 in chicken breasts. Similar results were obtained by Hugas *et al.* (23). The increasing amino acid content observed in the chicken breasts during the 6 days storage when compared to minced pork meat (Table 1) could in part explain the higher growth of *Listeria* in chicken breasts (17). Indigenous competitive microflora have also been proposed as a factor to explain the differential in growth of *Listeria* in minced pork meat while compared to chicken breasts (17, 26, 27). Other reported parameters linked to the growth of *Listeria* such as the higher water activity of the chicken breast and the higher thiamine, fat, and salt content of the minced pork meat could not be directly related to the growth of *Listeria* in the different products assayed in this study (17, 20, 41). In the batches treated with nisin a smaller amount of bacteriocins was needed to keep the *Listeria* counts under 3 MPN/g in the minced pork meat than in chicken breasts. Minced pork was fatter and had better bacteriocin adsorption than whole chicken breasts. In cooked ham and pate, *Listeria* grew very well and reached higher counts in cooked ham than in pate. In pasteurized products, the absence of competitive microflora seems to be the most important factor facilitating the growth of secondary contaminants, as there is no competition with the indigenous microflora of the raw meat (24). Besides, both products had a high aw and a pH over 6.0 (6.3), factors that have been described as benefiting the growth of *Listeria* (17). The higher fat and amino acid content of the pate did not promote the growth of *Listeria* in this product when compared to cooked ham. Without competitive microflora and in favorable physicochemical conditions, the small amino acid content of the cooked ham was enough to support a high outgrowth of *Listeria*. High numbers of *Listeria* are usually found in pate, 10^4 to 10^5 CFU/g (32). The batches of pate treated with 4,800 AU/g of cacteriocin were able to keep the *Listeria* counts under 3 MPN/ g while the batches of cooked ham treated with the same amount of bacteriocin reached 2 x 10^2 CFU/g. The results obtained in the raw (minced pork meat and raw chicken breasts) and cooked meat products (pate and cooked ham) seem to confirm that fat is important for the in situ activity of bacteriocin but not for Listeria growth. In espetec, *Listeria* grew 1 log cycle during the first 4 days of processing and then decreased to the initials counts. Thus, the physicochemical characteristics of the product, a_w (0.83 to 0.86), pH (6.0), nitrate (150 ppm), and nitrite (150 ppm) together with NaCl concentration (2.2%) and pepper (2‰) were able to inhibit the growth without eliminating *Listeria*. In dry fermented sausages, Hugas *et al.* (21, 22) observed 1-log reduction of *L. innocua* CTC1014 counts in the uninoculated batches. The lower pH (5.05) of dry fermented sausages was probably the main factor explaining the reduction of *Listeria* counts when compared to espetec. Grau and Vanderlinde (20) observed that Listeria grew better at pH 6.0 or higher than at 5.5 or less. The espetec sausages treated with 648 AU/g of nisin obtained an extra reduction of 3 log cycles in *Listeria* counts when compared to the control batch. With 400 AU/g of sakacin, Hugas *et al.* (21) obtained a 2.64 log reduction in *L. innocua* counts

when compared to the initial counts. Thus, bacteriocins may be considered as an additional anti-*Listeria* hurdle that could act cooperatively with the other additives and the global drying process typical in sausage manufacture.

CONCLUSION

Thus, bacteriocins may be considered as an additional anti-*Listeria* hurdle that could act cooperatively with the other additives and the global drying process typical in sausage manufacture.

Conflict of interest:
The authors declare that they have no conflict of interest.

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