Potravinarstvo, vol. 6, 2012, no. 4, p. 32-35
doi: 10.5219/218
Received: 9 May 2012. Accepted: 5 October 2012.
Available online: 13 December 2012 at www.potravinarstvo.com

APPLICATION OF NISIN INTO SLOVAK FERMENTED SALAMI PÚCHOV EXPERIMENTALLY INOCULATED WITH *LISTERIA INNOCUA*

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ABSTRACT

Púchov salami is favorite fermented salami among Slovak consumers. Nisin is the only bacteriocin accepted by European Commission for a commercial use as additive for food preservation (although not commonly used in meat products). Because of its possibility to prolonge shelf-life of the products and its antimicrobial activity, its effect in dry fermented Slovak salami Púchov experimentally inoculated with *Listeria innocua* Li1 strain was checked. The initial number of *L. innocua* Li1 in the inoculated salami mixtures was 10^4 CFU/g (log 10; 4.04 ± 0 07). After nisin addition, the count of Li1 strain in the meat samples (inoculated with Li1 and treated by nisin) was 1.36±0.07 CFU/g; difference 2.68 logarithmic cycle was noted between Li and Li/Ni samples. At day 2, the difference 3.23 log cycle was detected between Li1 and Li/Ni samples (Li:5.46±0.08, Li/Ni: 2.14±0.07 CFU/g); at weeks 3, 4, it was 1.69 and 1.80 log cycle. Activity of nisin itself was not recovered from the experimental salamis by the analytical method; however, its inhibitory effect was shown by Li1 count decrease. The pH in salamis during processing was almost at the same level (5.52, 5.53, 5.55). Water activity was not negatively influenced. Water content in Li/Ni salamis reached almost requested levels (maximum percentage of water requested is 34 %).

Keywords: nisin, *Listeria innocua*, fermented salami

INTRODUCTION

Púchov salami is a smoked fermented meat product having a flat shape form, a brown-red colour, a strong, spring consistency, with smoked, pepper smell without moulds on its surface; it is smooth and presents homogenous cuts with eventual few air bubbles; its taste is salty and spicy. It is one of favorite fermented salami among Slovak consumers. Nisin is the only bacteriocin (lantibiotic) accepted by European Commission for a commercial use as additive in food preservation (although not commonly used in meat products). It was admitted into the European food additive list, where it was assigned the number E234 (1983). Nisin was also approved by the Food and Drug Administration in the USA as Generally Recognized As Safe. It is a peptide composed of 34 amino acid residues with a molecular mass of 3.5 kDa with an inhibitory activity against Gram-positive, spoilage bacteria **(Sahl & Bierbaum, 1998).** However, its *in vitro* activity towards Gram-negative bacteria was also reported **(Delves-Broughton et al., 1996; Lauková, 2000; Boziaris & Adams, 2001)** due to permeabiliziation of outer membrane cell layer as caused by sublethal heating, freezing and chelating agents. Nisin is a highly surface-active molecule that can bind to different compounds e.g. fatty acids of phospholipids. This feature makes it suitable for adsorption to solid surfaces and killing bacterial cells that adhering there **(Sobrino-López & Martín-Belloso, 2008).** Several authors reported successful use of nisin to reduce the growth of *Listeria monocytogenes* or *Staphylococcus aureus* on meats **(Chung et al., 1989)** or to prolonge shelf-life of the products **(Scannel et al., 1997).** Therefore, we decided to check nisin effect in dry fermented salami Púchov which was experimentally inoculated with *Listeria innocua* Li1 strain as a model meat contaminant. Although more frequently *Listeria monocytogenes* has been found as meat contaminant; because of sausage preparing in the meat manufacture, here *Listeria innocua* Li1 instead *L. monocytogenes* was used. The experiment was conducted to have possibility to compare nisin effect in meat with those previously obtained with nisin in dairy products such as cow cheese lump where *S. aurues* SA5 was reduced by nisin treatment; and also to compare use of other bacteriocins (enterocins) in the same or similar niches as previously tested in dairy products **(Lauková & Czikková, 1999; Lauková et al., 1999a; Lauková et al., 2001a).**

MATERIAL AND METHODOLOGY

Bacterial strains, media, experimental salami manufacture

Listeria innocua Li1 was kindly supplied by Dr. Hans Blom (Matforsk, As, Norway). It was incubated in Trypticase soy broth enriched with 0.6 % yeast extract

(TSB, Becton & Dickinson, Cockeysville, USA) at 32 ºC for 18 h before use. Li1 strain (10^7 CFU/ml) was used for direct inoculation into a salami during the manufacture experiment as well as in the bacteriocin activity testing.

The meat mixture for Púchov salami contained the following components (g): pork lean meat (800); pork without skin (650); beef back without bones (130); nitrite curing salt (32); glucose (1.0); black pepper (3.5); red pepper (7.0); chilli pepper, (4.0); garlic (1.0); muscat nutmeg (0.4); grind caraway seeds (1.0). Starter culture "Flora Carn" (Christian Hansen Laboratory, a.s. Copenhagen, Denmark, containing *Lactobacillus pentosus* and *Staphylococcus carnosus*) was added at 25 g per 100 kg (i.e.1.87g per 7.5 kg-3 trials in each 2.5 kg of meat mixture (10^{11} CFU/ml). The strains involved in Flora Carn were not inhibited by nisin. The initial pH of the meat mixture was 6.19. The bulk salami mixture was prepared in the pilot plant and 2.5 kg for each of three trials was transferred to the laboratory for the experiment. Three independent trials were conducted, each trial comprising then five salamis (0. 500 g). Trial A (reference control), involving only untreated salami mixture. Trial B represents salami mixture inoculated with *Listeria innocua* Li1 (10^7 CFU/ml). For trial C, nisin (commercial product Nisaplin, *Aplin & Barrett*, Dorset, England) in concentration 1mg/g was added to the salami mixture inoculated with *L. innocua* Li1 strain. Nisin for use was prepared and maintained as described **Kišidayová et al. (2009).** The meat mixtures were stuffed into 50 mm diameter collagen casings and the flat shape salamis prepared by this way were transferred back to the pilot plant, kept separately and ripened at 2-4 ºC for 2 days in the cool room. The salamis smoking procedure was performed according to the technological parameters; the products were smoked permanently at 20 ºC for 24h, kept for 14 days under 12º C and relative humidity 80 % in the dry chamber. They were stored (ripened) for 4 weeks.

Salami sampling and analyses

Sterile lancet, removing 10 g from the middle of the each product was used to take samples for the microbiological determination by the standard microbiological method according to International Organization for Standardization (ISO). The samples were homogenized in Stomacher (Stomacher 80, Seward Laboratory Stems, England) with 90 ml of peptone water (Oxoid) for several minutes. Then, serial dilutions were prepared and spread onto Fraser agar base supplemented with Fraser additive (Becton & Dickinson) and simultaneously on Mc Bridge Listeria agar/Oxford agar (Becton & Dickinson) and cultivated at 29-31º C for 48 h. The counts of bacteria were enumerated in CFU/g ±SD. In the reference salami samples, the total bacterial count was estimated using Columbia blood agar and Trypticase soy blood agar (Becton & Dickinson) continually in the time of the experiment. Sampling was provided at day 1 (before and after bacteriocin addition), at day 2 and at weeks 3, 4. All samples (from each trial) were examined in duplicate (five salamis in each trial in duplicate). The bacteriocin activity in the salami samples was checked according to **Coffey et al. (1998).** Briefly, 5g of salami was mixed with 70% of *iso*-propanol to a total volume of 10 ml and homogenized. The homogenate was diluted and 50 µl aliquots of it were applied into wells in an agar plates which had been seeded with *L. innocua* Li1 strain. Plates were incubated at 37º C for 4 h (first check-up), then for 16 h. The pH measurement was carried out by inserting the pin electrode of pH-meter Hanna Checker^R (Fischer Scientific Ltd. Pardubice, Czech Republic). Water activity (a_w) and water content were determined by the standard norm STN 56 0030.

RESULT AND DISCUSSION

The initial number of *L. innocua* Li1 in the inoculated meat mixtures was 10^4 CFU/g (log 10; 4.04 ± 0 07, Table 1). After nisin addition, the *Listeria* count detected in the samples infected with Li1 strain and treated by nisin was 1.36 ± 0.07 CFU/g; difference 2.68 logarithmic cycles was noted between Li samples and Li/Ni samples. At day 2, the difference 3.23 log cycles was noted between Li1 and Li/Ni samples (Li:5.46±0.08, Li/Ni: 2.14±0.07 CFU/g). Although, at weeks 3 and 4 slight increase in Li1 cells was determined in Li samples, the difference of Li1 cells in Li samples and Li/Ni samples was still noted (difference 1.69; 1.80 log cycles, Table 1). The microbial backround of the reference salami (concerning an unsuitable microbiota) was under detection limit (<1.0 respectively 0.60 CFU/g). Inhibitory activity of nisin was demonstrated by the decrease of Li1 strain in Li/Ni samples; however, the bacteriocin activity of nisin itself was not possible to measure in the product by the analytical method. Nisin action can either be bactericidal or bacteriostatic depending on the nisin concentration, bacteria concentration, physiological state of the bacteria and the prevailing conditions. Nisin will show a more pronounced bactericidal effect when conditions to test are optimum for the growth of the bacteria, e.g. optimum temperature, pH, water activity, redox potential and nutrient availability and the bacteria are in an energised stage **(Sahl, 1991**; **Maisner-Patin et al., 1992).** So, there exist contradictory results of nisin use in meat; e.g. **Rayman et al. (1983)** who have underlined an interference of nisin by meat components. This could be explanation why bacteriocin has been not detected in salami or meat; however in our experiment decrease of Li1 cells was demonstrated. **Davies et al. (1999)** reported higher effect of nisin in bologna-type sausage to control lactic acid bacterial spoilage when lower fat content was detected in the product. Loss of nisin activity in meat has been ascribed, in part, to the formation of a nisin-glutathione adduct. Activity is lost more quickly in raw meat than in cooked meat, and this has been taken as evidence that the reaction is enzyme mediated. Formation of the nisin-glutathione adduct has been confirmed but is shown not to be enzyme mediated. Retention of activity in cooked meat is shown to be due to the loss of free sulfhydryl groups during cooking as a result of the reaction of glutathione with proteins and not a result of the inactivation of endogenous enzymes. Microbial enzymes do not appear to play a role, as similar losses are seen in raw and cooked meat extracts, both of which contained undetectable levels of microorganisms **(Sergiou et al., 2006).** Lost of activity could be also the response of environmental factors on nisin activity; that is, the activity may be affected by changes in solubility, binding of the bacteriocin to food components, etc. **(Gänzle et al., 1999).** Cells reduction but

no activity was also reported in our studies with enterocin addition to Hornád salami **(Lauková et al,. 1999b).** Under *in vitro* conditions an inhibitory effect of nisin was demonstrated by **Delves-Broughton et al. (1996)**; **Lauková & Juriš , 1998; Lauková et al,. 2001b).** To compare effect of nisin with the effect of *e.g.* enterocin 4231 after its addition in the same type of the product-Púchov salami; at the beginning higher decrease of Li1 cells was achieved between untreated samples and samples treated with nisin than after enterocin addition; then at weeks 3, 4, lower decrease of Li1 cells was achieved in the salamis with nisin than in salamis with enterocin 4231 (Ni:1.69, 1.80; Ent:4.2.36, 2.38 log cycles; **Lauková et al., 2011).**

The initial pH of the meat mixture was quite high-6.19. This value was decreased in Li/Ni salami, Li1 salami and the reference salamis almost to the same level (R-5.52, Li-5.53, Li/Ni-5.55, Table 2). The values of a_w were only slightly decreased. At week 4, the initial a_w 0.92 was decreased to 0.84 in Li/Ni, Li and R as well (Table 2). That is, pH in the salamis was not influenced by nisin addition as well as by Li1 contamination. Moreover, a_w was not influenced by nisin. There are not so many studies concerning the study of the effect of bacteriocins and/or their producers on a_w or pH values in meat products. They are more focused on their antimicrobial effect or sensory character. The similar pH and a_w as in our study were reported **by Lauková et al. (1999b)** in Hornád salami processed with enterocin 4231 (produced by *Enterococcus faecium* CCM 4231) and in the salami Štart processed with bacteriocin-producing strain *Staphylococcus xylosus* SX S03/1M/1/2, **Lauková et al., 2010).**

Although no specific sensory analyses were provided, the salamis kept their typical character. Water content in Li/Ni salamis possess 23.4 % comparing to the reference control salamis (24.9%) and Li salamis (23.5 %). The salamis processed in our experiment reached almost requested levels (maximum percentage of water requested is 34 %). In conclusion, nisin treatment has led to *L. innocua* cells decrease. The value of pH and water activity were not influenced. In our experiment nisin seems to be promising additive in this type of fermented salami. Because of some contradictory results reported previously, of course, further more detail studies and more parameters to search are requested.

Tab.1 The counts of *Listeria innocua* Li1 in Púchov salami after nisin treatment

(expressed as log10 CFU/g, colony forming units per g ± SD)

Sampling	Li[a]	Li/Ni[b]
Day 0-1	4.04 ± 0.07	1.36 ± 0.07
Day 2	5.46 ± 0.08	2.14 ± 0.07
Week 3	6.40 ± 0.11	4.71 ± 0.09
Week 4	6.50 ± 0.08	4.70 ± 0.08

Li[a] - the samples with *Listeria innocua* Li1, Li/Ni[b]- the experimental samples with Li1 strain and nisin, Day 0-1: the start of the experiment, Day 2, weeks 3, 4: sampling at day 2, at weeks 3,4; at day 0-1, after nisin addition into mixture difference 2.68 log cycle was noticed between samples infected with Li1 strain and those also treated by nisin; at day 2 this difference was 3.32 log cycles, at weeks 3,4 it was 1.69, 1.80

Tab. 2 The pH values and water activity (a_w) in Slovak fermented salami Púchov treated by nisin and experimentally inoculated with *L. innocua* Li1

Sampling	pH			a_w	
	R[a]	Li[b]	Li/Ni[c]	R[a]	Li[b]
Li/Ni[c]					
Day 0-1	6. 19	6.19	6.19	0.92	0.92
0.92					
Week 1	5.35	5.42	5.41	0.91	0.92
0.92					
Week 2	5.38	5.33	5.38	0.90	0.90
0.90					
Week 3	5.47	5.47	5.55	0.90	0.90
0.89					
Week 4	5.52	5.53	5.55	0.84	0.84
0. 84					

[a]Reference control samples, [b]samples infected with *L. innocua* Li; [c]experimental samples-infected with Li1 strain and treated with nisin, pH values, a_w-water activity

CONCLUSION

Although nisin is the only bacteriocin accepted by European Commission for a commercial use as additive for food preservation (however not commonly used in meat products) the results achieved confirmed its effectivity in dry fermented Púchov salami experimentally inoculated with *Listeria innocua* Li1 strain. Inhibitory effect if nisin in the experimental salamis was demonstrated by Li1 count decrease. The pH in salamis during processing was almost at the same level. Water activity was not negatively influenced.

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Acknowledgments:

The experiment was performed in continuity with EU-Copernicus project ERBCIPACT940160. Following further study, the search has been also partially provided by the project TRADISAUSAGE QLK1 CT-2002-02240.

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