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EFFECT OF ENTEROCIN 4231 IN SLOVAK FERMENTED SALAMI PÚCHOV AFTER ITS EXPERIMENTAL INOCULATION WITH *LISTERIA INNOCUA* Li1

Andrea Lauková[1], Peter Turek[2]
[1]Slovak Academy of Sciences in Košice, Slovakia
[2]University of Veterinary Medicine and Pharmacy in Košice, Slovakia

Background. Enterococci occur and may compete well in fermented sausages and *Enterococcus faecium* represents that species of the lactic acid bacteria which can be found in the fermented sausages. The representatives of this species can produce bacteriocins with predominant anti-listerial effect. Therefore, the effect of enterocin (*Ent*) 4231 produced by *Enterococcus faecium* CCM 4231 strain with probiotic properties was tested in a dry fermented salami Púchov (Slovak product) experimentally inoculated with *L. innocua* Li1 strain (10^7 cfu/ml).
Material and methods. The bulk salami mixture was prepared in the pilot plant and 2.5 kg for each of three trials were transferred to the laboratory for the experiments. Three independent trials were conducted, each comprising then five salami samples (0.500 g). Trial A (reference control) involved only untreated salami mixture. Trial B represented salami mixture inoculated with *Listeria innocua* Li1 (10^7 cfu/ml). For trial C, *Ent* 4231 possessing activity 6400 AU/ml was added into the salami mixture inoculated with *L. innocua* Li1 (Li/*Ent*). The mixtures were stuffed into collagen casings and the flat shape salamis were transferred back to the pilot plant and treated according to conditions typical for this product and stored for 4 weeks.
Results. The initial number of *L. innocua* Li1 in the inoculated salami mixture was 10^4 cfu/ml. After *Ent* 4231 addition, the count of *Listeria* detected in the salami samples inoculated with Li1 and treated with *Ent* 4231 was 3.64 ±0.14 cfu/ml; difference 0.40 logarithmic cycles was noted between Li samples and Li/*Ent* samples. On day 2, the difference 1.86 log cycles was noted between Li1 and Li/*Ent* samples. Although, in weeks 3 and 4, slight increase in Li1 cells was determined in Li salamis, the difference in the detection of Li1 cells in Li salamis and Li/*Ent* samples was even higher than that immedially after *Ent* addition (difference 2.30; 2.48 log cycles). Bacteriocin activity itself was not recovered from Li/*Ent* salamis. The pH of the all salamis was almost at the same level. Water activity and water content were not influenced.

Corresponding author – Adres do korespondencji: Ph.D. Andrea Lauková, Institute of Animal Physiology of Slovak Academy of Sciences, Šoltésovej 4-6, 04001 Košice, Slovakia, e-mail: laukova@saske.sk

Conclusion. Addition of *Ent* 4231 during processing of salami Púchov experimentally inoculated with *L. innocua* Li1 has lead to decrease of Li1 cell growth, although the bacteriocin activity of *Ent* itself was not possible to detect in salami samples. The pH value, water activity, as well as sensory character of the final products were not negatively influenced.

Key words: enterocin, dry-fermented salami, *Listeria*, cells reduction

INTRODUCTION

Food safety is a top priority for authorities and consumers worldwide. Although the established microbiological criteria must be accomplished, the prevalence of food--borne pathogens is documented [European Food Safety Authority 2005]. According to the newest references [Bergey's Manual 2009], enterococci are representatives of the division *Firmicutes*, the *Enterococcus* family (Enterococcaceae), genus *Enterococcus*. They occur in many ecosystems, food including [Devriese et al. 1991, Lauková et al. 1993, Franz et al. 2003]. Enteroccocci occur and may compete well in fermented sausages [Talon et al. 2007]. According to Hugas et al. [2003] *Enterococcus faecium* represents that species of the lactic acid bacteria which can be found in the fermented sausages. The representatives of this species can produce bacteriocins with predominant anti-listerial effect [McKay 1990, Giraffa et al. 1994, Lauková et al. 1999 a, Callewaert et al. 2000] but also against spoilage bacteria and some Gram-negative species [Cintas et al. 1997, Garcia et al. 2004]. The potential of non-meat origin enterococci and/or their bacteriocins to be used in meat/food has been studied only in limit up to now [Callewaert et al. 2000, Lauková et al. 1999 a]. However, their real potential to reduce spoilage organisms lead us to study the effect of enterocin (*Ent*) 4231 (produced by *Enterococcus faecium* CCM 4231 strain, our isolate possessing also probiotic properties) in controlling *Listeriae* growth in dry-fermented salami Púchov (favourite among Slovak consumers) after its experimental inoculation with *L. innocua* Li1 strain. Púchov salami is a smoked fermented meat product presented in a flat shape form, brown-red colour, strong and spring consistency and smoked and pepper smell without moulds on its surface; it is smooth and presents homogenous cuts with possible few air bubbles; its taste is salty and spicy. There are several differences between previously tested Hornád salami in the composition content and in the starter culture used, as well as in some parameters during the ripening [Lauková et al. 1999 a]. *Ent* 4231 is thermo-stable bacteriocin, with a broad anti-microbial spectrum, it is small peptide, size in the range 3-10 kDa. Its production culminates in late logarithmic phase of its producer strain growth [Lauková et al. 1993]. The decision to use *Ent* 4231 in dry-fermented salami was based on our previous results because of its beneficial experimental application in dairy products [Lauková and Czikková 1999, Lauková et al. 1999 b, 2001].

MATERIAL AND METHODS

Partially purified Enterocin 4231 preparation

The supernatant of an 18 h culture of *Enterococcus faecium* CCM 4231 which produces *Ent* 4231 was treated as previously described [Lauková et al. 1999 a, b]. Briefly, the cells were removed by centrifugation at 10.000 g for 30 min. The supernatant fluid adjusted with the addition of phosphate buffer (pH 7.2) to 50 mM was applied to a phenyl-Sepharose high performance liquid chromatography column (HPLC) at a flow-rate of 30 ml/h. The fraction was eluted in distilled water at flow rate of 60 ml/h. Tris-HCl was added to this fraction which was applied to a DEAE-Sepharose HPLC column. The *Ent* was eluted with buffer adjusted with the addition of 350 mM sodium chloride (NaCl). The bacteriocin activity was determined by the agar spot test using the critical dilution method [De Vuyst et al. 1996]. Activity was defined as the reciprocal of the highest two-fold dilution demonstrating complete inhibitory activity (zone of inhibition) of the indicator and was expressed in activity units (AU) per ml of culture medium. The bacteriocin for use was kept at 4°C. The remainder was stored at –20°C. *Ent* 4231 used for application possessed activity 6400 AU/ml.

Bacterial strains, media, salami manufacture

Listeria innocua Li1 strain was supplied by Dr. Blom (Matforsk, As, Norway). It was incubated in Trypticase soy broth enriched with 0.6% yeast extract (TSB, Becton and Dickinson, Cockeysville, USA) at 32°C for 18 h before use. Broth culture of Li1 strain (10^7 cfu/ml) was used for inoculation of the salami mixtures during their manufacturing. *Ent* 4231 producing strain *Enterococcus faecium* CCM 4231 is our isolate [Lauková et al. 1993]. It was incubated in Todd-Hewitt broth (Imuna, Šarišské Michaľany, Slovakia) at 37°C for 18 h.

The salami mixture 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 (3.50), red pepper (7.0), chilli pepper (4.0), garlic (1.0), muscat nutmeg (0.40), grind caraway seeds (1.0). Starter culture FloraCarn (Christian Hansen Laboratory, a.s. Copenhagen, Denmark, containing *Lactobacillus pentosus* and *Staphylococcus carnosus*) was added at 25 g per 100 kg (i.e. 1.87 g 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 sensitive to *Ent* 4231. 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 experiments. Three independent trials were conducted, each comprising then five salami samples (0.500 g). Trial A (reference control) involved only untreated salami mixture. Trial B represented salami mixture inoculated with *Listeria innocua* Li1 (10^7 cfu/ml). For trial C, *Ent* 4231 possessing activity 6400 AU/ml was added into the salami mixture inoculated with *L. innocua* Li1 (Li/*Ent*). The 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 where they were kept separately and ripened at 2-4°C for 2 days in the cool room. The salami smoking procedure was performed according to the technological parameters; the products were smoked permanently at 20°C for 24 h; in the dry chamber they were kept for 14 days under the temperature 12°C, relative humidity 80%. They were stored for 4 weeks.

Salami sampling and analyses

Sterile lancet, removing 10 g from the middle of the product was used to take samples for the microbiological determination by the standard microbiological method according to the International Organization for Standardization. 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 broth additive (Becton & Dickinson) and simultaneously on Mc Bridge Listeria agar/Oxford agar (Becton & Dickinson) and cultivated at 29-31°C for 48 h. The counts of bacteria were enumerated in cfu/ml ±SD. In the reference salami samples, the bacterial backround was estimated on Columbia blood agar and Trypticase 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, 5 g 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 37°C for 4 h (first checking), 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 using the standard norm STN 56 0030 (previously Slovak norm).

RESULTS

The initial number of *L. innocua* Li1 in the inoculated salami mixture was 10^4 cfu/ml (log 10, 4.04 ±0.07; Table 1). After *Ent* 4231 addition, the count of *Listeria* detected in the salami samples inoculated with Li1 and treated with *Ent* 4231 was 3.64 ±0.14 cfu/ml; difference 0.40 logarithmic cycles was noted between Li samples and Li/*Ent* samples. At day 2, the difference 1.86 log cycles was noted between Li1 and Li/*Ent* samples (Li: 5.46 ±0.08, Li/*Ent*: 3.60 ±0.0814 cfu/ml). Although, in weeks 3 and 4, slight increase in Li1 cells was determined in Li samples (Table 1), the difference in the detection of Li1 cells in Li salami samples and Li/*Ent* samples was even higher than that immediatelly after *Ent* addition (difference 2.30; 2.48 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/ml). Bacteriocin was not recovered from our experimental salamis by the analysis applied; in spite of demonstrating decrease in Li1 cells.

The initial pH of the meat mixture was 6.19. However this value was decreased in the experimental, control, as well as reference salamis almost to the same level (Table 2). The water activity (a_w) was decreased from the initial level 0.92 to 0.83 in Li/*Ent* salamis and 0.84 in Li and R salami samples at week 4 (Table 2).

Table 1. Effect of *Ent* 4231 against *Listeria innocua* Li1 in Púchov salami (expressed in colony forming unit per g and ml – cfu/ml) log 10

Sampling	Li	Li/*Ent*
Day 0-1	4.04 ±0.07	3.64 ±0.14
Day 2	5.46 ±0.08	3.60 ±0.08
Week 3	6.40 ±0.11	4.04 ±0.06
Week 4	6.50 ±0.08	4.02 ±0.05

Li – the salami samples with *Listeria innocua* Li1.
Li/*Ent* – the samples inoculated with Li1 and treated with *Ent* 4231.

Table 2. The pH values and water activity (a_w) in fermented salami Púchov experimentally inoculated with *Listeria innocua* Li1 and treated *Ent 4*231

Sampling	pH values			Water activity a_w		
	R	Li	Li/*Ent*	R	Li	Li/*Ent*
Day 0-1	6.19	6.19	6.19	0.92	0.92	0.92
Week 1	5.35	5.42	5.40	0.91	0.92	0.92
Week 2	5.38	5.33	5.53	0.90	0.90	0.91
Week 3	5.47	5.47	5.48	0.90	0.90	0.89
Week 4	5.52	5.53	5.55	0.84	0.84	0. 83

R – reference control samples.
Li – samples inoculated with *L. innocua* Li1 strain.
Li/*Ent* – samples inoculated with *L. innocua* Li1 strain and treated with *Ent* 4231.

Although no specific sensory analyses were provided, the salamis kept character prescribed for Púchov salami. The salamis processed in our experiment possess water content 23.8% (Li/*Ent*), 23.5% (Li) comparing with the reference control salamis (24.9%)

DISCUSSION

Enterocins could be considered as extra biopreservative hurdles to reduce/protect *Listeria* growth in dry-fermented sausages. Aymerich et al. [2000] reported significant decrease of *Listeria* counts by 1.13 log ($P < 0.001$) in sausages treated by *Ent* A, B comparing with the control samples, as well as to compare with sausage produced with the *Ent*- producing strain *E. faecium* CTC 492T [Aymerich et al. 2000]. Moreover, *Ent* 4231 strongly inhibited the growth of *Listeriae* when applied in Spanish-style dry-fermented sausages [Callewaert et al. 2000]. The possibility to control *Listeria* counts by *Ent* was also demonstrated in model sausages by Ananou et al. [2005]; there a significant decrease of *Listeria* cells was noted even in the lowest concentration of *Ent* AS-48

addition. On the other hand, in our previous study with *Ent* M used in Gombasek sausage to reduce *Listeria* counts after the experimental contamination of sausage, a decrease in cells of Li1 strain was noted at day 2 and at week 1; but at week 2 re-growth of cells was noted [Lauková et al. 2003]. The effect of *Ent* even in meat ecosystem is probably dependent on the *Ent* purity, its concentration and dose, as well as on the sensitivity of the indicator-inoculant used. On the base of our previous *in vitro* and *in vivo* results, under *in vitro* conditions inhibition of sensitive indicator (inoculant) strain by *Ent* has not been corresponded with its *in vivo* inhibition because of its adsorption to meat vehicules and/or *Ent* activity could be diminished or inhibited by influence of sausage ingredients and additives – except nitrate [Aymerich et al. 2000]. Sabia et al. [2003] referred bacteriocin 416 K1 produced by *E. casseliflavus* IM 416K1 as a natural antagonist to control *L. monocytogenes* in Italian sausages "cacciatore". Additionally, antimicrobial activity of *Ent* was also reported in the other foods; e.g. zucchini pureé [García et al. 2004], Cheddar or soft cheese [Foulquié Moreno et al. 2003, Izquiérdo et al. 2009]. When nisin was applied in Púchov salami, the reduction of Li1 cells was also noted; however with higher differences immediatelly after nisin addition and with lower difference in weeks 3, 4 [Lauková and Turek 2011] opposite to the results achieved here.

Bacteriocin was not recovered from our experimental salamis, in spite of demonstrating decrease in Li1 cells in Li/*Ent s*alamis. It could be explained by the same conditions formerly mentioned by Aymerich et al. [2000]. No bacteriocin detection, but decrease in the counts of *Listeria* cells, was noted in our previous experiment using *Ent* 4231 in dry-fermented salami Hornád [Lauková et al. 1999 a].

The pH in the Li/*Ent* and Li salami samples was not influenced by *Ent* 4231 addition, as well as by Li1 contamination. Moreover, a_w was also not negatively influenced. There are not so many studies concerning 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. However, Hugas et al. [1995] or Tyopponen et al. [2003] reported the decrease of pH in sausage as a consequence of the fermentation of carbohydrates to lactic acid. The decrease in pH causes a decrease in water binding capacity of the meat, which accelerates the drying process of dry sausages; it results in low water activity of the end product. The similar pH or a_w were reported by Lauková et al. [1999 a, 2010] in Hornád salami processed with *Ent* 4231 produced by *E. faecium* CCM 4231 and in salami Štart processed with bacteriocin-producing strain *S. xylosus* SX S03/1M/1/2. In general, the pH and/or a_w values have been not negatively influenced by bacteriocin or probiotic additives. The water content was at the level of the requested parameters provided for Púchov salami (34%). There is, it was repeatedly confirmed in different types of meat products, a promising use of *Ent* control *Listeria* growth. Moreover, the strains which produce enterocins often possess probiotic properties. It is supposed they can be directed for the use in functional food/feed. However, the problem is legislation; but it has been started to be processed by EFSA [Morelli 2009]. On the other hand, in Japan already in 1991 the concept of functional foods was established including 9 meat products [Arihara 2006]. Even in the year 1996, the British Advisory Committee on Novel Foods and Processes (ACNFP) accepted the use of *E. faecium* strain K77D as a starter culture in fermented dairy products.

CONCLUSION

Addition of *Ent* 4231 during processing of salami Púchov experimentally inoculated with *L. innocua* Li1 has lead to decrease of Li1 cell growth, although the bacteriocin activity of *Ent* itself was not possible to be detected in salami samples. The pH value, water activity, as well as (however, not specifically analysed) sensory character of the final products were not negatively influenced. Although more further detail studies are requested, the results achieved contribute to knowledge concerning the anti-listerial control of enterocins in food ecosystem.

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WPŁYW (SKUTECZNOŚĆ) ENTEROCYNY 4231 W SŁOWACKIEJ FERMENTOWANEJ SALAMI PÚCHOV ZASZCZEPIONEJ *LISTERIA INNOCUA* Li1

Wstęp. Enterokoki występują i są mikroflorą konkurencyjną w kiełbasach fermentowanych, a *Enterococcus faecium* reprezentuje gatunek bakterii kwasu mlekowego, wykrywany w kiełbasach fermentowanych. Przedstawiciele tego gatunku mogą produkować bakteriocyny hamujące rozwój listerii. Dlatego sprawdzono wpływ enterocyny (*Ent*) 4231, produkowanej przez szczep *Enterococcus faecium* CCM 4231 o właściwościach probiotycznych, na rozwój *L. innocua* Li1. Bakteriami *L. innocua* Li1 zaszczepiono fermentowane salami 'Puchov' (produkt słowacki) w ilości 10^7 jtk/g.
Materiał i metody. Farsz salami został wyprodukowany w zakładzie pilotowym i po 2,5 kg każdej z trzech prób przekazano do laboratorium. Wykonano trzy niezależne powtórzenia, każde składające się z pięciu próbek salami (0,500 g). Próba A (kontrolna) obejmowała tylko farsz salami nie poddany żadnemu działaniu. Próba B przedstawiała farsz salami zaszczepiony *Listeria innocua* Li1 (10^7 jtk/g). W przypadku próby C dodano *Ent* 4231 o aktywności 6400 AU/ml do farszu salami, zawierającego *L. innocua* Li1 (Li/*Ent*). Farszem nadziano osłonki kolagenowe i salami o spłaszczonym kształcie przewieziono z powrotem do zakładu pilotowego, poddano zabiegom typowym dla danego produktu i przechowywano przez 4 tygodnie.
Wyniki. Początkowa liczba *L. innocua* Li1 w zaszczepionym farszu salami wynosiła 10^4 jtk/g. Po dodaniu *Ent* 4231 liczba *Listerii* wykrywanej w próbkach salami zaszczepionych Li1 i poddanych działaniu *Ent* 4231 wynosiła 3,64 ±0,14 jtk/ml; różnica 0,40 cykli logarytmicznych została stwierdzona między próbką Li a Li/*Ent*. W 2 dniu między próbkami Li1 i Li/*Ent* zanotowano różnicę 1,86 cykli logrytmicznych. Chociaż po 3 i 4 tygodniu zaobserwowano niewielki wzrost liczby komórek Li1 w salami Li, różnica między liczbą stwierdzonych komórek Li1 w próbkach salami Li i Li/*Ent* była nawet większa niż bezpośrednio po dodaniu *Ent* (różnica 2,30; 2,48 cykli log). Aktywność bakteriocyn nie została odzyskana w salami Li/*Ent*. Wartość pH wszystkich próbek salami była prawie na takim samym poziomie. Nie zmieniały się aktywność wody i zawartość wody.
Wnioski. Dodatek *Ent* 4231 w procesie produkcji salami 'Puchov', doświadczalnie zaszczepionej *L. innocua* Li1, prowadził do zmniejszenia rozwoju komórek Li1, chociaż aktywność bakteriocyn *Ent* nie była możliwa do wykrycia w próbkach salami. Zarówno wartość pH, jak i aktywność wody i właściwości sensoryczne gotowego produktu nie zmieniły się negatywnie.

Słowa kluczowe: enterocyna, salami fermentowane, *Listeria*, redukcja komórek

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