

Anti-Listerial Activity Control of Postbiotic in Cow Milk Lump Cheese Processing

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ABSTRACT

Nowadays, a new approach has been focused on the food safety maintenance using postbiotics (antimicrobial peptides-bacteriocins). This approach involves also use of postbiotics where bacteriocins are included. Here Enterocin A/P (isolated and studied at our laboratory) was applied in cow milk lump cheese inoculated with *Listeria innocua* LMG 13568. Enterocin A/P was found with reducing tendency against *L. innocua* LMG 13568 in cow milk lump cheese (differences 1.0 - 0.42 log cycle) using the standard microbial method. Direct anti-listerial activity of Enterocin A/P in cheese was not found. The acidity parameters (pH, °SH) were well balanced, not influenced (pH) and/or continually increased (°SH) with Enterocin A/P treatment and LMG 13568 strain inoculation. Additional studies are requested; however, postbiotic-dipeptide Enterocin A/P showed beneficial antimicrobial potential tendency in cow milk lump cheese.

Keywords: Enterocin, effect, cow milk lump cheese, anti-*Listeria*

INTRODUCTION

Taking into account the strong impact of foodborne diseases on human health and the growing consumers attention on the use of natural substances (antimicrobial peptides) to maintain food safety, a new approach has been focused on this object [1]. This approach involves also use of postbiotics [2, 3], among which bacteriocins are also included. Our laboratory has already possessed a beneficial experience with the use of bacteriocins to protect food since 1999 year [4-5]. Therefore, we decided to apply Enterocin A/P against *Listeria innocua* in cow milk lump cheese. Dipeptide Enterocin (Ent) A/P belongs in a group of thermo-stable, small peptide-enterocins, possessing a broad inhibitory spectrum against more and less related bacteria [6]. Ent A/P was purified into homogeneity [7] and its beneficial effect was experimentally checked also in animals, e.g. gnotobiotic Japanese quails [8]. There inhibition of *Salmonella* infection was reported and better therapeutic than preventive effect was demonstrated. Application of Ent A/P before and after *Salmonella* infection had a protective effect on the duodenal epithelium.

The damage to microscopic and submicroscopic structure of enterocytes and goblet cells of the intestinal epithelium and its subsequent necrosis was less intensive after the treatment with Ent A/P [9]. Also, its effect against methicillin-resistant staphylococci was demonstrated [10]. Moreover, that effect was also experimented in broiler rabbits [11]; there it was reported that Ent A/P could be used as a preventive supplement in rabbit husbandry. Moreover, phagocytic activity as non-specific parameter of immunity was stimulated and gut microbiota were optimized in broiler rabbits due to reduction of coliforms and also methicillin-resistant staphylococci [12]. That it is why we decided for Ent A/P application.

Listeria spp. are potentially pathogenic bacteria. Through the ingestion of contaminated food products, *Listeria* spp. can cause serious infections [13, 1]. Biocontrol of listeriae by bacteriocins has attracted a great attention during last decade [14]. Cheese is considered to be one of the dairy foods most frequently contaminated with *Listeria* spp., especially with the species *L. monocytogenes* [15]. It is also isolated from dairy facilities due to its ability to adapt and proliferate in low temperatures and pH, high salt content and its ability to form biofilm [15]. Therefore, listerial growth prevention and/or reduction/elimination is requested. And when it is via a new approach such as postbiotic it is more welcomed not only by scientific/producers' community but also by a broad public. In addition, it is following a sense of One-Health concept. Moreover, a product supplemented with postbiotic can fulfill a character of functional food [16].

MATERIAL AND METHODS

Cow Milk Lump Cheese Manufacturing Process

Cow milk lump cheese was manufactured from milk in 20-L (liter) vats using the standard technology for this type of cheese as previously described by Grieger and Burdová [17] and Lauková et al. [18]. Pre-heated milk vats were divided into the reference/control vat/cheese (RCV), the experimental vat/cheese E1 (10^7 cfu/ml of *Listeria innocua* LMG 13568, kindly provided by prof. Luc De Vuyst from University in Brussel, Belgium), the experimental vat/cheese 2 (E2= inoculation with LMG 13568 strain and treated with postbiotic-dipeptide Ent A/P in its partially purified form (precipitate). Ent A/P possessed initial inhibitory activity 12 800 AU/ml against the principal indicator *Enterococcus avium* EA5 (using agar spot test [19]. The appropriate amount of mesophilic cream culture (150 ml), rennet (20 ml), and 40% CaCl_2 (20 ml) was added to each vat calculated for 20 L. Curds were cut (15 min), scalded, pressed, cheese lumps were formed. They were put to drop off (18-20° C) and cheeses were stored in cold room for 7 days. Sampling (10 g) for microbiota detection was provided at day 0/1 (start of the experiment), at day 2, 5 and 7. LMG 13568 strain was controlled using Fraser broth, and also Fraser broth enriched with agar supplemented with Fraser additive (Becton and Dickinson, Cockeysville, USA). Cultivation was performed for 2 days at 29°- 30 °C. The most probable technique was used [20].

The pH values, °SH acidity and lactic acid were also checked. Before cheese experiment, *in vitro* inhibitory activity of Ent A/P was checked using diffusion agar spot test [19] against LMG 13568 strain. This method was also used during experiment. Inhibitory activity was expressed in AU/ml (6 400 AU/ml). The principal indicator strain *E. avium* EA5 (our isolate) was positive control (inhibitory activity 12 800 AU/ml).

Enterocin (Postbiotic) Preparation and its Inhibitory Activity

Non-autochthonous strain *Enterococcus faecium* EK13 deposited in Czech Culture Collection in Brno (Czech Republic) under number CCM 7419 is dipeptide Enterocin A/P [7, 18]. The strain and Enterocin were isolated and characterized in our laboratory [7,18]. For experiment precipitate of Enterocin (Ent) was prepared according to Mareková et al. [7]. Briefly, pre-inoculum of the producer strains EK13=CCM 7419 in MRS broth (pH.7,0 Merck, Darmstadt, Germany) was inoculated into 500 ml of MRS broth and incubated overnight at 37 °C. After growth, culture was centrifuged at 10,000 x g for half hour. The supernatant was adjusted for pH 5.0. It was precipitated with ammonium sulphate (40% saturation) by stirring at 4 °C for 4-7 h. Then it was centrifuged again at 10, 000 x g for half hour. The precipitate was re-suspended in the minimal volume of 10 mM phosphate buffer (pH 5.0). Bacteriocin activity was checked against the principal indicator *Enterococcus avium* EA5 using diffusing agar spot test [19]. Precipitate was stored at -20 °C until its use. Activity reached 12 800 AU/ml.

Inhibitory Activity Control in Cheese

Inhibitory activity in cheese was checked as previously described Lauková et al. [5, 18]. The homogenized samples in sterile 0.1% trisodium citrate solution were heated at 80 °C for 10 min and centrifuged (10, 000 x g) at 4 °C for 10 min. The enterocin/inhibitory activity was tested by the diffusion agar spot test [19] against the principal indicator *Enterococcus avium* EA5, and against *L. innocua* LMG 13568 as well. The titer of bacteriocin activity was quantified and expressed in arbitrary units (AU/ml) meaning the reciprocal of the highest sample dilution showing inhibition.

Lactic Acid Evaluation, And Acidity Measurement (pH and °SH)

Lactic acid (LA) was measured using the isotachophoric method (YKi-001) with a detector and leading and terminating electrolytes as previously described by Lauková et al. [18]. As the leading electrolytes, 10-2 M HIS, 10-2 M HIS Cl, 0.1 % MHEC 10 mL, pH 6.0 were used, and as the terminating electrolyte 5 x 10⁻³ glutaric acid and 5 x 10⁻³ TRIS, pH 7-9 were used. The standard was lactate calcium. The pH measurement was carried out by inserting the pin electrode of the pH meter Jenway 3310 (England) at 24 h, 72 h, and 96 h (cheese manufacturing). Acidity (°SH/100/ml) was checked by Soxhlet-Henkel method, in °SH/100/ml [21].

RESULTS AND DISCUSSION

Standard Microbial Analysis

Prior starting the experiment, milk was controlled for listerial contamination. It was found absent of cultivated cells. Immediately after Ent A/P addition, difference one (1) log cycle was found between E1 (only LMG 13568 strain) and E2 (LMG and Ent A/P, Table 1). At day 2 (48 h) this difference was slightly higher (1.16 log cycle) (Table 1). This decreasing effect on LMG 13568 cells due to Ent A/P was almost stable in E2 also at day 5 (Table 1) comparing day 2 and day 5 (difference 0.63 log cycle). The count of LMG 13568 in E2 was the same, 1.77 (0.20) at day 7 as it was at day 5 but slightly decreased count of LMG 13568 cells was noted in E2 comparing with E1 (difference 0.42 log cycle). It seems, tendency to reduce LMG 13568 cells was indicated. In cheese direct bacteriocin/enterocin activity was not detected.

Table 1. *Listeria innocua* LMG 13568 counts in milk vats and cheeses experimentally inoculated with LMG 13568 strain and treated with postbiotic-Enterocin A/P expressed in colony forming unit per ml/g (cfu/ml/g log 10) $\pm$ SD

Milk/cheese	Day 0/1	Day 2	Day 5	Day 7
E1 (LMG)	2.30 (0.18)	3.00 (0.20)	2.40 (0.19)	2.19 (0.21)
E2 (EntA/P +LMG)	1.30 (0.12)	1.84 (0.15)	1.77 (0.15)	1.77 (0.20)

CV-control vat was *Listeria* spp. absent.

Lactic Acid Evaluation, and Acidity Measurement (pH and °SH)

Values of lactic acid value (LA) were well balanced at day 0/1; a slightly lower value was noted in E2 milk vat immediately after Ent A/P addition. At day 2, oppositely higher LA value was measured in E2 milk/cheese vat comparing with E1 and RCV vats. Even higher LA value was noted at day 2 in E2 comparing with day 0/1 (mathematical difference 1.95). At day 5, the lowest LA value was noted in E1 (LMG 13568 strain) and mathematical difference was found between E1 and E2 (0.15 log cycle); however, LA in E1 was higher than in E1. LA was lower in E1 and E2 at day 5 also comparing with RCV at day 5. At day 7 in all vats LA values decreased; the lowest value was measured in E1 vat and then in the others (Table 2). The pH values were well balanced during whole controlled processing. It seems, they were not influenced with Ent A/P treatment and LMG 13568 strain inoculation as well (Table 2).

The highest acidity values (°SH) were measured in E2 (Table 2) reaching 91 °SH/100/ml at day 0/1 up to 116 °SH/100/ml at day 7. However, in RCV was found the highest °SH value at day 7 (118 °SH/100 ml). In summary, in individual checking the values of °SH were well balanced and continually increased (Table 2). It seems, that LMG 13568 strain did not negatively influenced °SH acidity in milk/cheese vats.

Table 2 Lactic acid (g/l), pH value and °SH value (SH/100ml) during processing cow milk lump cheese inoculated with *Listeria innocua* LMG 13568 and treated with Enterocin A/P.

Milk/cheese	Day 0/1	Day 2	Day 5	Day 7
		Lactic acid (g/l)		
RCV	3.80	4.10	3.58	2.96
E1 (LMG)	3.85	4.90	1.85	0.65
E2 (EntA/P +LMG)	3.10	5.05	2.40	1.70
		pH value		
RCV	6.69	4.66	4.44	4.43
E1 (LMG)	5.06	4.94	4.92	4.98
E2 (EntA/P +LMG)	5.05	4.91	4.80	4.83
		°SH value		
RCV	56	100	102	118
E1 (LMG)	90	95	100	109
E2 (EntA/P +LMG)	91	97	104	116

Recently, the term postbiotics has been developed for a health-promoting compounds [2]. As formerly mentioned, among those compounds are also involved bacteriocins-enterocins. One

of postbiotic definition formed speaks about the complex of mixture of metabolic products secreted by probiotic/beneficial bacteria in cell-free supernatants. This means also effect of bacteriocins/enterocins [2]. Moreover, when a product is supplemented and/or enriched with this kind of additive, it can be marked as functional food [16]. In spite of the fact, that not bactericidal effect of Ent A/P in cheese was demonstrated, there was found reduction tendency against LMG 13568 cells meaning bacteriostatic effect was demonstrated. Direct enterocin activity in cheese was not shown; however, reduction of cells was noted. Also, Lauková et al. [5] detected enterocin activity only in milk samples and curd (100 AU/ml) against *L. monocytogenes* Ohio; however, it was not detected in cheese, when Saint-Paulin cheese was manufactured and Ent CCM 4231 was applied. But *L. monocytogenes* Ohio cells were reduced in cheese. Enterocin activity of Ent 4231 was also demonstrated in yoghurt but immediately after Enterocin 4231 addition (400 AU/ml); again, bacteria, in this case staphylococcal cells were reduced [4]. Moreover, anti-staphylococcal effect of Ent A/P was reported in goat milk lump cheese [22, 18] and also in cow milk lump cheese not only with Ent A/P but there also Ent M was applied.

The lowest values of LA in E1 can be explained such as LMG 13568 strain did not belong among lactic acid bacteria. And in E2 vat it was lower because LMG strain with Ent A/P were in competition. But still not so low as in E1 vat. No influence on LA was also reported in our previous experiments with Ents application in cheeses [18, 22]. Also, pH values were not influenced. Acidity in °SH was the highest in RCV (reference control cheese-118 °SH), then in cheeses treated with Ent after LMG inoculation (116 °SH) and the lowest value was measured in E1 cheese (109 °SH) meaning that Ent optimizes °SH in E2. The use of bacteriocins in food offers several advantages such as decrease the risk of transmission of food-borne pathogens through the food chain, extends shelf-life of foods, provides extra protection during temperature abuse conditions, etc. [23]. Bacteriocins/enterocins can be incorporated in food products without changing their organoleptic properties since bacteriocins are colorless, odorless and tasteless [24].

An advantage is also the fact, that they can be incorporated in the food products as semi-purified compounds and/or in the form of bioactive molecules [24]. In spite of the fact that more additional studies are requested, indicative possibilities of Ents application have been several times clearly shown and this effect has been recently spread also in seafood biopreservation [25]. However, as it has been already mentioned in introduction, the beneficial complex effect of our enterocins in food-producing animals [9, 11,12, 26] and in horses [27] as well was previously reported. Moreover, enterocins have been studied and shown as a part of pharmacological strategy in fight with *Trichinella spiralis* zoonosis [28].

CONCLUSION

Ent A/P was found with reducing tendency against *L. innocua* LMG 13568 strain in cow milk lump cheese with differences from 1.0, 1.16, 0.63, and 0.42 log cycle. In spite of this tendency, direct anti-listerial activity of Ent A/P was not found in cheese. The acidity parameters such as pH and °SH were well balanced. The values of pH were not influenced and the values of °SH were continually increased after Ent A/P treatment and LMG 13568 strain inoculation. In spite of the requested additional studies, postbiotic-dipeptide Ent A/P showed beneficial potential tendency in cow milk lump cheese.

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