

Article

In Situ Interaction of Enterocin A/P with *Staphylococcus aureus* SA5 in Goat Milk Lump Cheese

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Featured Application: Enterocin A/P is promising stabilizing additive for dairy products.

Abstract: In Slovakia, goat milk is used for the production of traditional/local products, for example goat milk lump cheese. Healthy and safe dairy products are one of the major objectives for producers and consumers. Staphylococci are found in frequent contaminants of raw milk and dairy products. A promising approach of how to prevent/eliminate them is represented by bacteriocins. The aim of this study was testing in situ the anti-staphylococcal effect of Enterocin A/P, characterized in our laboratory and produced by the non-autochthonous strain *Enterococcus faecium* EK13 = CCM7419 against the contaminant strain *Staphylococcus aureus* SA5 experimentally inoculated in a goat milk vat during processing of goat milk lump cheese to show the application potential of Enterocin A/P. In addition, the parameters influencing the ripening of the cheese (lactic acid-LA), the acidity °SH and the pH were also checked. The 2.5 log cycle difference was found in the EV comparing to the CV at day 168 (1 week), which indicates a reduction of SA5 cells due to Ent A/P. The acidity value (pH and °SH) and LA were not negatively influenced. These preliminary results indicate that Enterocin A/P seems to be a promising additive for dairy products. Moreover, this is the first study showing in situ (in goat milk cheese) the anti-staphylococcal effect of Ent A/P.

Keywords: enterocin; cheese; goat milk; safety; quality; prevention; elimination



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1. Introduction

In Slovakia, goat milk is used for the production of traditional/local products, for example goat milk lump cheese. In this specific livestock sector, most goats are concentrated in the small-scale breeding of white shorthair goats [1]. Milk for the production of goat milk cheeses made at farm does not always have to be pasteurized [2]. That means that concern over its microbial safety is important. It can be achieved, for example, by focusing on prevention, following hygiene conditions and the principles of hazard analysis and critical control points (HACCP) [1]. However, following prevention, the utilization of beneficial bacteria and their metabolic substances and bacteriocins can also be recommended for this purpose. In dairy products, the most important bacterial group represent lactic acid bacteria (LAB). Those bacteria mostly belong to the phylum Firmicutes, and due to lactic acid production they have the ability to inhibit the growth of spoilage bacteria [3,4] but also due to their antimicrobial substances (bacteriocins) [5]. Among bacteriocins, enterocins have been previously used in dairy experimental processes with beneficial effects [6–8]. Enterocins are bacteriocins produced mostly by the representatives of the genus *Enterococcus*, which belongs in lactic acid bacteria from the phylum Firmicutes. On the other side, staphylococci, the species involving *Staphylococcus aureus*, are the most frequent contaminants of milk and/or dairy products [9–11]. For example, coagulase-positive staphylococci were detected

in goat cheese (at day 0/1) 4.41 ± 0.41 cfu/g log 10 [1]. The genus *Staphylococcus* within various species was also detected as the most abundant genus in goat milk [11,12]. Following the above information, the aim of this work was testing in situ the anti-staphylococcal effect of Enterocin A/P (characterized in our laboratory and produced by the non-autochthonous strain *Enterococcus faecium* EK13 = CCM7419) against the contaminant strain *S. aureus* SA5 experimentally inoculated in a goat milk vat during the processing of goat milk lump cheese to show the possible application potential of enterocin. In addition, the most important parameters for ripening (lactic acid, acidity °SH and pH) were checked.

2. Materials and Methods

2.1. Goat Milk Lump Cheese Manufacturing Process and Sampling

Goat milk lump cheese was manufactured in 10-L (L) vats using the standard technology for this type of cheese [4]. Pre-heated milk vats were divided into the control vat (CV) experimentally contaminated with the *S. aureus* SA5 strain (isolated from mastitis milk in our laboratory, concentration of inoculum was 10^7 cfu/mL = 7.0 cfu/mL (log 10), the experimental vat (EV) inoculated with the SA5 strain and treated with Enterocin A/P (its inhibitory activity 12,800 arbitrary unit per mL, AU/mL against the SA5 strain) [13], and the reference vat (RV) (without the SA5 strain and Enterocin A/P). Appropriate amount of cream culture, rennet as well and 40% CaCl_2 , was added in the amount calculated for 10 L. Curds were cut (15 min), scalded, pressed, and cheese lumps were formed. They were placed to drop off (18–20 °C) and cheeses were stored in a cold room for 7 days. Two independent replicates were performed (each cheese was checked in duplicate) and the experiment was reproduced once. Bacterial counts were expressed in colony forming units per gram. Sampling for microbial analysis was performed during cheese manufacturing, at the start of experiment at time S/0 h (meaning after inoculation and before the addition of Ent A/P), at 0h when Ent A/P was added, at 4 h, 24 h, then after storage at days 4 (96 h) and 7 (168 h). To check lactic acid, acidity (°SH/100/mL) and pH values, sampling was provided at 24 h, 72 h, and 96 h (cheese manufacturing).

2.2. Enterocin A/P Preparing

Enterocin-dipeptide A/P is produced by the non-autochthonous strain *Enterococcus faecium* EK13, which was deposited in the Czech Culture Collection in Brno (Czech Republic) CCM7419. For the experiment, the precipitate of Enterocin A/P (Ent) was prepared as previously described by Lauková et al. [11] and Mareková et al. [13]. Briefly, the pre-inoculum of the producer strain EK13 = CCM7419 in MRS broth (pH 7.0 Merck, Darmstadt, Germany) was inoculated into 500 mL of MRS broth and incubated overnight at 37 °C. Then the culture was centrifuged at $10,000 \times g$ for 30 min. The supernatant was adjusted to have a pH of 5.0 and precipitated with ammonium sulphate (40% saturation) by stirring at 4 °C for 4–7 h. Then it was centrifuged again at $10,000 \times g$ for 30 min and the precipitate was re-suspended in the minimal volume of a 10 mM phosphate buffer (pH 5.0). Bacteriocin activity was checked against *Enterococcus avium* EA5 using the diffusion agar spot test [14]. Precipitate was stored at −20 °C until its use.

2.3. Standard Microbial Analysis

Before the experiment, raw goat milk was analyzed for staphylococci using the Baird–Parker agar (BP, ISO 6888-1) with supplement and yolk tellurite (Oxoid, Ltd., Basingstoke, UK) as well as on the plate count agar (pH 7.0 Biomark Laboratories, Pune, India). Bacteria counted were under detection limit. Before the cheese experiment, in vitro inhibitory activity of Ent A/P was checked using the diffusion agar spot test [13,14] against the SA5 strain and activity was expressed in AU/mL (1600 AU/mL). The principal indicator strain *E. avium* EA5 (our strain) was the positive control (inhibitory activity 12,800 AU/mL). However, Ent A/P also inhibited a different staphylococcal species from Slovak local ewe milk lump cheeses with inhibitory activity up to 12,800 AU/mL [11]. Then the cheese samples (10 g) were homogenized in 90 mL of sterile peptone water (Merck, Germany)

and treated in a Stomacher-Masticator PK400, IUL (Spain). Decimal dilutions (in Ringer solution, pH 7.0) were prepared according to the standard microbiological method (ISO, International Organization for Standardization). Appropriate dilutions were spread on the BP agar (Oxoid) and cultivated at 30 °C for 48 h. Two independent replicates were performed (each cheese was checked in duplicate) and the experiment was reproduced once. Bacterial counts were expressed as the average amount of colony forming units per gram of cheese (cfu/g $\pm$ SD). The SA5 strain was also confirmed using PCR with the following primers: Sau1-F, 5'-TCT TCA GAA GAT GCG GAA TA-3' and Sau2-R, 5-TAA GTC AAA CGT TAA CAT ACG-3' with 30 cycles at 58 °C, 30 cycles at 72 °C, finally 5 min at 72 °C according to Forsman et al. [15]. The positive control was strain ATCC25923. PCR product was visualized using 1.5% (v/w) agarose gel (420 bp).

2.4. Detection of Inhibitory Activity in Cheese

Samples were treated as previously described by Lauková et al. [6]. The homogenized samples (in sterile 0.1% of trisodium citrate solution) were heated at 80 °C for 10 min and centrifuged (10,000 $\times$ g) at 4 °C for 10 min. The bacteriocin/inhibitory activity was tested by the diffusion agar spot test [14] against the principal indicator *Enterococcus avium* EA5 as well as against *S. aureus* SA5. The titer of bacteriocin activity was quantified and expressed in arbitrary units (AU/mL), meaning the reciprocal of the highest sample dilution showing inhibition.

2.5. Lactic Acid, Acidity (°SH) and pH Values Measurement

Lactic acid (LA) analysis was performed by the isotachophoric method (YKi-001) with a detector and leading and terminating electrolytes as previously described by Lauková et al. [4]. 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 electrolytes 5×10^{-3} glutaric acid and 5×10^{-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) [16].

3. Results

3.1. Microbial Analysis and pH Values

The difference in staphylococcal count between the CV (inoculated with the SA5 strain) and the EV (SA5 and Ent A/P) was 1.1 log cycle at the start of the experiment (0 h, Table 1). This mathematical difference (1.5 log cycle) was slightly higher at 4 h, which was almost stable up to 24 h. During processing and storing (at 96 h), a 1.7 log cycle difference was found comparing the CV and the EV. Finally, at day 168 (1 week) the highest difference was noted (2.5 log cycle). It appears that Ent A/P indicated a reductive effect against the amount of SA5 cells, although direct bacteriocin activity of Ent A/P in cheese has not been detected. Colonies picked up from the BP agar were genotyped using PCR and the appropriate primers. Taxonomical allocation to the species *Staphylococcus aureus* was confirmed (420 bp).

Table 1. The counts of staphylococci during goat cheese manufacturing and storing expressed in colony forming units per mL and/or per gram $\pm$ SD.

Goat Milk/Cheese Vat	S/0 h	0 h	4 h	24 h	96 h	168 h
CV	5.2 $\pm$ 0.3	5.2 $\pm$ 0.3	5.6 $\pm$ 0.3	5.6 $\pm$ 0.6	5.6 $\pm$ 0.8	4.6 $\pm$ 0.6
EV	5.2 $\pm$ 0.3	4.1 $\pm$ 0.2	4.1 $\pm$ 0.2	4.1 $\pm$ 0.2	3.9 $\pm$ 0.4	2.1 $\pm$ 0.3

CV—control vat milk/cheese (with *S. aureus* SA5 strain); EV—experimental vat milk/cheese (with *S. aureus* SA5 and Enterocin A/P, 12,800 AU/mL); in RV (reference vat milk/cheese-no additives) bacterial counts were under detection limit. Initial concentration/count of *S. aureus* SA5 strain inoculum was 10^7 cfu/mL = 7.0 cfu/mL (log 10). The time S/0h means start of experiment after inoculation with SA5 before adding Ent A/P in EV.

The values of pH were well balanced during goat cheese processing; the pH value measured at 24 h was 6.69 in the CV, EV, and RV (Table 2). Reduction in pH was noted in all vats/cheeses at the 72 h compared to 24 h; the lowest pH values (4.38, 4.39) were noted in the EV cheese and the RV to be almost the same. In the CV (with SA5), the pH reached the value 4.66. At 96 h, the pH was 3.93, which was the lowest pH that was measured in the EV; it was lower by comparison than in both the CV and the RV.

Table 2. The values of pH, lactic acid (g/100 g), and acidity (°SH) during cheese processing.

Sampling	CV	EV	RV
24 h/pH	6.69	6.69	6.69
72 h/pH	4.66	4.38	4.39
96 h/pH	4.43	3.93	4.19
24 h/LA	0.21	0.21	0.21
72 h/LA	4.66	4.38	4.39
96 h/LA	3.58	3.71	2.58
24 h/°SH	56	56	44
72 h/°SH	94	93	98
96 h/°SH	115	90	110

CV—control vat milk/cheese (with *S. aureus* SA5 strain); EV—experimental vat milk/cheese (with *S. aureus* SA5 and Enterocin A/P, 12,800 AU/mL); RV—reference vat milk/cheese (no additives); LA—lactic acid in g/100 g; °SH—acidity (Soxleth–Henkel method).

3.2. Lactic Acid and Acidity Measurements

Surprisingly, the lowest production of lactic acid was detected in the RV cheese (2.58 g/L) at 96 h, while in the EV and the CV, the LA amount was almost the same and there was no significant difference between measurements at 72 h and 96 h. The LA values in the CV and EV were also well balanced.

The lowest acidity (90 °SH/100 mL) was measured in the EV. In the CV and the RV, almost the same values were found (Table 2). Acidity was continually increased from 24 h up to 72 h and 96 h, respectively.

4. Discussion

Healthy and safe dairy products are one of major objectives for producers and consumers who are increasingly demanding healthier and safer dairy products [17]. As formerly mentioned, staphylococci are found in frequent contaminants of raw milk and products made from it [9,11]. Some strains of *S. aureus* can produce hemolysins or enterotoxins, which can influence the technological process but also the health of consumers [10]. A promising approach of how to prevent unrequested parameters is represent by bacteriocins. For example, in our previous studies using enterocins it was reported that Enterocin CCM4231 showed anti-staphylococcal effects in yogurt and also in Sunar (milk nourishment for suckling babies) [18]. The reduction of *S. aureus* Oxford 209P in Sunar and *S. aureus* SA1 in yogurt was found with a difference of up to 3.0 log cycles. However, direct inhibitory activity of Enterocin 4231 was detected only immediately after its addition (400 AU/mL) in yogurt and after three and a half hours (200 AU/mL). Another problem of staphylococci is their increasing antibiotic resistance. When staphylococci cause mastitis in dairy and are also antibiotic resistant, milk can be influenced negatively. It is known that staphylococci form 24% of all mastitis cases in dairy [19]. Toxinogenic *S. aureus* has regularly appeared in milk in low numbers and its growth during uncontrolled fermentation of milk and/or young cheese may be intensive [20]. De Buyser et al. [21] reported that milk and cheeses were implicated in 1–5% of the total bacterial outbreaks in food-borne diseases with the most frequently identified *S. aureus* as a causative agent. Therefore, utilizing the antimicrobial effect of bacteriocins and enterocins has an advantage not only for the maintenance of suitable technological conditions during product processing but also for protecting the health of consumers. Alvarez-Cisneros [22] reported enterocins as perspective bacteriocins

to be used in the food industry. However, it is also necessary to add that the optimal use of bacteriocins within a multi-barrier system to inhibit spoilage microbiota requires a detailed knowledge of their nature and of those factors that may limit their effectiveness. Those factors may include the food structure and composition as well as the interaction of bacteriocin with the other microbiota [21].

Active acidity (pH) and titrable acidity ($^{\circ}\text{SH}$) and LA are parameters that can influence the ripening process during goat milk lump cheese processing [23]. During our experiment, the changes in acidity ($^{\circ}\text{SH}$) were noted in the period from 24 h up to 72 h. The lowest value was in the RV (44 $^{\circ}\text{SH}$), while in the EV and CV it was the same (56 $^{\circ}\text{SH}$). However, almost the same values were measured at 72 h and then also at 96 h in the CV and RV. In the EV cheese (with SA5 and Ent M), a lower $^{\circ}\text{SH}$ was measured than in the RV and CV (there were the same values measured). This acidity was comparable with that measured in cow lump cheese treated with Enterocin CCM4231 at day 7 (129 $^{\circ}\text{SH}$) [23]. It seems that the pH was not negatively influenced in our cheeses; however, it was lower in the EV than in the RV and CV. LA was not influenced by the SA5 strain inoculation and the addition of Ent A/P, reaching almost the same values (3.58-CV, 3.71 g/100 g-EV) at 96 h. The lowest LA production was measured (2.58 g/100 g) in the RV. This information corresponds with the values reported in cow lump cheese with the same strain SA5 and enterocin as reported by Burdová et al. [23].

The results achieved indicate the promising application potential of Enterocin A/P in dairy processing, although some disadvantages can appear. One could be that they are hydrophobic molecules and bind to the hydrophobic phase of foods such as emulsions of food surfaces which notably reduces activity [22]. It could explain why bacteriocin activity cannot be detected directly in food. However, enterocins are mostly small molecules and usually can diffuse through the aqueous phase of food products. Altogether, in practice bacteriocins have shown effectiveness in cheese [22], although continual studies are required to find the more detailed effects of enterocins.

5. Conclusions

Based on the results, it seems that the addition of Ent A/P indicated the reduction of *S. aureus* SA5 cells in goat milk lump cheese. However, the direct bacteriocin activity of Ent A/P in cheese has not been detected. Parameters influencing the ripening process, such as active acidity (pH), titrable acidity ($^{\circ}\text{SH}$) and lactic acid, were not negatively influenced. Enterocin A/P appears to be a promising protective additive for the processing of dairy products. Of course, additional studies will need to be undertaken. Moreover, this is the first study showing in situ (in goat milk cheese) the anti-staphylococcal effect of Ent A/P.

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