LUPINE PUBLISHERS

Concepts of Dairy & Veterinary Sciences

DOI: 10.32474/CDVS.2022.05.000207

ISSN: 2637-4749

Research Article

Relation of Dipeptide Enterocin A/P and Enterocin M with *Staphylococcus aureus* SA5 during Processing of Cow Milk Lump Cheese

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Received: October 11, 2022 **Published:** October 18, 2022

Abstract

Microbial contamination of milk can be problem during its technological processing. Especially *Staphylococcus aureus* can produce enterotoxins and they can influence technological processing and health of consumers as well. Bacteriocins represent a promising approach to control bacteria during cheese processing. The aim of this study was checking relation of dipeptide bacteriocin, Enterocin A/P and Enterocin M with S. *aureus* SA5 during processing of cow milk lump cheese. The count of controlled bacteria in reference milk vat/cheese was under detection limit. The count of SA5 was high in control vat (CV) and in E2 vat (SA5 strain and Ent M addition) at day 0/1, meaning that Ent M did not influence SA5 counts. However, in E1 vat (SA5 strain and Ent A/P addition) decrease of SA5 cells was noted (difference 1.94 log cycle), which was prolonged up to day 7 (reduction from 4.1 cfu/ml/g up to 2.1 cfu/ml/g between CV and E1). At day 6 was found difference 1.0 log cycle between CV and E2. It seems, that Ent A/P showed anti-staphylococal effect, while Ent M did not possess anti-staphylococcal activity. Lactic acid and pH were not influenced by SA5 strain and enterocins addition. The acidity values were the highest in E1 vat at day 7 (127 °SH/100/ml); they were the lowest in E2 vat/cheese (90 °SH/100/ml).

Keywords: Cow milk lump cheese; enterocins; staphylococci; treatment

Introduction

In spite of the high nutritional qualities of bovine milk, it is also a good growth matrix for a variety of spoilage microbiota [1]. Spoilage bacteria can cause contamination in food industry [2]. In dairy, microbial contamination of milk can be problem during its technological processing. Among the most frequently appeared contaminants of raw milk belong staphylococci [1-4]. Especially *Staphylococcus aureus* species is the major pathogen which is often involved in foodborne outbreaks [5]. Some strains of *S. aureus* can produce e.g. hemolysins or enterotoxins which can influence technological processing but also health of consumers [6]. Therefore, control of these bacteria in food is essential to food safety [5]. Different physical and chemical methods have been used to control foodborne pathogens or spoilage bacteria such as pasteurization, cooking, drying, radiation, acidification, salting or addition preservatives [5]. Producers and consumers prefer mainly natural additives such as phytochemicals, and/or plant extracts. However, also bacteriocins represent natural additives. These antimicrobial proteinaceous substances are produced by some bacterial strain species and showed antimicrobial effect not only in situ or in vitro [7,8]. They also beneficially influence animals health [9] as well as safety of animal-derived food products [10]. Therefore, the aim of this study was to test effect of bacteriocins-enterocins Ent A/P and

Ent M in situ - in cow milk lump cheeses which were inoculated with *S. aureus* SA5 strain. The idea followed further possible enterocins utilization in protection of cheeses. Regarding the enterocins, mostly those enterocins produced by *Enterococcus faecium* strains are the best studied [11-13]. However, nowadays also enterocins produced by the other species are known [14, 15]. Ent A/P is dipeptide with a broad antimicrobial spectrum [12] which has been shown as beneficial in rabbit broilers [9]. After its administration in rabbits was noted increase in weight gain, reduction of feed conversion, phagocytic activity was stimulated and gut microbiota was optimized due to reduction of coliforms and methicillin-resistant staphylococci as well. Similarly, Ent M is a thermo-stable, small peptide with a broad antimicrobial activity [13] as well as with benefit application in poultry, broiler rabbits or horses [16-18]. Therefore, we decided for these two enterocins to be used in presented study.

Materials 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 et al. [19,20].Pre-heated milk vats were divided into the reference vat/cheese (RV), the control vat/cheese CV (10^7 cfu/ml of *Staphylococcus aureus* SA5, isolated in our laboratory from mastitis milk), the experimental vat/cheese 1 (E1= inoculation with SA5 strain and dipeptide Ent A/P addition in its precipitated form (partialy purified) with its initial inhibitory activity 12 800 AU/ml against the principal indicator *Enterococcus avium* EA5). The experimental vat cheese 2(E2) was incoculated with SA5 strain and Ent M was added (its partial purified form-precipitate) possessing initial activity 12 800 AU/ml against EA5 strain. Then, appropriate amount of mesophilic cream culture (150 ml), rennet (20 ml) as well, 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 (10g) for microbiota detection was provided at day 0/1 (start of the experiment), at day 2, 4, 6 and 7. The pH values, °SH acidity and lactic acid were checked at day 0/1, day 3, 6 and 7.Before cheese experiment *in vitro* inhibitory activity of Ent A/P and Ent M was checked using diffusion agar spot test [21] against SA5 strain. Inhibitory activity was expressed in AU/ml (1 600 AU/ml). The principal indicator strain *E. avium* EA5 (our isolate) was positive control (inhibitory activity 12 800 AU/ml).

Standard Microbial Analysis and SA5 Strain Confirmation Using PCR

Before the experiment, milk was analyzed for staphylococci using Baird-Parker agar (ISO 6888-1) with supplement and yolk tellurite (Oxoid, Ltd. Basingstoke, United Kingdom) as well as on plate count agar (pH 7,0 Biomark Laboratories, Pune, India). Bacterial counts in cheese milk were under detection limit. Then samples/or cheese homogenates (10g) were homogenized in 90 ml of sterile peptone water (Merck, Germany) treated in Stomacher-Masticator PK400, IUL (Spain). Decimal dilutions (in Ringer solution, pH 7.0) were prepared according to ISO (standard microbiological method-International Organization for Standardization). Appropriate dilutions were spread on BP agar (Oxoid) and cultivated at 30 ˚C for 48 h. Each cheese was checked in duplicate. Bacterial counts were expressed in colony forming units per gram/milliliter of milk/cheese (cfu/ml/g). SA5 strain was also confirmed using PCR with the following primers: Sau1-F,5´-TCT TCAGAA 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. [22]. Positive control was strain ATCC 25923. PCR product was visualized using 1.5% (v/w) agarose gel (420 bp) as previously described by Lauková et al. [23].

Enterocins Preparing and Their Activity

Dipeptide enterocin A/P is produced by non-autochthonous strain *Enterococcus faecium* EK13 which was deposited in Czech Culture Collection in Brno (Czech Republic) CCM 7419, and Ent M is a new type of enterocin produced also by non-autochthonous strain *E. faecium* AL41 deposited in CCM with number CCM 8558. Both strains were isolated in our laboratory. For experiment precipitate of enterocins (Ent) were prepared as previously described by Mareková et al. [12,13]. Briefly, pre-inoculum of the producer strains EK13=CCM 7419 and AL41=CCM 8558 in MRS broth (pH.7,0 Merck, Darmstadt, Germany) was inoculated into 500 ml of MRS broth and incubated overnight at 37 ˚C. Then cultures were centrifuged at 10,000 x g for 30 min. The supernatants were adjusted to have pH 5.0 (for Ent M pH 5.5) and precipitated with ammonium sulphate (40% saturation) by stirring at 4 ˚C for 4-7 h. In case of Ent M precipitation was at laboratory temperature for 1 h. Then it was centrifuged again at 10,000 x g for 30 min and precipitates were re-suspended in the minimal volume of 10 mM phosphate buffer (pH 5.0, pH 6.5 for Ent M). Bacteriocin activity was checked against the principal indicator *Enterococcus avium* EA5 using diffusing agar spot test [21]. Precipitates were stored at -20 ˚C until its use.

Inhibitory Activity in Cheese

Samples were treated as previously described Lauková et al. [24]. The homogenized samples (in sterile 0.1% of trisodium citrate solution) were heated at 80 ˚C for 10 min and centrifuged (10,000 x g) at 4 ˚C for 10 min. The bacteriocin/inhibitory activity was tested by the diffusion agar spot test [23] against the principal indicator *E. 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.

Lactic Acid, 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. [25]. 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 [26].

Results and Discussion

The bacterial count in RV was under detection limit. At day 0/1, SA5 cells count was high in control vat (CV) and in E2 (SA5/Ent M) meaning that Ent M did not start with influencing SA5 strain or no competitive relation was appeared between SA5 strain and Ent M (Table 1). On the other hand, in E1 (SA5/Ent A/P) decrease in SA5 cell count was noticed with difference 1.94 log cycle. This decrease in SA5 cells was prolonged up the end of checking day 7. SA5 strain was reduced from 4.1 cfu/ml/g up to 2.1 cfu/ml/g with differences ranged from 1.9, 2.3 up to 2.5 log cycles between CV and E1 (Table 1). In E2 slight difference (0.4 log cycle) was found at day 2 between CV and E2 and difference 1.0 log cycle at day 6. It seems, that Ent A/P showed anti-staphylococal effect, while Ent M did not possess this inhibitory activity in cheese. Survived SA5 cells grown on media were confirmed by PCR.

Microbial quality of raw milk is of particular importance. Fotua et al. [27] represented that in 24% cases of controlled sheep milk *S. aureus* was detected. Therefore, to avoid this bacterial contamination is requested. Promising approach for these purposes represents bacteriocins. E. g. enterocin CCM 4231 showed anti-staphylococcal effect in yoghurt and also in Sunar (milk nourishment for suckling babies) as reported in our previous studies [28]. Experimentally inoculated *S. aureus* Oxford 209P in Sunar and SA1 in yoghurt were found reduced with difference up to 3.0 log cycles. However, direct inhibitory activity of Enterocin 4231 was detected only immediately after its addition in yoghurt (400 AU/ml) and after 3 and half hour (200 AU/ml) testing against the principal indicator *E. avium* EA5 using agar spot test. Toxinogenic *S. aureus* has been regularly appeared in milk in low numbers and its growth during uncontrolled fermentation of milk and/or young cheese may be intensive [29]. De Buyser et al. [29] reported that milk and cheeses were implicated in 1-5 % of the total bacterial outbreaks in foodborne diseases with the most frequently identified *S. aureus* as a causative agent. Therefore, utilizing antimicrobial effect of enterocins has an advantage from both processing/technological condition during products processing and also from aspect of consumers health protecting. In general, enterocins have been reported as perspective to be used in food industry [29]. However, 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. These factors may include the food structure and composition, and the bacteriocin interaction with the other microbiota [30].

The values of LA in cheeses were not influenced by additives; these values were well balanced. However, they were higher at day 6 and 7 in E1, E2, and CV than in RV. The pH values were also balanced in all samples with the lowest pH in E2 at day 7. The highest acidity values were in E1 (Table 2) with the highest value at day 7 (127˚ SH/100 ml), and the lowest value in E2 (90 ˚SH/100ml). The active acidity (pH) and titrable acidity (˚SH) are parameters which can influence the most ripening process during cheese processing and LA production as well [31]. However, it looks, that bacteriocins did not have impact on these parameters. Vanegas-Ortega et al. [32] even reported production of bioactive peptides from lactic acid bacteria as a sustainable approach for healthier foods. The interaction between food-derived peptides and microorganisms is very promising. Bacteriocins can be also encapsulated which can increase their stability [32]. Muruzovic et al. [33] mentioned microbial effect of enterocins against a number of food pathogens meaning to use them as food protecting substances. It means, it is requested to continue in this testing to support all findigs.

Table 1: Staphylococcal count (*S. aureus* SA5) in milk vats and cheeses experimentally inoculated with SA5 strain treated with enterocins in log 10 cfu/ml/g.

Milk/Cheese	Day 0/1	Day 2	Day 4	Day 6	Day 7
CV	6.4 (0.18)	6.0 (0.12)	5.6 (0.8)	5.6 (0.8)	4.6 (0.6)
E1	4.1 (0.2)	4.1 (0.2)	3.9 (0.4)	3.9 (0.4)	2.1 (0.3)
E2	6.0 (0.13)	5.6 (0.03)	5.5 (0.16)	4.6 (0.06)	4.6 (0.19)

RV, Reference raw milk vat/cheese, CV-Control vat, E1, Experimental vat-inoculated with *Staphylococcus aureus* SA5 strain and treated with Enterocin A/P, E2, Experimental vat-inoculated with *S.aureus* SA5 strain and treated with Enterocin M, Before the experiment, milk was analyzed for staphylococci using Baird-Parker agar (ISO 6888-1) with supplement and yolk tellurite (Oxoid, Ltd. Basingstoke, United Kingdom) as well as on plate count agar (pH 7,0 Biomark Laboratories, Pune, India**).** Bacterial counts in milk for cheese production were under detection limit. Day 0/1: E1 (SA5 and Ent A/P) decrease in SA5 cell count was noticed with difference 1.94 log cycle. This decrease in SA5 cells was prolonged up the end of checking day 7. SA5 strain was reduced from 4.1 cfu/ml/g up to 2.1 cfu/ml/g with differences ranged from 1.9, 2.3 up to 2.5 log cycles between CV and E1 (day 2-7). In E2 slight difference was found at day 2 between CV and E2 with difference 1.0 log cycle at day 6.

Table 2: The pH, ˚SH/100/ml and lactic acid values during processing cow milk lump cheeses inoculated with *Staphylococcus aureus* SA5 and treated with enterocins.

Milk/cheese	Day 0/1	Day3	Day6	Day 7
Lactic acid				
RV	0.21	4.33	3.58	2.96
CV	0.21	3.58	5.46	4.83
E1	0.21	4.89	5.08	4.83
E2	0.21	3.71	4.83	4.83
Value pH				
RV	6.61	4.96	4.86	4.84
CV	6.69	4.66	4.44	4.43
E1	6.69	4.65	4.51	4.4
E2	6.69	4.38	4.53	3.93
Value ˚SH				
RV	76	100	101	118
CV	56	94	102	115
E1	56	109	122	127
E2	56	93	90	90

RV, Reference raw milk vat/cheese, CV-Control vat, E1, Experimental vat-inoculated with *Staphylococcus aureus* SA5 strain and treated with Enterocin A/P, E2, Experimental vat-inoculated with *S.aureus* SA5 strain and treated with Enterocin M, Lactic acid expressed in g/l, ˚SH/100/ml: Soxhlet-Henkel method [26]**.**

Conclusion

Enterocin A/P was found to reduce *S. aureus* count in cow milk lump cheese with differences up to 2.5 log cycles. On the other hand, Ent M did not possess anti-staphylococal activity. The acidity parameters (pH and ˚SH) and lactic acid production were not influenced by enterocins. In spite of prelimanary experiment, Ent A/P looks as promising additive in cheese processing to avoid contamination.

Funding

This research was partially financed by the Slovak Research and Development Agency under contract No. APVV-17-0028 and partially APVV-20-0204. We thanks Mrs. Margita Bodnárová for her laboratory work.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Slovak Research and Development Agency under contract No. APVV-17-0028 and partially APVV-20-0204.

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DOI: 10.32474/CDVS.2022.05.000207