ANTILISTERIAL EFFECT OF BACTERIOCIN ISOLATED FROM ENTEROCOCCUS FAECALIS DURING THE FERMENTATION OF SOFT WHITE CHEESE

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Abstract

Until recently, genus Enterococcus is considered as indicator of fecal contamination. Nowadays, strains of this genus are considered as common microbiota, particularly in autochthonous kinds of cheese, in which they contribute to the development of specific sensory properties. Also, numerous Enterococcus strains, isolated from fermented and non-fermented foodstuffs, produce many types of bacteriocins that induced many investigations due to perceiving its use in foodstuff protection. In this paper, it is investigated the antilisterial effect of bacteriocins produced by Enterococcus faecalis, isolated from autochthonous Serbian white cheese (Zlatar cheese), in the aim of Listeria monocytogenes growth control during soft white cheese manufacturing.

Isolation of semi purified bacteriocin from E. faecalis (enterocin) was done by the method of saturated precipitation with ammonium-sulphate, adjusted to the individual laboratory conditions. The activity of the isolated, semi purified enterocin was determined by the critical dilution method against the selected test microorganism - L. monocytogenes ATCC 19111. Its strength was approximately 1280 AU/mL. After milk coagulation on traditional way by commercial rennet with strength of 1:5,000 (1.5 - 2 mL/L), the total amount was divided into three equal parts: the first part was control group (K), the second part was inoculated with L. monocytogenes (ca. 10⁴ cells/mL) (O-1), in the third part was added L. monocytogenes and bacteriocin isolated from E. faecalis (1280 AU/mL (O-2)). Manufacturing of these cheese was repeated three times. Samples for laboratory examinations were taken on days: 0, 2, 4, 7, 14 and 21. Determination of the presence of Listeria spp. was carried out following the procedure of the ISO 11290 - 1, 2 ([26] and [27]). The software package “Statistica for Windows” (StatSoft, Inc., USA) was used for statistical analysis. Differences between average values are presented on the level of 95% (P ≤ 0.05).

L. monocytogenes was not detected in any sample of the control group. In the experimental group of cheeses with added bacteriocins (group O-2), at the end of maturation, there was the statistically significant level of reduction of L. monocytogenes number (P ≥ 0.05). From the initial number of 10⁴ CFU/g, at the end of the maturation, the number of this pathogen was reduced under 100 CFU/g.

These results show that enterocin was successful in preventing L. monocytogenes growth, despite the possible inactivation by various interactions within the food system. A very important fact is that the strain tested in the present work is isolated from a traditional Zlatar cheese, represents the natural bioprotector isolated from a similar matrix.

Key words: Enterococcus faecalis, Bacteriocin, Antilisterial effect, Cheese.

1. Introduction

Lactic acid bacteria (LAB) a heterogeneous group of acid-tolerant, Gram-positive, catalase- and oxidase-negative microorganisms which produce lactic acid during homofermentative or heterofermentative metabolism (Klaenhammer et al., [1], Holzapfel and Wood, [2]). Due to their metabolic properties (in addition to lactic acid, they produce: other volatile organic acids, H₂O₂,CO₂, diacetyl, reuterin and bacteriocins - specific antibacterial compounds), LAB are responsible for lactic acid fermentation, the ripening process, flavor development and nutritional characteristics of the fermented product, and have direct effect on the finished product shelf-life (Holzapfel et al., [3]; Beresford et al., [4]; Leroy and De Vuyst, [5]). Due to their positive metabolic and antimicrobial effects, LAB are recognized as “a safe microflora” (Tagg et al., [6], Stiles, [7], De Vuyst
& Vandamme [8], and Caplice and Fitzgerald [9]). The antibacterial spectra of LAB and their metabolites, bacteriocins, are generally associated with Gram-positive spoilage microorganisms and food-borne pathogenic bacteria, with antilisterial effect as the major effect they exert (Drosinos et al., [10]).

Until recently the genus Enterococcus was considered an indicator of fecal contamination. Today, however, species within this genus are considered as normal microflora, primarily of autochthonous cheeses, having a positive effect on the development of their specific organoleptic characteristics (Giraffa et al., [11], Fuller, [12], Bulajić and Mijačević, [13]). Numerous Enterococcus strains, isolated from different fermented and non-fermented food products, produce more bacteriocins per strain, and this finding has prompted interest in their use as protective cultures in foods (Nes et al., [14]).

Listeria monocytogenes is considered as one of the most frequent food-borne diseases causative. Numerous outbreaks and sporadic cases of listeriosis associated with foods have been reported since 1980 in various parts of the world (Norton and Braden [15]). In European countries the annual incidence of reported listeriosis cases can vary between 0.3 and 7.5 cases/million inhabitants (Swaminathan & Gerner-Smidt [16], EFSA [17]). L. monocytogenes is a ubiquitous bacteria usually found in the environment. The pathogen has been found in various food matrices, such as ready-to-eat foods, milk and cheeses, cold-cut meats, smoked fish, seafood, and vegetables (Chao et al., [18], EFSA [17], and Chen et al., [19]). Its presence in foods generally results from post-processing contamination due to the manipulation of foods and contact with contaminated surfaces or other foods from the storage area (Lunden et al., [20]) and due to biofilm formation on the food processing equipment surfaces (Borucki et al., [21]). It is generally known that once introduced into the processing plants, it is able to survive and persist for a long time under adverse conditions (Gram et al., [22]).

The aim of this study was to test bacteriocin isolated from Enterococcus faecalis with regard to control L. monocytogenes growth during the manufacture of soft white cheese.

2. Material and Methods

2.1 Bacterial strains and growth conditions

Enterococcus faecalis isolated from autochthonous Serbian white cheese (Zlatar cheese), was used throughout this study. The strain E. faecalis was kept frozen at -20 °C in GM17 broth (M17 broth, Merck, KGaA, Darmstadt, Germany, and 0.5% (w/v) glucose) supplemented with 20% glycerol. Prior to use, the microorganism was subcultured twice in 10 mL of GM17 broth (1% inoculum, 24 h, 30 °C).

2.2 Isolation of semi purified bacteriocin from Enterococcus faecalis and determination of its spectrum of antimicrobial activity and strength

Isolation of semi purified bacteriocin from E. faecalis was done by the method of saturated precipitation with ammonium-sulphate (Schillinger and Lücke, [23]), adjusted to the individual laboratory conditions. Several days after planting broth culture with the objective of achieving the required E. faecalis concentration (10^9 - 10^11 CFU/g), it was centrifuged at 10,000 g for 30 minutes at 4 °C (MSE, “High Speed 18”; England). After the separation and neutralization up to pH 6.5 - 7.0 of the supernatant with 10 N NaOH, the precipitation of bacteriocin was achieved with ammonium-sulphate. Separated bacteriocin in the shape of whitish pellets was suspended in 0.05 M of sodium-phosphate buffer pH 7.

The spectrum of antimicrobial activity of isolated bacteriocin was determined using the indicator stains (Listeria monocytogenes ATCC 19111, Staphylococcus aureus ATCC 6538, and Escherichia coli ATCC 11303).

Isolated bacteriocin, as well as its series of suitable dilutions in the amount of 50 μL, after the microbiological filter (Acrodisc, Gelman) of a diameter of 0.22 μm, were spotted into the previously made and marked basins in agar. Dilutions were made with sterile deionized water. After one hour incubation at 4 °C, in order to stimulate the examined bacteriocin diffusion, the plates were incubated at 30 °C for 24 h.

The activity of the isolated bacteriocin was expressed as the absolute value, marked as the arbitrary unit (AU/mL). Arbitrary values were determined by the following formula: AU/mL = 2^n x (1,000 μL/50 μL), where n represents the maximum dilution of the bacteriocin portion of 50 μL which gives the growth inhibition zone for L. monocytogenes (test-microorganism in this research) as being higher than 2 mm (Barefoot and Klaenhammer, [24]; Ennahar et al., [25]).

2.3 Cheese production and sampling procedure

Raw cow’s milk was heat-treated (5 minutes at temperatures above 90 °C) and then it was cooled to 30 °C (the optimum temperature for coagulation). Milk coagulation was performed using traditional method i.e. the commercial “Maja” rennet (Čačak, Serbia), strength 1 : 5,000, in the amount of ca. 1.5 - 2 mL/L. The rennet is a mixture of enzymes isolated from the stomach of young ruminants, typically calves (chymosin for the most part, and pepsin in part). The total amount was divided into three equal parts:

i) the first part was control group (K),

ii) the second part was inoculated with L. monocytogenes (ca. 10^9 cells/mL) (group E-1), and

iii) in the third part was added L. monocytogenes and bacteriocin isolated from E. faecalis (1280 AU/mL (group E-2)).
Upon milk coagulation, it took 80 min. for curd formation. After curdling, the curd was placed on a piece of cheese cloth to drain. The draining of the curd was spontaneous at first, without pressing, for about 30 min., and then the curd was kept under pressure (the curd was pressed with a 3 kg stone) for 3 to 4 h. The cheese was sliced, salted and stored in containers during analysis (21 days) at a cool room temperature of 15 - 18°C. Due to the specificities of the added pathogen, the study was carried out under strictly controlled conditions. Samples for laboratory examinations were taken on days: 0, 2, 4, 7, 14 and 21. Three samples were collected at each step of sampling and used for analysis. The cheese manufacture and fermentation process were repeated three times.

2.4 Microbiological analyses

Determination of the Listeria spp. presence was carried out following the ISO 11290 - 1, 2 (11290 - 1, 2, [26], [27]) procedure. 25 g of each sample were homogenized with 225 mL sterile Fraser broth base (Biolife, Italy) in a stomacher for 2 min. The homogenates were incubated at 20°C for 1 h, in order to resuscitate stressed microorganisms. For L. monocytogenes enumeration, a volume of 0.1 mL from each homogenate was directly streaked onto each of 2 Palcam Agar (Oxoid, UK) plates and incubated at 37°C for 24 - 48 h. The homogenates were then supplemented by Fraser half selective supplement (primary enrichment) (Biolife, Italy) and incubated at 30°C for 24 h for detection of L. monocytogenes. Afterwards, 0.1 mL of the primary enrichment was inoculated in 10 mL of Fraser broth supplemented (Biolife, Italy) by Fraser selective supplement (secondary enrichment) (Biolife, Italy) and incubated at 37°C for 24 h. Cultures were streaked onto Oxford (Oxoid, UK) plates and incubated at 30°C. From each plate of the primary and secondary enrichment, 5 colonies presumed to be Listeria spp. were streaked onto TSYEA (Tryptone soya yeast extract agar) plates (Biolife, Italy) and incubated for 24 h at 37°C. Colonies were selected for typical appearance on TSYEA and submitted to Gram staining, catalase and oxidase test. Haemolytic activity and CAMP tests on sheep blood agar were performed for the L. monocytogenes confirmation.

The results (a number of colony forming units, CFU) were expressed as average number of CFU g⁻¹ cheese. The arithmetic means and standard deviations were calculated.

2.5 Statistical analysis

The results were analyzed using statistical methods to determine measures of central tendency (the arithmetic mean) and measures of variability or dispersion (standard deviation, SD). The results were subjected to a two-way analysis of variance (experimental variants, fermentation period), and the significance of differences was determined by the LSD test (Statistica SPSS 5). The statistical package “Statistica for Windows” (StatSoft Inc., USA [28]) was used for the analysis.

3. Results and Discussion

3.1 Detection, spectrum of antimicrobial activity and strength of the isolated bacteriocin

Interestingly, three strains of Enterococcus faecalis that produce bacteriocin-like antimicrobial compounds were isolated from the traditional Serbian cheese (Zlatar cheese). Inhibitory effects of most of the isolates were attributed to the production of extracellular metabolites, mainly organic acids. Hydrogen peroxide could also act as an anti-Listeria agent, but supernatants treatment with catalase allowed the activity of H₂O₂ to be excluded. Test for bacteriocin production revealed a proteinaceous nature of antimicrobial compounds, indicating the possibility that they could be a bacteriocin-like substances. The proteinase test (using the proteolytic enzyme proteinase K) led to bacteriocin inactivation, thus indirectly confirming its proteinaceous nature (Joerger et al., [29]).

Compared to test microorganisms, LAB bacteriocins showed marked antibacterial activity against Listeria monocytogenes ATCC 19111, whereas their action against Staphylococcus aureus ATCC 6538 and Escherichia coli ATCC 11303 was not detected. These results are supported by findings of other authors (Schilinger, [30], Abbe, [31], Vesković Moracanin et al., [32], and [33]) who pointed to the fact that the inhibitory activity of LAB bacteriocins is dominant mostly against Gram-positive bacteria.

Since two bacteriocins very rapidly lost their antilisterial activity in the environment, a bacteriocin whose producer based on 16S rRNA sequencing, was designated as E. faecalis ATCC 19433 was used in the research that followed.

The maximum dilution of bacteriocin isolated from E. faecalis, which produced an antilisterial effect was 1 : 64, giving a calculated activity of approx. 1,280 AU/mL.

3.2 The antilisterial activity of the added bacteriocin

L. monocytogenes was not detected in any sample of the control group of soft white cheese (K-1) in all stages of testing (days: 0, 2, 4, 7, 14 and 21) during the triplicate fermentations. The results of this group are not presented. The changes in the number of the total viable count of L. monocytogenes in the examined experimental groups of cheese (from groups E-1, and E-2) are presented in Table 1 and Figure 1.
The antimicrobial effect of enterococcal bacteriocins (the so-called enterocins) was detected more than fifty years ago in different strains of the genus *Enterococcus* widely distributed in nature (Brock *et al*., [36]). Most of enterococcal bacteriocins are class II bacteriocins, which are: heat stable, cationic, hydrophobic and low molecular weight peptides with antilisterial activity and with an interesting technological potential (Giraffa, [37], Khan *et al*., [38], Gálvez *et al*., [39], and Franz *et al*., [40]), especially those with a broad-spectrum of inhibition like the cyclic peptide AS-48 (Maqueda *et al*., [41], Edalatian *et al*., [42]). To date, a large number of enterococcal bacteriocins that show an antimicrobial effect against foodborne pathogens such as *Listeria* spp. and *Clostridium* spp. have been described (McAuliffe *et al*., [43], Mendoza *et al*., [44], Moreno *et al*., [45], Cocolin *et al*., [46], Izquierdo *et al*., [47]). Bacteriocins isolated from enterococci have a very strong antilisterial effect which makes them potential control agents against this pathogen in foods. However, their direct use and the use of *Enterococcus* cells generally lead to reduction or loss of their antibacterial activity due to interactions with food components (Vesković Moračanin, [48], Vesković Moračanin *et al*., [49]).

Certainly, for the direct application of these bioprotectors and entry into the full production process, in addition to receiving GRAS compounds status (Generally recognized as safe) they must be well studied and harmonized with other technological factors in the production (pH, temperature, salt and nitrite) (Cheveland *et al*., [50], Vesković and Đjukić, [51]). Their implementation should be viewed in terms of good alternatives, especially when combined with other natural protectors (Leroy and de Vuyst, [52]) and, of course, good hygienic and manufacturing practices. However, reduction of *Listeria* cells in this way could be a promising tool, although it should be considered only as an additional measure to implement *Listeria* contamination control.

### 4. Conclusions

- Growing need for naturally safe and healthy food has led to increased interest in the introducing bacteriocins as a factor in the hurdle technology approach to food protection, and has attracted scientists in this food safety field.
- The use of bacteriocins can be interesting and quite desirable since consumers’ trust in chemical preservatives has been shaken and even questioned. Food composition i.e. properties (pH, temperature, ingredients and supplements, type and counts of microorganisms) and the technology used during the production process can affect the stability and activity of the added bacteriocins. Future research in this field

### Table 1. The total viable count (log CFU/g ± SD)* of *Listeria monocytogenes* in samples of experimental groups E-1 and E-2 soft white cheeses during fermentation

<table>
<thead>
<tr>
<th>Days</th>
<th>Group E-1</th>
<th>Group E-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.19bc ± 0.19</td>
<td>4.47ab ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>4.38bc ± 0.08</td>
<td>4.45bc ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>5.35bc ± 0.35</td>
<td>3.25cd ± 0.55</td>
</tr>
<tr>
<td>7</td>
<td>4.24cd ± 0.23</td>
<td>2.74cd ± 0.04</td>
</tr>
<tr>
<td>14</td>
<td>4.14bc ± 0.24</td>
<td>2.77cd ± 0.17</td>
</tr>
<tr>
<td>21</td>
<td>4.29bc ± 0.33</td>
<td>1.78cd ± 0.01</td>
</tr>
</tbody>
</table>

*each result is expressed as the average of measurements from three samples ± standard deviation (SD).

CFU - colony forming units.

Values followed by different small letters within columns are significantly different (P ≥ 0.05) according to the LSD test.

### Figure 1. Growth of *L. monocytogenes* ATCC 19111 in the samples of soft cheese during the fermentation period

E-1 (○) - Experimental group 1 with added *L. monocytogenes*. E-2 (■) - Experimental group 2 with added *L. monocytogenes* and bacteriocin isolated from *E. Faecalis*.

Results on changes in the total viable count of *L. monocytogenes* during cheese ripening indicate a high concentration of this pathogen in group E-1 cheese which was on day 21 of ripening close to initial contamination (ca. 10<sup>7</sup> CFU/g). The maximum cell count of *Listeria* was determined on day 4 of testing.

In both experimental group of cheese supplemented with bacteriocin isolated from *E. faecalis* (group E-2), at the end of the ripening process, a statistically significant level of reduction in the count of *L. monocytogenes* (P ≥ 0.05) was determined. The initial count of this pathogen (ca. 10<sup>7</sup> CFU/g) was reduced to below 100 CFU/g (group E-2) at the end of the cheese ripening process.

The obtained results show that the enterocin is successful in preventing the growth of *L. monocytogenes*, despite the possible inactivation by various interactions with the food system (Ennahar *et al*., [34], Izquierdo *et al*., [35]).

A very important fact is that the strains tested in the present work were isolated from a traditional Zlatar cheese, and that represents natural bioprotectors isolated from a similar matrix.
should elucidate this unknown aspect regarding their use, thus providing information on the optimization of environmental conditions in order to contribute to the maximum antimicrobial effect of bacteriocins added to food systems and facilitate the search for new producers.

- Moreover, our further research in this field will focus on improving the understanding bacteriocins nature, their antimicrobial activities and potential applications, and searching for new bacteriocin-producing strains of LAB originating from autochthonous fermented products whose controlled and planned use would make them act as natural preservatives or food bioprotectors.

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5. References


