The probiotic potential and evaluation of the safety aspects of Enterococcus sp. strains isolated from traditionally made Serbian cheese

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ABSTRACT

The purpose of this research paper was the evaluation of the safety aspects and probiotic potential of six isolates from the genus Enterococcus isolated from traditionally made cheese. The isolates represented part of the unexplored microflora of the cheese. The tolerance of Enterococcus isolates to different gastrointestinal conditions (low pH, the presence of pepsin, pancreatin and bile salts) were investigated. Using the microdilution method, sensitivity to clinically relevant antimicrobial agents (tetracycline, ampicillin, gentamicin, vancomycin and polymyxin B) was evaluated. The adhesion ability of autochthonous Enterococcus isolates to solvents, as well as the ability of auto-aggregation and co-aggregation between them and Escherichia coli clinical isolates, was investigated. The results indicated that isolates showed tolerance to the simulated gastrointestinal condition in a high percentage. Isolates were sensitive to all the tested antibiotics, especially to ampicillin, with MIC values obtained from 0.19-2.5 μg/mL. The isolates showed the ability of growth in medium with phenol and showed no ability to synthesize histamine and tyramine. The highest percentage of adhesion was detected with chloroform, and the lowest with xylene. The isolates showed moderate auto-aggregation ability, while a different degree of co-aggregation with E. coli was observed. The results indicated that the potential application of investigated Enterococcus isolates is selective and limited.

Key words: antibiotics; adhesion ability; aggregation ability; probiotics; safety aspect; enterococci

Introduction

One of the major criteria for autochthonous bacteria to be considered as probiotics is their resistance to gastrointestinal conditions (HERNANDEZ-HERNANDEZ et al., 2012). However, BOTES et al. (2008) indicated that one more property of a good probiotic is its adhesion to mucus and epithelial cells. The reason for this can be found in the fact that adhesion, particularly in intestinal epithelial cells, is a very important prerequisite for colonization of the gastrointestinal tract by probiotic bacteria. Also, good bacterial adhesion can prevent their immediate elimination by peristalsis, and provide a competitive advantage in this ecosystem (KOS et al., 2003).

SOLIERI et al. (2014) indicated that the beneficial effects of probiotics are strain specific. Some studies indicated the potential use of members of the genus Enterococcus as safe probiotic candidates (FRANZ...
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et al., 2011; FERREIRA ARAÚJO and DE LUCES FORTES FERREIRA, 2013). The main species of this genus found in food are Enterococcus faecalis and Enterococcus faecium (HOSSEINI et al., 2009; MURUZOVIĆ et al., 2018a). Some authors indicated that enterococci have some desirable characteristics for this purpose, such as resistance to gastric juices and bile salts, and the production of antimicrobial compounds such as enterocins (FRANZ et al., 1999).

It has been shown that LABs, with auto-aggregation ability, act on the hydrophobicity of the cell surface and had a better ability to adhere to intestinal cell surfaces (DEL RE et al., 2000). Many authors indicated that LABs, with co-aggregation ability, may form a barrier that prevents colonization by pathogenic microorganisms (DEL RE et al., 2000; KOS et al., 2003; ASLIM et al., 2007; YONES et al., 2012; PRINGSULAKA et al., 2015). LABs, which use co-aggregation ability with some pathogenic strains, which mostly belong to the gastrointestinal and urogenital tracts, could inhibit their growth (BOTES et al., 2008). LI et al. (2015) showed that LABs (Lactobacillus, Lactococcus, and Enterococcus), isolated from Chinese traditional fermented food, had a good co-aggregation ability with Salmonella spp. STEVENS et al. (2015) demonstrated the ability of co-aggregation between E. faecalis MF328, isolated from food, with bacteria isolated from different environmental sources.

However, very few Enterococcus strains have been used as probiotics or feed additives because of the safety concerns associated with their pathogenic traits as opportunistic microorganisms. Numerous enterococcal strains are known for carrying virulence factors, which includes resistance to antibiotics. This is the most dangerous characteristic for probiotics, related to the ability of horizontal transfer of genes from beneficial bacteria to pathogens (BILSTROM et al., 2008; BRAIEK and SMAOUI, 2019).

The aims of this study were the evaluation of the tolerance of indigenous Enterococcus isolates to different gastrointestinal conditions, their sensitivity to antibiotics, their ability to synthesize biogenic amines and to grow on media with phenol, as well as the detection of hemolysis on blood agar. Also, the aims were the evaluation of the auto-aggregation, co-aggregation and adhesiveness abilities of indigenous isolates.

Materials and methods

Microorganisms used in study. Six isolates from the genus Enterococcus (Enterococcus hirae KGPMF9, Enterococcus durans KGPMF0, Enterococcus faecium KGPMF14, Enterococcus faecalis KGPMF47, E. faecalis KGPMF48, E. faecalis KGPMF49) were used in this study. All tested bacteria were isolated from cheese from Sokobanja (Southeastern Serbia), and provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The cheese was made in a traditional way, without adding any bacterial starter culture, so the isolates tested in this study represent the natural cheese microflora. The isolates were chosen according to their previously investigated biochemical characteristics and antagonistic potential against enterobacteria isolated from the same cheese (MURUZOVIĆ et al., 2018a; GRUJOVIĆ et al. 2019). The bacterial strains were kept in glycerol stock at −80 °C. Enterococcus faecalis ATCC 29211 was used as a reference strain. Escherichia coli, a clinical isolate, was a generous gift from the Institute of Public Health, Kragujevac, Serbia. Before experimental use, working cultures were subcultured twice in MRS broth.

Simulated gastrointestinal transit tolerance assay. The acid tolerance of LAB was studied in different pH solutions, which were prepared by adjusting hydrochloric acid (HCl) (Zorka Šabac, Šabac, Serbia) to pH levels of 3, 4 and 5, as described in GRUJOVIĆ et al. (2019a). The bacterial strains were kept in glycerol stock at −80 °C. Enterococcus faecalis ATCC 29211 was used as a reference strain. Escherichia coli, a clinical isolate, was a generous gift from the Institute of Public Health, Kragujevac, Serbia. Before experimental use, working cultures were subcultured twice in MRS broth.

Simulated gastric and small intestinal juice tolerance assays were performed according to the method described in HUANG and ADAMS (2004), with some modifications described in GRUJOVIĆ et al. (2019a). Gastric juice was prepared by suspending 0.22% (w/v) pepsin (Merck, New Jersey, USA) in sterile filtered 0.5% (w/v) NaCl solution, with the pH adjusted to 2. Incubation was conducted at 37°C/3 h. The results were determined by an ELISA plate reader at 600 nm, in triplicate (BASSYOUNI et al., 2012).
Small intestinal juice was prepared by suspending 0.2% (w/v) of pancreatin (Sigma-Aldrich, St. Louis, USA) in filter sterile 0.5% NaCl (w/v) solution with 0.4% bile salts (Sigma-Aldrich, St. Louis, USA) and adjusting the pH to 8 by adding sterile 0.1 M NaOH. Ninety-six-well microtiter plates were incubated at 37 °C/4 h. The results were performed in triplicate. The number of viable enterococci was determined by transferring the appropriate samples onto the MRS agar plates. The percentage of survival was calculated using the following formula:

\[ \% \text{ survival} = \left( \frac{\beta}{\alpha} \right) \times 100 \]

\( \alpha \) - CFU/mL of the assayed strain (uninoculated MRS (pH 6.5), at 37 °C/48 h); \( \beta \) - CFU/mL of the same strain after incubation under different gastrointestinal conditions.

**Synthesis of biogenic amines and growth in the presence of phenol.** The ability of the isolates to synthesize biogenic amines (histamine and tyramine) from histidine and tyrosine was analyzed by the method described in JEONG and LEE (2015). The growth of isolates in the presence of phenol was determined as described in ŠUŠKOVIĆ et al. (2001).

**Evaluation of auto-aggregation and co-aggregation ability.** The auto-aggregation ability of Enterococcus isolates, as well as the co-aggregation ability with E. coli was monitored by the method described in TUO et al. (2013), with the modification described in GRUJOVIĆ et al. (2019a).

**Microbial adhesion to solvents.** Microbial adhesion to solvents (MATS) was measured according to the method described in COLLADO et al. (2008), with modifications described in GRUJOVIĆ et al. (2019a). Three different solvents were tested in this study: xylene (Sineks, Belgrade, Serbia), which is an apolar solvent; chloroform (Alkaloid, Skoplje, Macedonia), a monopolar and acidic solvent; and ethyl acetate (Zorka Šabac, Šabac, Serbia) a monopolar and basic solvent. Only bacterial adhesion to xylene reflects cell surface hydrophobicity or hydrophilicity. The values of MATS obtained with the two other solvents, chloroform, and ethyl acetate, were regarded as a measure of the electron donor (basic) and electron acceptor (acidic) characteristics of the bacteria, respectively.

**Safety assessment.** The safety of enterococci was assessed by detection of hemolysis on blood agar plates, with an evaluation of their sensitivity to clinically relevant antibiotics.

The hemolytic activity of Enterococcus isolates was examined by culturing fresh overnight cultures on Columbia agar plates (Oxoid) containing 7% (v/v) sheep blood (Oxoid), incubated for 48 h at 37 °C. Hemolytic activities were detected as the appearance of a halo around the colony: a greenish zone was considered α-hemolysis, a clear zone β-hemolysis and no halo γ-hemolysis (ABEDI et al., 2018).

The antibiotic sensitivity of enterococci was tested using the microdilution method with resazurin (SARKER et al., 2007), by determining the minimum inhibitory concentration (MIC) The followed antibiotics were used for this study: tetracycline, ampicillin, gentamicin, vancomycin and polymyxin B (Sigma Chemicals Co., USA), in a concentration range from 0.05-4000 μg/mL. The method was described in detail in MURUZOVIĆ et al. (2016).

**Statistical analysis.** All data were presented as means ± standard deviations, using Microsoft Excel (Redmond, Washington, DC, USA). Differences between bacterial survival in gastrointestinal conditions, as well as sensitivity to the tested antibiotics, were tested using one-way ANOVA and Paired-T test. A paired T-test was used for statistical processing of the results of adhesion to different solvents. Spearman's correlation coefficient was used for determination of a correlation between the auto-aggregation and hydrophobicity of the tested bacteria. All statistical analyses were performed using SPSS (IBM SPSS Statistics 20).

**Results**

**Simulated gastrointestinal transit tolerance assay.** It is desirable that potential probiotic strains show tolerance to low pH, since they pass through stomach conditions. In this test, all the isolates showed the ability of growth at low pH, although the optical densities of bacterial growth were reduced (Table 1).
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Table 1. Resistance of Enterococcus isolates to low pH

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Growth control</th>
<th>pH 5</th>
<th>pH 4</th>
<th>pH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. hirae KGPMF9</td>
<td>0.22 ± 0.04</td>
<td>0.16 ± 0.01 (72.71)*</td>
<td>0.15 ± 0.02 (68.18)*</td>
<td>0.14 ± 0.01 (63.64)*</td>
</tr>
<tr>
<td>E. durans KGPMF10</td>
<td>0.25 ± 0.01</td>
<td>0.16 ± 0.01 (64)*</td>
<td>0.14 ± 0.02 (56)*</td>
<td>0.14 ± 0.00 (56)*</td>
</tr>
<tr>
<td>E. faecium KGPMF14</td>
<td>0.28 ± 0.00</td>
<td>0.26 ± 0.00 (92.85)</td>
<td>0.24 ± 0.01 (85.71)*</td>
<td>0.17 ± 0.03 (60.71)*</td>
</tr>
<tr>
<td>E. faecalis KGPMF47</td>
<td>0.26 ± 0.04</td>
<td>0.22 ± 0.02 (84.62)</td>
<td>0.18 ± 0.01 (69.23)*</td>
<td>0.17 ± 0.01 (65.38)*</td>
</tr>
<tr>
<td>E. faecalis KGPMF48</td>
<td>0.28 ± 0.02</td>
<td>0.25 ± 0.02 (89.29)</td>
<td>0.17 ± 0.01 (60.71)*</td>
<td>0.15 ± 0.01 (53.57)*</td>
</tr>
<tr>
<td>E. faecalis KGPMF49</td>
<td>0.26 ± 0.06</td>
<td>0.22 ± 0.04 (84.62)</td>
<td>0.20 ± 0.00 (76.92)*</td>
<td>0.18 ± 0.00 (69.23)*</td>
</tr>
<tr>
<td>E. faecalis ATCC 29211</td>
<td>0.28 ± 0.05</td>
<td>0.08 ± 0.05 (28.57)*</td>
<td>0.07 ± 0.04 (25.00)*</td>
<td>0.04 ± 0.03 (14.29)*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD measured at 600 nm; growth percentages are given in parentheses; *statistical significance (P<0.05) of the growth of bacteria compared with growth control

Table 2. Tolerance of Enterococcus isolates to simulated gastric juice tolerance

<table>
<thead>
<tr>
<th>Isolates</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>% of surviving after 3 h(CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. hirae KGPMF9</td>
<td>0.12 ± 0.00 (92.31)</td>
<td>0.12 ± 0.00 (92.31)</td>
<td>0.12 ± 0.00 (92.31)</td>
<td>90 ± 0.64</td>
</tr>
<tr>
<td>E. durans KGPMF10</td>
<td>0.27 ± 0.02 (90)</td>
<td>0.24 ± 0.01 (80)</td>
<td>0.20 ± 0.02 (66.67)*</td>
<td>56.9 ± 0.60</td>
</tr>
<tr>
<td>E. faecium KGPMF14</td>
<td>0.15 ± 0.00 (100)</td>
<td>0.13 ± 0.01 (86.67)</td>
<td>0.12 ± 0.00 (80)*</td>
<td>82.9 ± 2.33</td>
</tr>
<tr>
<td>E. faecalis KGPMF47</td>
<td>0.15 ± 0.00 (100)</td>
<td>0.14 ± 0.03 (93.33)</td>
<td>0.13 ± 0.01 (86.67)</td>
<td>85.5 ± 0.20</td>
</tr>
<tr>
<td>E. faecalis KGPMF48</td>
<td>0.18 ± 0.02 (85.71)</td>
<td>0.18 ± 0.02 (85.71)</td>
<td>0.17 ± 0.02 (80.95)*</td>
<td>80.9 ± 4.56</td>
</tr>
<tr>
<td>E. faecalis KGPMF49</td>
<td>0.15 ± 0.01 (100)</td>
<td>0.14 ± 0.04 (93.33)</td>
<td>0.13 ± 0.02 (86.67)*</td>
<td>86.4 ± 0.36</td>
</tr>
<tr>
<td>E. faecalis ATCC 29211</td>
<td>0.10 ± 0.01 (66.67)</td>
<td>0.10 ± 0.01 (66.67)</td>
<td>0.09 ± 0.02 (60)*</td>
<td>72.8 ± 2.12</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD measured at 600 nm; percentages of growth compared with growth of control are given in parentheses; *Significant differences (P<0.05) at 0 h

Table 3. Tolerance of Enterococcus isolates to stimulated small intestinal juice

<table>
<thead>
<tr>
<th>Isolates</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>% of surviving after 4 h(CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. hirae KGPMF9</td>
<td>0.13 ± 0.02 (54.17)</td>
<td>0.14 ± 0.03 (58.33)</td>
<td>0.14 ± 0.02 (58.33)</td>
<td>0.14 ± 0.02 (58.33)</td>
<td>56.3 ± 0.88</td>
</tr>
<tr>
<td>E. durans KGPMF10</td>
<td>0.24 ± 0.01 (82.76)*</td>
<td>0.25 ± 0.01 (86.21)*</td>
<td>0.21 ± 0.02 (72.41)</td>
<td>0.21 ± 0.00 (72.41)</td>
<td>30.4 ± 0.12</td>
</tr>
<tr>
<td>E. faecium KGPMF14</td>
<td>0.12 ± 0.00 (52.17)</td>
<td>0.13 ± 0.00 (56.52)*</td>
<td>0.11 ± 0.01 (47.82)</td>
<td>0.10 ± 0.00 (43.47)</td>
<td>40.5 ± 0.36</td>
</tr>
<tr>
<td>E. faecalis KGPMF47</td>
<td>0.15 ± 0.00 (60)</td>
<td>0.16 ± 0.00 (64)*</td>
<td>0.15 ± 0.01 (60)</td>
<td>0.14 ± 0.01 (56)</td>
<td>52.3 ± 0.64</td>
</tr>
<tr>
<td>E. faecalis KGPMF48</td>
<td>0.19 ± 0.02 (82.61)*</td>
<td>0.21 ± 0.02 (91.30)*</td>
<td>0.18 ± 0.00 (78.26)*</td>
<td>0.17 ± 0.00 (73.91)*</td>
<td>52.7 ± 0.16</td>
</tr>
<tr>
<td>E. faecalis KGPMF49</td>
<td>0.11 ± 0.01 (44)</td>
<td>0.12 ± 0.00 (48)*</td>
<td>0.11 ± 0.00 (44)</td>
<td>0.11 ± 0.00 (44)</td>
<td>40.4 ± 0.26</td>
</tr>
<tr>
<td>E. faecalis ATCC 29211</td>
<td>0.10 ± 0.01 (66.67)</td>
<td>0.08 ± 0.01 (53.33)</td>
<td>0.05 ± 0.00 (33.33)*</td>
<td>0.05 ± 0.02 (33.33)*</td>
<td>26.7 ± 0.54</td>
</tr>
</tbody>
</table>

Absorbance values are presented as mean ± SD measured at 600 nm; growth percentages are given in parentheses; *Significant differences (P<0.05) at 0 h
KGPMF9, KGPMF10, and KGPMF48 isolates showed a slight decrease in growth after 1 h of incubation. After 3 h of incubation, only the KGPMF47 isolate showed continuous growth. The percentage of survival was in the range of 56.9-90% (Table 2).

The growth of all isolates after 4 h of incubation in stimulated small intestinal juice showed a further decrease. The percentage of survival of enterococci was in the range of 30.4-56.3% (Table 3).

Synthesis of biogenic amines and growth in the presence of phenol. Enterococcus isolates showed no ability to synthesize histamine and tyramine (biogenic amines), but showed the ability of growth in the medium with 0.1, 0.2 and 0.3% of phenol.

The adhesion, auto-aggregation and co-aggregation ability of Enterococcus isolates. The auto-aggregation ability of Enterococcus sp. strains isolated from traditionally made cheese from Southeastern Serbia was measured over a period of 5 h. The results showed that the tested strains exhibited a selective auto-aggregating phenotype. The tested Enterococcus isolates showed moderate auto-aggregation ability (Fig. 1). The percentage of auto-aggregation attained after 5 hours range from 22.9 to 46.64%.

The co-aggregation ability of Enterococcus isolates with E. coli also was examined (Table 4).

The results are expressed as the percentage of co-aggregation after 2 h in the absorbance of a mixed suspension. All tested Enterococcus isolates showed selectively and strain specific co-aggregation ability, with results ranging from 10.53 to 23.32%.

The MATS method was used to evaluate the hydrophobic/hydrophilic cell surface properties of members from the genus Enterococcus, isolated from the cheese. E. faecalis ATCC 29211 was used for comparative purposes. In order to assess the Lewis acid-base characteristics of the bacterial cell...
surfaces, bacterial adhesion to chloroform and ethyl acetate was tested. The results indicated that the tested bacteria had a stronger affinity to chloroform, which is an acidic solvent and electron acceptor, than to ethyl acetate, which is a basic solvent and electron donor (Table 5). On the basis of these results, it may be concluded that the adhesion of the tested enterococci was selective and strain-specific. No affinity for xylene was observed.

Safety assessment. The results of the hemolytic activity indicated that Enterococcus isolates showed α-hemolysis. None of the tested isolates showed β-hemolysis.

The sensitivity of isolates against antibiotics was tested (Table 6). The results were checked according to the LAB resistance criteria proposed for antibiotics of human and veterinary importance by the European Food Safety Authority (EFSA).

Enterococcus isolates showed significant sensitivity to ampicillin and tetracycline, compared to the other tested antibiotics (P<0.05). MIC values obtained were from 2.5 to 12.5 μg/mL for tetracycline and from 0.19 to 2.5 μg/mL for ampicillin.

Discussion

The presence of enterococci in milk and raw milk cheeses has been traditionally considered as a result of fecal contamination, but BRAIEK and SMAOUI (2019) reported that this occurrence is not always related to fecal contamination. Moreover, it is important to note that members of the genus Enterococcus play a beneficial role in

![Table 5. The adhesion ability of Enterococcus isolates to xylene, chloroform and ethyl acetate](attachment:table5.png)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Xylene</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. hirae KGPMF9</td>
<td>0</td>
<td>14.21 ± 0.08</td>
<td>0</td>
</tr>
<tr>
<td>E. durans KGPMF10</td>
<td>0</td>
<td>3.85 ± 1.56</td>
<td>0</td>
</tr>
<tr>
<td>E. faecium KGPMF14</td>
<td>0</td>
<td>13.17 ± 0.41a</td>
<td>9.41 ± 0.12a</td>
</tr>
<tr>
<td>E. faecalis KGPMF47</td>
<td>0</td>
<td>23.97 ± 0.63a</td>
<td>15.86 ± 0.74b</td>
</tr>
<tr>
<td>E. faecalis KGPMF48</td>
<td>0</td>
<td>22.48 ± 0.84a</td>
<td>6.24 ± 0.24b</td>
</tr>
<tr>
<td>E. faecalis KGPMF49</td>
<td>0</td>
<td>5.4 ± 0.04a</td>
<td>3.17 ± 1.36a</td>
</tr>
<tr>
<td>E. faecalis ATCC 29211</td>
<td>0</td>
<td>19.66 ± 1.24a</td>
<td>12.13 ± 1.05b</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SD from three separate experiments; Means in the two solvent columns or each particular isolate, with superscript with different letters, are significantly different (P<0.05)

![Table 6. Antibiotic sensitivity of Enterococcus isolates](attachment:table6.png)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Tetracycline</th>
<th>Gentamicin</th>
<th>Polymyxin B</th>
<th>Ampicillin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. hirae KGPMF9</td>
<td>6.25</td>
<td>25</td>
<td>250</td>
<td>2.5</td>
<td>125</td>
</tr>
<tr>
<td>E. durans KGPMF10</td>
<td>2.5</td>
<td>25</td>
<td>125</td>
<td>2.5</td>
<td>125</td>
</tr>
<tr>
<td>E. faecium KGPMF14</td>
<td>2.5</td>
<td>25</td>
<td>125</td>
<td>0.19</td>
<td>125</td>
</tr>
<tr>
<td>E. faecalis KGPMF47</td>
<td>12.5</td>
<td>25</td>
<td>250</td>
<td>0.19</td>
<td>125</td>
</tr>
<tr>
<td>E. faecalis KGPMF48</td>
<td>12.5</td>
<td>25</td>
<td>250</td>
<td>2.5</td>
<td>125</td>
</tr>
<tr>
<td>E. faecalis KGPMF49</td>
<td>2.5</td>
<td>50</td>
<td>250</td>
<td>2.5</td>
<td>125</td>
</tr>
<tr>
<td>E. faecalis ATCC 29211</td>
<td>8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
<td>250</td>
</tr>
</tbody>
</table>

MIC-minimal inhibitory concentration; ’Values are given in μg/mL; n.d. - not determined
cheese fermentation, as well as in cheese ripening and the development of its specific flavor, texture, and taste. Previous research by MURUZOVIĆ et al. (2018a) and GRUJOVIĆ et al. (2019) was aimed at the isolation, identification and characterization of Enterococcus sp. strains from traditionally made Serbian cheese (Southeastern Serbia). So far their antimicrobial potential has been demonstrated against some members of the fam. Enterobacteriaceae isolated from the same cheese, as well as their ability for biofilm formation. In the present paper, for the first time, the probiotic potential and antimicrobial sensitivity were investigated, as well as the adhesion and aggregation ability of six selected isolates from the genus Enterococcus, isolated from the cheese in question.

The probiotic characteristics of Enterococcus strains have already been investigated in many studies and their beneficial and significant health-promoting effects reported (ZOMMITI et al., 2018; NASCIMENTO et al., 2019; NAMI et al., 2019). However, investigation of enterococci as potential probiotics is still a controversial issue because they may have detrimental traits that make it difficult to establish a clear decision relating to enterococcal strains between emerging pathogens and potential probiotics.

The ability of enterococci to grow in broth supplemented with 40% of bile salts and to hydrolyze esculin is well known. Also, they are able to grow in a huge range of pH, from 4.4 and 9.6, and in hyper salty media with 6.5% NaCl (FOULQUIÉ MORENO et al., 2006). In our study, the survival rate of Enterococcus isolates in low pH, and bile salts displayed a significant variability. This might be due to the fact that mechanisms of acid and bile tolerance are species- and strain-dependent. The tolerance to different gastrointestinal conditions (acid and bile tolerance) of the Enterococcus isolates is consistent with the results reported by NAMI et al. (2014), GUO et al. (2016), AYYASH et al. (2018) and NAMI et al. (2019).

The tested Enterococcus isolates showed no ability to synthesize histamine and tyramine (biogenic amines), which is a desirable characteristic when selecting possible probiotics (AMMOR and MAYO, 2007). They also showed ability of growth in media with 0.1, 0.2 and 0.3% of phenol. Physiological levels of phenols in humans are low. That is why it is important to analyze the sensitivity of potential probiotics to this substance.

One of the major characteristics of LABs, which can potentially be used as probiotics, is their ability for adhesion and aggregation. Auto-aggregation and co-aggregation ability are defined as the bacterial accumulation of the same species and of different species, respectively (CAMPANA et al., 2017). Auto-aggregation is correlated with adherence to epithelial cells (COLLADO et al., 2008), while coaggregation represents a defensive barrier for the colonization of pathogenic microorganisms (KOS et al., 2003; ABUSHELAIBI et al., 2017). Specific probiotic strains usually show a higher auto-aggregation ability that pathogen strains (ELHADIDY and ZAHRAN, 2014). There are studies which indicate that the biofilm formation of LABs is associated with adhesion properties, which is an important feature in gut colonization and the probiotic potential of LABs (ELHADIDY and ZAHRAN, 2014; ŽIVKOVIĆ et al., 2016; POPOVIĆ et al., 2018). Previous studies of Enterococcus sp. strains isolated from Sokobanja cheese indicated that they had the ability to produce a moderate biofilm (MURUZOVIĆ et al., 2018a, 2018b; GRUJOVIĆ et al., 2019). In the current paper, their auto- and co-aggregation ability were demonstrated.

According to AYYASH et al. (2018) and NAMI et al. (2019), hydrophobicity is one of the indicative parameters of the cell surface properties of probiotics, and it is in correlation with the adhesion ability of probiotics to epithelial cells. Selection of probiotic LABs was primarily done on the basis of their hydrophobicity against xylene (PALOMARES et al., 2007), hexadecane (PRINGSULAKA et al., 2015) and toluene (DOWARAH et al., 2018). In the current study, the tested Enterococcus isolates showed a better affinity to chloroform (acidic solvent) than to ethyl acetate (basic solvent) (P<0.05). LI et al. (2015) indicated that selecting LABs with higher adhering ability, according to their aggregation ability, is not a desirable method.

Lee and SALMINEN (2009) and LI et al. (2015)
showed that these characteristics were strain specific, which was confirmed in our research, too.

NAMI et al. (2019) indicated that E. durans isolates from different artisanal dairy products showed no β-hemolysis, which is agreement with the results of our study. The antibiotic sensitivity of LABs isolated from Serbian and Croatian cheeses was investigated by UROIĆ et al. (2014). They showed that all the isolates were susceptible to the antibiotics, which was confirmed in our study. NAMI et al. (2014) indicated that E. durans 6HL was sensitive to vancomycin, tetracycline, ampicillin, and gentamicin. The Enterococcus isolates tested in our study were sensitive to these antibiotics too, and showed no resistance to the tested antibiotics, according to EFSA (2012) breakpoints.

Conclusion

Enterococcus isolates from Sokobanja cheese showed tolerance to different gastrointestinal conditions (mainly to bile extracts and gastric pH values), as well as surviving in the presence of phenols. They showed no ability for biogenic amines production, which is a desirable characteristic when selecting possible probiotics or starter cultures. Sensitivity to tested antibiotics was also noted. Adhesion ability is an important characteristic, because LABs, which process this ability, can be used as the mechanical barrier to adhesion by other bacteria, such as enterobacteria, to the epithelium. All the isolates showed moderate auto-aggregation and co-aggregation ability. Enterococcus sp. strains, isolated from the cheese from Serbia, showed probiotic potential, but they also showed the ability for α-hemolysis. On the basis of the results presented in this paper, it could be concluded that their probiotic application is selective and limited. Further studies need to include an investigation of adherence and colonization of intestinal epithelium cells, possible in vivo studies, and a more detailed investigation of the safety aspect. Enterococcal strains investigated for potential use as probiotics in human and veterinary medicine must be well-characterized and completely assessed regarding safety aspects.

Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SAŽETAK

Cilj ovoga rada bio je procjena sigurnosnog aspekta i probiotičkog potencijala šest izolata roda Enterococcus izoliranih iz tradicionalno rađenog sira. Izolati su predstavljali dio neistražene mikroflore sira. Analizirana je tolerancija izolata Enterococcus na različite gastrointestinalne uvjete (nizak pH, prisutnost pepsina, pankreatina i soli žućkih kiselina). Mikrodilucijskom metodom procijenjena je osjetljivost na sve testirane antibiotike; sposobnost adhezije; sposobnost agregacije; probiotici; sigurnosni aspekt; bakterije Enterococcus. Utkriveno je da je najveći postotak adhezije s kloroformom, a najmanji sa ksenolom. Izolati su pokazali umjerenu toleranciju izolata (tetraciklin, ampicilin, gentamicin, vankomicin i polimiksin B). Istraživana je adhezivna sposobnost autohtonih žučnih kiselina. Mikrodilucijskom metodom procijenjena je osjetljivost na klinički relevantne antimikrobne agense (tetraciklin, ampicilin, gentamicin, vankomicin i polimiksin B). Istraživana je i adhezivna sposobnost autohtona izolata Enterococcus na otapala, kao i sposobnost autoagregacije i koagregacije između njih i kliničkih izolata Enterococcus faecium. Izolati su pokazali toleranciju na simulirane gastrointestinalne uvjete u velikom postotku. Utvrđena je osjetljivost na sve testirane antibiotike, posebno na ampicilin, s MIC vrijednostima od 0,19 do 2,5 μg/mL. Izolati su pokazali sposobnost rasta na hranjivoj podlozi s fenolom, a nisu pokazali sposobnost sintetizacije histamina i tiramina. Otkriveno je da je najveći postotak adhezije s kloroformom, a najmanji sa ksenolom. Izolati su pokazali umjerenu sposobnost autoagregacije, a opažen je i različit stupanj koagregacije s E. coli. Rezultati pokazuju da je potencijalna primjena istraživanih izolata bakterije Enterococcus selektivna i ograničena.

Ključne riječi: antibiotici; sposobnost adhezije; sposobnost agregacije; probiotici; sigurnosni aspekt; bakterije Enterococcus