Introduction

Probiotic microorganisms are extensively studied and implemented in a wide range of applications such as prevention of food poisoning or treatment of certain gastrointestinal disorders (1–4). They are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (5). One of the most frequent functional properties for probiotics concerns the putative reduction and prevention of infectious disease in the gastrointestinal tract (1,2). Human gastrointestinal tract houses countless bacterial species (6) and beneficial role of intestinal microbiota is still intensively investigated. Probiotic microorganisms react to intestinal pathogens by producing antibacterial compounds such as organic acids, hydrogen peroxide, diacetyl, bacteriocins and antimicrobial peptides, with a variable spectrum of action (7,8). They can induce changes in enzyme activity, compete for nutrients and adhesion sites and/or increase levels of antibody and macrophage activity (9).

While selecting a preferable probiotic strain several aspects of functionality have to be considered such as tolerance to human gastric juice and bile tolerance as an im-
important property for survival in the small bowel (10). Another important criterion for selecting probiotic strains is adherence to intestinal epithelium. This is a crucial step in colonisation of the gastrointestinal tract and is important for competitive exclusion of enteropathogens (11,12). An important feature is also antagonistic activity against gut pathogens such as Helicobacter pylori, Salmonella spp., Listeria monocytogenes and Clostridium difficile (13–15).

Salmonella enterica serotype Typhimurium (S. Typhimurium), belonging to the diverse Salmonella genus, is one of the leading causes of self-limiting non-typhoidal gastroenteritis in humans. Most infections arise from oral ingestion of contaminated food or water (16,17). Adhesion to mammalian epithelial cells is a crucial step for bacteria to colonise the gastrointestinal tract. Epithelial cells of gastrointestinal tract are protected from pathogenic bacteria by several mechanisms exerted by commensal microbiota: competition for adhesion sites, and production of components with antimicrobial activity (18,19). The initial step of adhesion of Salmonella spp. is mediated by bacterial fimbriae which recognise certain receptors on eukaryotic cells (20). Salmonella enterica is known to adhere to and invade intestinal cells, including Caco-2 cells (21). Several studies indicate that different Lactobacillus strains could inhibit the adhesion of Salmonella and some other diarrhoeagenic bacteria (Escherichia coli, Yersinia pseudotuberculosis and Listeria monocytogenes), thus reducing colonisation and preventing infection (22,23).

The most commonly used probiotic microorganisms include various species of genera Lactobacillus and Bifidobacterium (24). Bacteria in genus Lactobacillus are aerobic, anaerobic, catalase-negative, rod-shaped lactic acid bacteria. Several strains of the genus Lactobacillus possess probiotic properties. The production of lactic acid together with some other products, like bacteriocins is responsible for the antimicrobial activity of these bacteria (25,26).

The objective of the present study is to test the effect of three potentially probiotic Lactobacillus plantarum strains isolated from traditional dairy products against the gut pathogen Salmonella enterica serotype Typhimurium.

Identification of L. plantarum strains was performed by classical biochemical and microbiological methods. Furthermore, viability of strains in the presence of bile salts, acidification ability, antimicrobial and anti-adhesion activity were examined.

Materials and Methods

Bacterial strains and growth conditions Three Lactobacillus plantarum isolates (strain A isolated from homemade cow’s cheese, strain B from homemade sheep’s cheese and strain S1 from whey), L. plantarum ATCC 1804 and clinical isolate of Salmonella enterica serotype Typhimurium 3064 were obtained from the culture collection of Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia. All tested bacteria were stored at −80 °C in 30% glycerol broth. Lactobacilli were grown in de Man, Rogosa and Sharpe (MRS) broth (Biolife Italiana Srl, Milan, Italy) in microaerophilic atmosphere (5% CO2) for 24 h at 37 °C. A culture of S. Typhimurium was prepared in nutrient or brain heart infusion (BHI) broth (Biolife Italiana Srl), and the number of bacteria was determined by plate counting on Salmonella Shigella (SS) agar (Biolife Italiana Srl) or Luria-Bertani (LB) agar (Biolife Italiana Srl). The number of bacteria in the inoculum was determined photometrically at λ=600 nm and the absorbance (A) was set to 1, corresponding to a concentration of 10^7 CFU/mL.

Human intestinal cells In order to examine bacterial adhesion to human intestinal cells, human colon adenocarcinoma cell line Caco-2 was used. Caco-2 cells were cultured in liquid nutrient Dulbecco’s Modified Eagle medium (DMEM; Lonza Group Ltd., Verviers, Belgium) supplemented with 10% foetal calf serum (FCS), 2 mmol/L of L-glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin (Lonza Group Ltd.), at 37 °C in the Co2 incubator MCO-20AC (Sanyo, Osaka, Japan). For the adherence assays, Caco-2 monolayers were prepared in 24-well tissue culture plates (TPP, Trasadingen, Switzerland). Cells were seeded at concentration of 2.5·10^5 cells/mL and incubated until the confluence was obtained. The culture medium was replaced by DMEM without any supplements at least 1 h prior to the adhesion experiments.

Characterisation of potential probiotic strains MALDI-TOF MS analysis Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF MS; Bruker Daltonik GmbH, Bremen, Germany) was used for identification of potential probiotic strains after biochemical identification by API test (bioMérieux, Marcy l’Etoile, France). Samples were prepared according to the manufacturer’s recommendation using a Microflex LT™ instrument (Bruker Daltonik). For the identification, the peaks from the generated mass spectrum were compared with reference spectra of the integrated database using MALDI Biotyper software package (Bruker Daltonik GmbH).

Viability of the cells in the presence of bile salts Resistance to bile salts was tested according to Babić et al. (3). Briefly, BHI broth was prepared by the addition of 1% (mass per volume) of bile salts (LP0055; Oxoid LTD, Basingstoke, UK). Overnight bacterial cultures were adjusted to initial A_600 nm=0.2 in BHI broth with bile salts. After incubation with agitation at 37 °C for 24 h, bacterial growth was measured using a photometer (Biophotometer, Eppendorf, Hamburg, Germany) at λ=600 nm.

The acidification ability of Lactobacillus isolates The ability of the isolates to reduce the pH of the medium was tested by overnight incubation of cultures at 37 °C with agitation in BHI and MRS broths, as well as in BHI broth with 1% bile salts. Initial pH of the media was 7.2 (BHI broth), 6.5 (MRS broth) or 7.2 (BHI broth with 1% bile salts).
Functional characterisation of Lactobacillus isolates

Agar well diffusion assay

Antibacterial activity of cell-free culture supernatants (CFS) of L. plantarum strains was examined by the agar well diffusion assay as described by Bilkova et al. (27). Culture supernatants were prepared by cultivation of lactobacilli in MRS broth overnight at 37°C in microaerophilic atmosphere (5 % CO₂) and the cells were removed by centrifugation at 2000g for 10 min. The supernatants were sterile-filtered through mixed cellulose ester membrane filter with 0.22-μm pores (Merck, Darmstadt, Germany). The equal fractions of the supernatant were adjusted to neutral pH=7.0 by adding 1 M NaOH and/or heat treated (5 min at 100°C). S. Typhimurium was diluted in physiological saline (McFarland No. 1; Densimat, bioMérieux) and spread on the surface of Müller Hinton (MH) agar (Oxoid Ltd.). After absorption wells of 5 mm in diameter were made with a sterile cork borer, portions of 50 μL of each untreated and treated CFS were added. The plates were left undisturbed for two hours and incubated for 24 h at 37°C, when the inhibition zones were measured.

Co-culture assay

Co-culture assay is a method for determination of antimicrobial effect of probiotic lactobacilli (28). Bacteria, 10⁵ CFU/mL of Lactobacillus and 10⁴ CFU/mL of S. Typhimurium (a ratio of 10:1), were co-incubated in BHI broth for 24 h at 37°C. The control was the monoculture of S. Typhimurium. The number of Salmonella was determined by plate counting on LB agar. Experiments were carried out in triplicates. The inhibition was calculated using the following equation:

\[
\text{Inhibition} = \left(\frac{N(\text{bacteria})}{N(\text{bacteria})} \times \frac{V(\text{control})}{V(\text{co-culture})}\right) \times 100 / \%
\]

Adhesion assay

Adhesion of L. plantarum strains to Caco-2 cells

The adhesion of tested Lactobacillus strains was examined by adding bacterial suspension (10⁶ CFU per well) to Caco-2 monolayers at the multiplicity of infection (MOI) of 1:1000. Each plate with Caco-2 cells was centrifuged at 240×g for 5 min, followed by 2 h of incubation at 37°C. After the incubation, the Caco-2 monolayers were washed three times with sterile phosphate-buffered saline (PBS) (pH=7.2) and treated with 0.05 % Triton X-100. The number of bacteria was determined by plating the 10-fold dilution on LB agar. The Trypan Blue (Sigma-Aldrich Chemie GmbH) test was used to examine the Caco-2 cell viability throughout the adhesion studies.

Statistical analysis

The data were analysed using STATISTICA commercial software, v. 12.0 (StatSoft, Tulsa, OK, USA). Results are expressed as mean value ± standard deviation (S.D.). Normality of the data distribution was assessed by the Kolmogorov-Smirnov normality test. The distribution qualified the normality test, so nonparametric tests were applied. Differences between groups of samples were analysed by the Kruskal-Wallis ANOVA on ranks test, while the influence of L. plantarum strains on Salmonella Typhimurium was tested by Mann-Whitney U test. Differences with p<0.05 were considered to be statistically significant.

Results

General characterisation of L. plantarum strains

Observed carbohydrate fermentation patterns confirmed the species identification according to the API system database with 99.5–99.9 % ID similarity (data not shown). MALDI-TOF MS analysis presented specific spectra allowing discrimination between closely related species and classification at subspecies level. Comparability of the match to the reference is expressed from 0 (no match) to 1000 (perfect identity) and converted into log score in the range of 0–3. L. plantarum A strain gave scores of 2.443, L. plantarum B strain of 2.448 and L. plantarum S1 strain of 2.403. Applying the recommended criteria, score 2.3 indicates highly probable L. plantarum species identification.

All tested lactobacilli were capable of growing in BHI broth containing 1 % bile salts, despite the lower growth
rate than the growth in plain BHI broth. All three \textit{L. plantarum} strains were able to reduce pH in the BHI and MRS broths to pH of approx. 4. The strains were also metabolically active in BHI broth with 1\% bile salts, reducing the pH to around 6.0, compared to control BHI where pH was adjusted to 7.2 (Table 1).

Table 1. Growth characteristics of \textit{Lactobacillus plantarum} isolates

<table>
<thead>
<tr>
<th>Lactobacillus plantarum</th>
<th>(A_{600})</th>
<th>Strain A</th>
<th>Strain B</th>
<th>Strain S1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI broth + 1% bile salts</td>
<td>1.5*</td>
<td>2.14*</td>
<td>3.59*</td>
<td></td>
</tr>
<tr>
<td>Initial pH</td>
<td>Medium pH after 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHI broth</td>
<td>7.2</td>
<td>(4.2±0.1)*</td>
<td>(4.1±0.1)*</td>
<td>(4.1±0.1)*</td>
</tr>
<tr>
<td>MRS broth</td>
<td>6.5</td>
<td>(3.9±0.1)*</td>
<td>(3.9±0.1)*</td>
<td>(3.9±0.1)*</td>
</tr>
<tr>
<td>BHI broth + 1% bile salts</td>
<td>7.2</td>
<td>6.1±0.1</td>
<td>6.1±0.1</td>
<td>6.1±0.1</td>
</tr>
</tbody>
</table>

Asterisk indicates differences between strains A, B and S1 and the control at p<0.05

### Functional characterisation of \textit{L. plantarum} strains

The antibacterial effect of CFS of each \textit{L. plantarum} strain on the \textit{S. Typhimurium} was tested by the agar well diffusion assay. There were no differences in the diameter of growth inhibition zones among the tested \textit{Lactobacillus} strains and all strains showed anti-\textit{Salmonella} activity (Table 2). Moreover, the antibacterial activity of CFS was evaluated after neutralisation to pH=7.0 and heat treatment. Neutralisation of the pH resulted in a complete loss of antibacterial activity of lactobacilli, while heat treatment had almost no impact on the inhibitory effect of CFS (Table 2).

Furthermore, the effect of \textit{L. plantarum} strains on \textit{S. Typhimurium} growth in BHI was examined. All tested \textit{L. plantarum} strains inhibited \textit{S. Typhimurium} growth (p<0.05) at all time points (6, 12 and 24 h) in co-culture assay in BHI broth and there were no statistically significant differences among the strains (Fig. 1). After 24 h of co-cultivation, the number of \textit{Salmonella} cells was reduced 1000 times in comparison with \textit{Salmonella} monoculture. The inhibition was most pronounced after 12 h of co-incubation and amounted to 97, 98 and 94\% by strains A, B and S1, respectively.

Table 2. Antibacterial activity of the cell-free supernatants (CFS) of \textit{Lactobacillus plantarum} strains against \textit{S. Typhimurium}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Zone of inhibition/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>8.0±0.0</td>
</tr>
<tr>
<td>pH=7.0</td>
<td>5.0±0.0</td>
</tr>
<tr>
<td>heat treatment (5 min at 100 °C)</td>
<td>7.6±0.6</td>
</tr>
</tbody>
</table>

The adhesive properties of \textit{L. plantarum} strains were examined on human enterocyte cell line, Caco-2 cells. The results showed that after 2 h, strains A and S1 showed higher adhesion ability to Caco-2 cells (approx. 10^7 and 10^8 CFU/mL, respectively), than strain B (10^7 CFU/mL) (Fig. 2). The number of adhered \textit{L. plantarum} strains A and S1 per eukaryotic cell was 5 to 10, and of strain B 1 to 5 (Fig. 2).

The same model was used to test the adhesion of pre-treated \textit{S. Typhimurium} or simultaneously incubated \textit{S. Typhimurium} with \textit{Lactobacillus} strains to Caco-2 cells. Results indicated that the pretreatment of \textit{L. plantarum} strain A (p=0.0045) and strain S1 (p=0.0067) significantly reduced the adhesion of \textit{Salmonella} cells to Caco-2 cells (Fig. 3), where the strain S1 was the most effective. The number of adhered \textit{Salmonella} after 2 h of simultaneous incubation with all \textit{L. plantarum} strains was significantly lower than the number of \textit{Salmonella} cells alone. There were no differences among \textit{L. plantarum} strains (Fig. 3). The comparison of the influence of \textit{Lactobacillus} strains in pretreatment and in simultaneous incubation with \textit{S. Typhimurium} showed that the simultaneous incubation with strain B was the most effective in the inhibition of \textit{Salmonella} adhesion to Caco-2 cells.
Discussion

Several representatives of Lactobacillus genus including L. plantarum species were found to exhibit probiotic activity (29). Many traditional lactic acid-fermented foods, dominantly dairy products are considered as ideal carriers for probiotic bacteria (5,30). Additionally, traditional dairy products may also be used as an ideal source for the isolation of novel probiotic lactic acid bacteria (2). However, as these bacterial strains differ in their characteristics, it is necessary to thoroughly examine their functional properties including antimicrobial activity and persistence in the gastrointestinal tract with reference to their ability to adhere to epithelial cells (31). In this study of relevant functional characteristics of three new L. plantarum strains (A, B and S1) and their activity against gut pathogen S. Typhimurium were evaluated in order to determine their probiotic potential. Candidate strains are supposed to meet the safety rules as they originate from popular types of traditional dairy products (strain A from homemade cow’s cheese, B from homemade sheep’s cheese and S1 from whey), which are commonly and widely used for food. Biochemical and phenotypic characterisation were performed confirming these are three different isolates of the same species of L. plantarum.

All three strains demonstrated the ability to reduce pH of different media, and were able to grow in the presence of bile salts. Lactobacilli ferment carbohydrates and produce high concentrations of lactic acid, which decreases the pH of their environment and enhances antimicrobial effectiveness (26).

Resistance to bile salts was evaluated under the conditions which mimic those in the gastrointestinal tract. Bacteria that are tolerant to bile salts are more promising as probiotic candidates, because it enables them to survive in the environment of small intestine (32).

In order to categorise certain bacteria as a probiotic, it is also necessary to exhibit antagonistic effect against pathogenic bacteria and adherence to the intestinal epithelium. Since Salmonella is one of the frequent causes of infection of the digestive tract (33), we decided to use it as a test microorganism for antibacterial properties of our potentially probiotic bacteria. Salmonella species are facultative intracellular bacteria, capable of invading, surviving, and often multiplying within diverse eukaryotic cell types, including epithelial and phagocytic cells (21). Adhesion to the intestinal epithelial surface is a key step in pathogenesis and is central to colonisation of the intestine (34,35).

The antimicrobial effect of CFS of all three L. plantarum strains against S. Typhimurium was first shown by well diffusion method, and the zone of inhibition ranged from 8.0 to 8.6 mm. The complete loss of their antibacterial activity was determined after neutralisation of pH, suggesting that the organic acids produced during fermentation were most probably responsible for the inhibitory effect. Furthermore, the significant acidification of the medium (pH 4.0) occurred after 24 h of incubation of L. plantarum strains in BHI broth. It seems that low pH of Lactobacillus CFS is most likely responsible for the inhibition of S. Typhymurium growth. The antimicrobial effect of organic acids has been observed when using several Lactobacillus species (26,36). The growth-inhibiting activity of different probiotic bacteria against pathogens such as Gram-negative S. Typhimurium, E. coli or Campylobacter jejuni, and Gram-positive bacteria Enterococcus faecalis and Clostridium difficile was attributed to a pH reduction and/or to the production of organic acids (37). According to Millette et al. (38) antimicrobial effect of lactobacilli in co-culture with pathogenic bacteria is mainly due to production of organic acids, which results in pH reduction, although they can produce some other substances as well.

The capability of L. plantarum isolates to inhibit the growth of Salmonella was evaluated in co-cultivation experiments in vitro. An inhibitory effect of all tested L. plantarum strains, regardless of the length of incubation, was shown. The results of the present study indicate that the inhibitory activity of all three Lactobacillus strains was mostly due to the pH effect, which is even more pronounced in Salmonella because they are sensitive to low pH. Besides the production of different inhibitory molecules during co-cultivation, lactobacilli have the ability to coaggregate with numerous pathogens, inhibiting the overgrowth and proliferation of pathogenic microorganisms (39). We have previously shown that the same three strains of L. plantarum are capable of high autoaggregation (≥80 %) and marked coaggregation with different enteropathogens including S. Typhimurium (around 20 %) (40). It can be assumed that due to very close proximity of both bacteria, antimicrobial substances released by lactobacilli can directly inhibit Salmonella.

Further important characteristics of probiotics are their own adherence to intestinal epithelial cells, as well as the prevention of the adhesion of pathogenic bacteria (41,42). Adherence of probiotic bacteria to the intestinal epithelium is an important characteristic as it promotes colonisation and long-time persistence. Our results showed that all strains were capable of adhering to Caco-2 cells, although L. plantarum strains A and S1 had better adhesive properties.

To test the influence of lactobacilli on Salmonella adhesion to Caco-2 cells, two types of experiments were done: pretreatment (L. plantarum was incubated with Caco-2 cells first, and then Salmonella was added), and co-incuba-
tion (both bacteria were added to Caco-2 cells simultaneously). In order to exclude the influence of the pH, to which Caco-2 cells are extremely sensitive, bacteria were prepared in DMEM medium supplemented with 10% FCS. The pH of the DMEM medium was monitored during each experiment and it was stable (7.2–7.5). Also, the viability of Caco-2 cells was tested during experiments and the percentage of survival was over 90% (data not shown). The results showed that all Lactobacillus strains interfered with S. Typhimurium adhesion to Caco-2 cells regardless of whether the Caco-2 cell were pretreated or co-incubated with the tested strains. Most probably Lactobacillus, similarly to Salmonella, uses receptor-type adhesion mechanism on Caco-2 cells and thus significantly attenuates its adhesion. The number of adhered Salmonella in the presence of any of the three Lactobacillus strains was significantly lower after the pretreatment (approx. 10^4 CFU per well) or after co-incubation (approx. 10^5 CFU/ well) than the number of Salmonella alone (approx. 10^6 CFU per well).

Jankowska et al. (43) have shown that the presence of L. paracasei affected S. enterica adhesion to Caco-2 cells (4- to 5-fold reduction), but the anti-adhesion activity was even more pronounced (8-fold) when L. paracasei was preincubated with Caco-2 cells prior to Salmonella addition. In the same study, Lactobacillus spent culture supernatants (SCS) acted as weaker inhibitors of Salmonella adhesion. In contrast, Lehto and Salminen (26) showed that only SCS of Lactobacillus strain GG, and not bacteria alone inhibited the adhesion of S. Typhimurium to Caco-2 cells and that the inhibition of SCS was most likely because of low pH. In our study, the influence of SCS of L. plantarum strains was not further tested because of the weak viability of Caco-2 cells in a preliminary experiment. We can postulate that inhibition of Salmonella adherence by all three Lactobacillus strains was because of the competition for eukaryotic cell receptors, although it may also involve some of produced or secreted antimicrobial compounds.

Conclusion

The results presented in this study demonstrated that all three Lactobacillus plantarum strains obtained from traditional dairy products exhibited favourable probiotic characteristics like the ability to grow in the presence of bile salts and good adhesion to Caco-2 cells, as well as good antibacterial activity and the ability to compete with Salmonella Typhimurium for adhesion sites on Caco-2 cells. Our results indicate that L. plantarum A, B and S1 strains are promising candidates with probiotic properties.

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