



EXOPOLYSACCHARIDES MAY INCREASE GASTROINTESTINAL STRESS TOLERANCE OF *LACTOBACILLUS REUTERI*

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ABSTRACT

This study investigated a possible relationship between exopolysaccharides (EPS) production and the resistance to bile salts and low pH in intestinal strains of *Lactobacillus reuteri*. The strains displayed a mucoid phenotype, when grown in the presence of 10 % sucrose. Scanning electron microscopy (SEM) revealed strands of exopolysaccharide linking neighbouring cells. The strains (except *L. reuteri* B1/1) produced EPS in the range from 15.80 to 650.70 mg.l⁻¹. The strains were tested for tolerance to bile salts (0.15; 0.3 %) and low pH (1.5–2.0–2.5–3.0). The survival rate, after the treatment with artificial gastric and intestinal juices, was determined by flow cytometric analysis. The strains of *L. reuteri* that produced 121–650 mg.l⁻¹ of EPS showed a significantly higher tolerance ($P < 0.001$) to the gastric juice at pH 3 and 2.5, throughout the entire exposure time, in comparison to the strains that produced less than 20 mg.l⁻¹ of EPS. *L. reuteri* L26, with the highest production of EPS, exhibited the highest survival rate (60 %) at pH 2 after the 120 minutes of in-

cubation and was able to tolerate pH 1.5 for 30 minutes. Higher production of EPS significantly ($P < 0.001$) increased the strains' tolerance against the intestinal juice in the presence of 0.15 and 0.3 % bile salts and was time dependent. *L. reuteri* L26 showed the highest tolerance ($P < 0.001$) against 0.3 % bile salts. This investigation revealed a positive correlation between the EPS production and the resistance of intestinal *L. reuteri* to the stress conditions of the gastrointestinal tract (GIT).

Key words: exopolysaccharides; gastric juice; intestinal juice; *Lactobacillus reuteri*; probiotic; resistance

INTRODUCTION

Lactobacilli, the major group of lactic acid bacteria (LAB), are the most commonly studied probiotics because of their strain-specific properties that are beneficial to health. Several studies have defined their mechanism of action based on: the modification of the immune system,

harmonization of local microenvironment and strengthening of the intestinal barrier through the production of antimicrobial agents, competition for nutrients and the inhibition of the adhesion of pathogenic bacteria [7, 19, 32].

According to the probiotic definition, a prerequisite of probiotic bacteria is that they need to reach the target location within the host, i. e., the gastrointestinal tract, in sufficiently high numbers to exert their healthful advantages [28]. The main factors to be considered that influence the viability of probiotics in the gastrointestinal tract (GIT) conditions are: very low pH in the stomach, bile salts and gastro-enzymes in the small intestine, lysozyme in saliva, and the colonic environments [29]. Even if bacteria can survive these stresses, exposure to such conditions can affect their probiotic properties. Various studies suggest synergistic as well as adverse relationships between the influence of gastrointestinal stress and the functional properties of probiotic strains [3]. Bacteria are equipped with several mechanisms to cope with hostile environments. These include: regulatory chaperone proteins, that act to repair proteins and DNA damage, proton translocation (extrusion) by the F1F0-ATPase, bile efflux pumps, changes in the energetic metabolism, decarboxylases and transporters to combat decreases in intracellular pH, bile salt hydrolase, changes in the lipid composition of the cell membrane and alteration of cell surface by production of exopolysaccharides [4, 26, 37, 39, 42, 45].

Many LAB, including *Lactobacillus*, have the ability to synthesize long chains of homopolysaccharides or heteropolysaccharides, consisting of (branched) repeating units of sugars or sugar derivatives, which may be substituted with various chemical moieties [35, 47]. Naturally, the bacterial exopolysaccharides (EPS) have a protective function within the natural environment. The EPS may protect bacteria against desiccation, phagocytosis, phage attack, antibiotics or toxic compounds and osmotic stress [17, 33]. Exopolysaccharides create condition for the initial steps during the colonization of abiotic and biotic surfaces and long-term attachment of biofilms [31]. In the gastrointestinal tract, biofilms could facilitate and promote the colonization and persistence of beneficial strains, which allow the expression of the probiotic properties [20]. Moreover, EPS exerts a blocking of specific receptors of the host cell membrane by competing and inhibiting the formation of biofilms by pathogenic bacteria [8, 12, 21]. The occurrence of EPS-producing LAB strains in the digestive tract could

indicate the importance of EPS production for adaptation of these bacteria in this type of stress conditions [18, 43].

The present study investigates the relationship between the EPS production and the resistance of intestinal *Lactobacillus reuteri* to bile salts and low pH. Artificial gastric as well as small intestinal juices were used to create conditions similar to the *in vivo* conditions.

MATERIALS AND METHODS

Bacterial strains

The EPS producing strains of lactobacilli, based on mucoid phenotype criteria observed in modified De Man-Rogosa-Sharpe agar (MRS; Carl Roth GmbH + CO. KG, Karlsruhe, Germany) containing 10 % sucrose (Mikrochem, Pezinok, Slovakia) at 37 °C, were isolated in our laboratory from the gut contents of healthy suckling piglets and pheasants. *Lactobacilli* were incubated anaerobically (Gas Pak Plus, BBL Microbiology systems, Cockeysville, Maryland, USA) at 37 °C for 48 h. In a previous study [38] the strains were identified by matrix-assisted laser desorption/ionization — time of flight mass spectrometry (MALDI-TOF MS) as *Lactobacillus reuteri* L26, B6/1, 2/5, 2/6, B1/1, 2/3, 4/2, 10/1. The strain *Lactobacillus reuteri* L26 was deposited in the Czech Collection of Microorganisms (CCM) as *Lactobacillus reuteri* L26 Biocenol™ CCM 8616.

Isolation and quantification of EPS

A semi-defined medium (SDM) [22], with low amounts of EPS equivalent ingredients, was used for the EPS culture from isolated lactobacilli strains. The SDM consisted of (g.l⁻¹): 10 g Bacto casitone, 5 g yeast nitrogen base, 1 g polysorbate 80.2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g dipotassium phosphate. Sucrose (10 % m/v) was used as the carbon source. After the inoculation (2 % v/v), samples were incubated for 24 h at 37 °C. The lactobacilli cultures were heated at 100 °C for 15 min to denature EPS-degrading enzymes and the cells were removed by centrifugation (9000 × g for 20 min, 4 °C). The EPS were isolated according to the method of Sims et al. [40]. Briefly, EPS along with proteins were precipitated with cold absolute ethanol at 4 °C and then dialyzed by using membrane tubing with a molecular weight cutoff of 12,000 to 14,000 (D-Tube™ Dialyzer, Merck) at 4 °C for 3 days. The proteins were

treated with proteinase K (6 mg.ml⁻¹, 37 °C, 24 h; Sigma-Aldrich, St. Louis, USA) and precipitated using 14 % (v/v) of 85 % trichloroacetic acid solution for 1 h on ice. Supernatant obtained by centrifugation (14.000 × g, 10 min, 4 °C) was dialyzed against water (4 °C, 72 h) to remove salts and other components in order to obtain pure EPS. Finally, the dialysates were freeze-dried and weighed. The total amount of carbohydrate (mg.l⁻¹) in the EPS was assessed using the phenol-sulfuric acid method [16] with glucose as the standard. The protein content was analyzed by Bradford method [10] with bovine serum albumin as a standard. The results are expressed as a mean from three independent experiments ± standard deviation.

Preparation of simulated gastric and small intestinal juice

Simulated sterile gastric juice and small intestinal juice was prepared with some modifications according to K o s et al. [23]. Gastric juice was prepared by resuspending pepsin (3 g.l⁻¹) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.4 mM Na₂HPO₄, 1.2 mM KH₂PO₄) and adjusting the pH to 1.5, 2.0, 2.5 and 3.0 with 8 M HCl. Small intestinal juice was prepared by resuspending pancreatin (1 g.l⁻¹) and bile salts (1.5 and 3.0 mg.ml⁻¹) in PBS and adjusting the pH to 8.0 with 0.1 M NaOH. Pepsin (from porcine stomach mucosa), pancreatin (from hog pancreas, 165 U.mg⁻¹) and bile salts (50 % sodium cholate and 50 % sodium deoxycholate) were obtained from Sigma-Aldrich.

Stress tolerance assays

One milliliter of the stock culture was diluted in 10 mL of MRS broth and incubated at 37 °C for 16 h. The overnight culture was then diluted 10 times with fresh MRS broth. The subcultured cells were allowed to grow to reach the midexponential phase, corresponding to a concentration of approximately 108 cells mL (OD at 620 nm = 0.6 – 0.7). The bacterial culture was centrifuged (3800 × g for 30 min at 4 °C), the supernatant was removed, and the pellet was resuspended in artificial gastric juice or small intestinal juice. For each stress assay, samples were incubated at 37 °C with agitation during the duration of the experiment. For all stress assays, aliquots were taken at selected time points, and viable cell counts were performed by flow cytometric analysis. Untreated cells resuspended in PBS (pH 6.8) served as control samples.

Flow cytometric analysis of viability of bacteria

The viability of lactobacilli in gastric juice was measured after the staining with propidium iodide (PI; Sigma-Aldrich). The bacterial suspensions in the gastric juice in the amount of 50 µl were mixed with 3 µl of a working solution of PI (1 mg PI ml) and with 447 µl of PBS pH 7.3, filtered through a 0.22 µl syringe filter. The samples were subsequently incubated for 15 min at 37 °C. In order to eliminate the non-specific reaction between PI and bile salts [30], the viability of bacteria in the intestinal juice was assessed with carboxyfluoresceindiacetate (cFDA) staining. A stock solution of cFDA (Sigma-Aldrich) was prepared and stored as described by Ben A m o r et al. [6]. The bacterial suspensions in the intestinal juice in the amount of 50 µl were mixed with 5 µl of 1 mM solution of cFDA and with 445 µl of PBS containing 1 mM dithiothreitol (Sigma-Aldrich). The samples were incubated for 30 min at 37 °C. Flow cytometric assessment of the viability of the lactobacilli was performed on a BD FACSCanto™ flow cytometer (Becton Dickinson Biosciences, USA) and analyzed with BD FACS Diva™ software. FSC vs. SSC dot plot was used to state the position of the bacteria. The fluorescence measurements were carried out using the 488 nm blue laser with FL-1 filter (530/30 nm) for cFDA and FL-3 filter (695/40 nm) for the PI. The numbers of viable and dead bacteria were evaluated on the basis of a SSC vs. FL-3 histograms for the PI and SSC vs. FL-1 histograms for the cFDA. All bacterial analyses were performed for 30 s at a low flow rate (10 µl.min⁻¹). The survival rate (in percentage) was calculated as follows: % survival = final (the number of viable cells)/control (the number of viable cells) × 100. The results are expressed as a mean from three replicates (three cultivations) ± standard deviation.

Statistical analysis

The data were analyzed with GraphPadPrism version 3.00 (GraphPad Software, San Diego California USA, www.graphpad.com.) by one-way analysis variance (ANOVA) followed by Tukey's multiple comparison test. Values of P < 0.05 were considered significant.

RESULTS AND DISCUSSION

EPS are high-molecular-weight sugar polymers, which can be either covalently associated with the bacterial cell

surface forming a capsule, or secreted into the environment of the cell in the form of slime or ropy EPS [11]. In this study, the EPS production by the intestinal *L. reuteri* isolated from the gut contents of piglets and pheasants was determined. The strains displayed non-ropy (slime), glistening colonies characteristic of exopolysaccharide production, when cultured on solid MRS medium supplemented with 10 % sucrose for 48 h at 37 °C anaerobically (Figure 1). Sucrose appears to be the best energy source for various lactobacilli [44], because the EPS are synthesized from sucrose by a single action of extracellular glucosyltransferases and fructosyltransferases enzymes [13]. The scanning electron microscopy (SEM) image indicated that the neighbouring cells were linked by strands of the exopolysaccharide (Figure 2). Our strains of *L. reuteri* produced exopolysac-

charides ranging from 15.80 to 650.70 mg.l⁻¹ when grown in a semi-defined medium with low amounts of EPS equivalent ingredients supplemented with 10 % (m/v) sucrose for 48 h at 37 °C, anaerobically. As presented in Table 1, the estimated EPS production for *L. reuteri* 2/6, *L. reuteri* B6/1, *L. reuteri* 10/1 and *L. reuteri* 2/5 ranged from 121 to 267 mg.l⁻¹. Under the same conditions, *L. reuteri* 2/3 and *L. reuteri* 4/2 produced low EPS levels (less than 20 mg.l⁻¹). Strain *L. reuteri* B1/1 showed no phenotypic expression of EPS formation on MRS medium with high sucrose content and EPS production was not detected. The strain *L. reuteri* L26 demonstrated the highest production of the EPS 650.70 mg.l⁻¹. The abundant literature validates, that the amount of EPS depends on the carbon and the nitrogen sources and physico-chemical conditions for bacterial growth as determined by temperature, pH, oxygen rate, etc. Generally, the yield of production is under 1 g.l⁻¹ for HoPSs when the culture conditions are not optimized [5].

The results of the recent studies demonstrated that EPS can play a role in the resistance to stress conditions by lactobacilli and bifidobacteria. The physical barrier created by the EPS on the cell surface is probably the mechanism by which EPS producer is protected from adverse environmental conditions [34]. Donoghue and Newman [15] concluded, that EPS affected the susceptibility of the

Table 1. The EPS production by strains of *L. reuteri*

Strains	EPS [mg.l ⁻¹]
<i>L. reuteri</i> L26	650.70 ± 1.00
<i>L. reuteri</i> 2/6	267.95 ± 1.00
<i>L. reuteri</i> B6/1	158.70 ± 0.95
<i>L. reuteri</i> 10/1	130.20 ± 0.97
<i>L. reuteri</i> 2/5	121.20 ± 1.01
<i>L. reuteri</i> 2/3	18.20 ± 0.97
<i>L. reuteri</i> 4/2	15.80 ± 1.09
<i>L. reuteri</i> B1/1	not detected

Each value in the table is the mean ± standard deviation of three trials



Fig. 1. Phenotypic expression of the EPS production by strain *L. reuteri* L26. The strain was grown overnight on MRS agar supplemented with 10 % sucrose

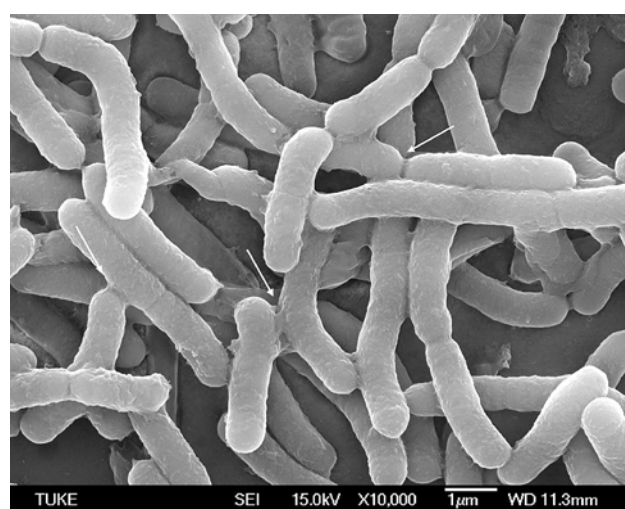


Fig. 2. SEM analysis of the EPS produced by strain *L. reuteri* L26. Arrows indicate EPS production. The strain was grown overnight in MRS medium supplemented with 10 % sucrose and subsequently processed by SEM analysis as described by Kubota et al. [25]. The sample was mounted in a brass holder and covered with a gold layer by using a metallizer (JEOL JFC-1100). The image was taken by JEOL JSM-7000F scanning electron microscope (Magn. ×10,000; high vacuum [HV], 15.0 kV; working distance [WD], 11.3 mm)

cells to acidic pH by virtue of anions associated with the EPS which could restrict the access of acids to the bacterial cells. The EPS producing strain *Lactobacillus mucosae* DPC 6426 exhibited a threefold increased survival during 90-min exposure to 0.7 % bile; a threefold increased survival when exposed to simulated gastric juice for 10 min; and a fivefold increased survival during a 60-min exposure to HCl compared with EPS non-producing *L. mucosae* DPC 6420 [27]. S t a c k et al. [41] reported the ability of beta-glucan-producing *Lactobacillus paracasei* NFBC 338 to survive in simulated gastric juice by 15-fold and its ability to survive in bile by 5.5 fold, compared to the control strain. Increased expression of gtf 01207, involved in EPS production, was observed in *Bifidobacterium animalis* subsp. lactis after the exposure to acid, bile and osmotic stresses [2, 36].

Our results revealed a correlation between the EPS production and tolerance to low pH and bile salts in the intestinal strains of *L. reuteri*. The time dependent survival of lactobacilli in gastric juice at pH 3; 2.5; 2 and 1.5 is shown in Figure 3. During this analysis, we considered

the fact that the passage through the GIT itself may be affected by various factors that could lead to the shorter or extended periods of bacterial presence in the particular GIT sections. As a result, bacteria are exposed to the aggressive GIT environment for a different period. This factor significantly affects lactobacilli survival. Bacterial strains with low EPS production (*L. reuteri* 4/2, *L. reuteri* 2/3) and without EPS production (*L. reuteri* B1/1) were sensitive to the gastric juices ($P < 0.001$) in comparison with the strains with higher EPS production. Incubation of bacteria for the period of 30 minutes at pH 3; 2.5; 2 and 1.5 caused more than 70 % mortality. Bacterial resistance against gastric juices was time dependent. The survival rate after 120 minutes of incubation was 0–16 %. On the contrary, bacterial strains with high EPS production such as *L. reuteri* L26, *L. reuteri* 2/6, *L. reuteri* 2/5, *L. reuteri* 10/1 and *L. reuteri* B6/1 presented more than 65 % survival rate ($P < 0.001$) in gastric juice at pH 3 and 2.5 in comparison with the strains with low EPS production and the non-EPS-producing strains. However, the lower pH acidity (2 and 1.5) affected

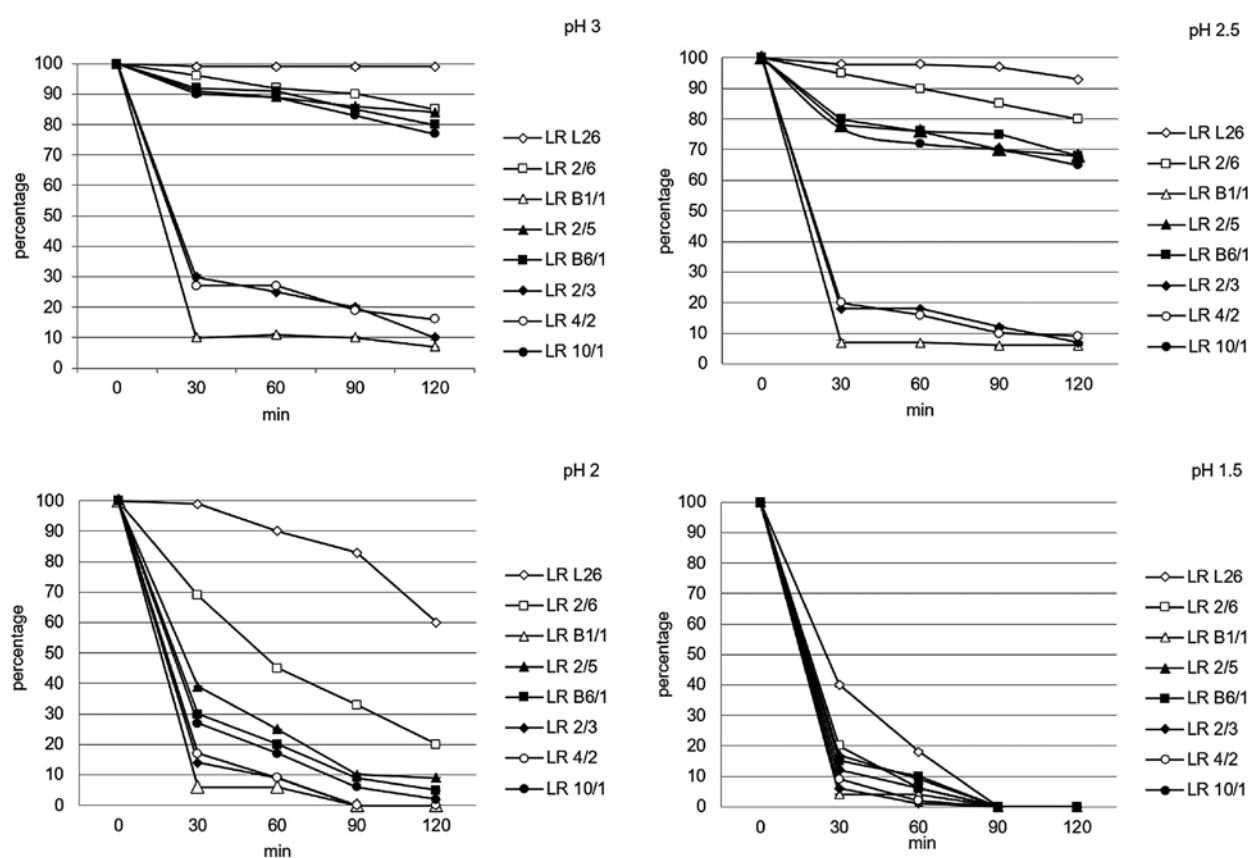


Fig. 3. Resistance of *L. reuteri* strains to artificial gastric juice tested at different pH. $P < 0.0001$. Data are expressed as percentage of bacterial survival. Values represent the means of triplicate measurements. Standard deviations are in the range from 0 to 0.82, therefore they are not visible in the figure

the bacterial survival rate to some extent. When compared with all bacterial strains, *L. reuteri* L26 showed the highest survival rate ($P < 0.001$) at pH 2. Its viability during the entire exposition time was in the range 60–100 %. The stronger effect of a lower pH on bacterial viability was detected at pH 1.5. Nevertheless, the *L. reuteri* L26 strain was able to tolerate this low pH environment for 30 minutes. Its viability was still about 40 %.

The effect of the incubation time on the viability of EPS producing lactobacilli strains was also examined in small intestinal juices at bile salts concentrations of 1.5 and 3 mg.ml⁻¹ (Figure 4). When compared with the strains with low EPS production and *L. reuteri* B1/1, high EPS producing strains such as *L. reuteri* L26, *L. reuteri* 2/6, *L. reuteri* 2/5, *L. reuteri* 10/1 and *L. reuteri* B6/1 showed an increased resistance ($P < 0.001$) against the small intestinal juice at 0.15 % concentration of bile salts. Bacterial resistance faded away over time and after the four hours of lactobacilli incubation the survival rate was 37–50 %. The only exception was strain *L. reuteri* L26 that showed

the most robust resistance ($P < 0.001$). When compared with the other bacterial strains the viability of *L. reuteri* L26, followed by four hour incubations, was 80 %. *Lactobacilli* strains with low EPS production (*L. reuteri* 4/2, *L. reuteri* 2/3) and non-EPS-producing strain (*L. reuteri* B1/1) were considerably sensitive to the small intestinal juice at the 0.15 % concentration of bile salts. One-hour exposure caused more than 75 % mortality. Strains producing high amounts of EPS survived even with the 0.3 % concentration of bile salts. The survival rate after one-hour of incubation was between 36–73 %. The *Lactobacilli* strain resistance was time dependent. When compared with the other strains, the highest resistance ($P < 0.001$) during the entire time of incubation was detected in *L. reuteri* L26. A one-hour exposure to the small intestinal juice at the 0.3 % concentration of bile salts in strains *L. reuteri* 4/2, *L. reuteri* 2/3 and *L. reuteri* B1/1, leads to almost 100 % mortality.

Based on our results, we determined that not only EPS formation but also the amount of EPS could significantly affect the ability of *L. reuteri* to survive in the harsh con-

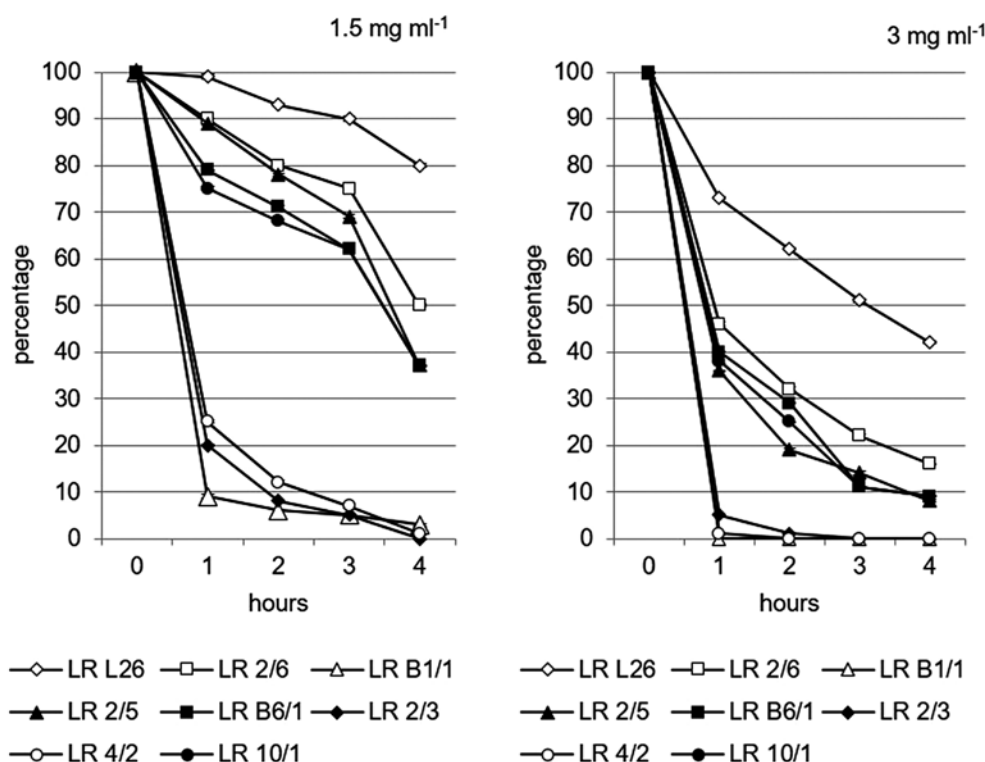


Fig. 4. Resistance of *L. reuteri* strains to the artificial small intestinal juice at 1.5 and 3.0 mg.ml⁻¹ bile salts' concentrations. $P < 0.0001$. Data are expressed as percentage survival. Values represent the means of triplicate measurements. Standard deviations are in the range from 0 to 0.49, therefore they are not visible in the figure

ditions of the GIT. This is in accordance with a similar study that investigated the relationship between the quantity of EPS and low pH or bile salts tolerance in yogurt isolates [9]. Highly producing EPS strains of *Lactobacillus delbruckii* subsp. *bulgaricus* B3, G12 and *Streptococcus thermophilus* W22 demonstrated a higher resistance to low pH 2 and 0.3 % bile than strains with low EPS production. Similarly, in bifidobacteria isolated from infants' faeces and breast milk, the positive correlation between the quantity of EPS production and resistance to bile salts or low pH was detected. The strains of bifidobacteria that produced high levels of EPS showed a higher tolerance to acid and bile [1]. Natural mutants of *Lactobacillus johnsonii* FI9785 with altered EPS profiles showed different survival rates under stressed conditions. Mutants with increased EPS production showed an improved resistance to antimicrobials and acid shock in comparison with wild type levels of EPS. On the other hand, a reduction in the EPS production made the bacteria less able to survive in the presence of 0.3 % bile salts, antimicrobials and heat shock, but significantly increased autoaggregation and bacterial adhesion; important factors for bacterial colonisation of the intestine [14]. Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *L. reuteri* TMW1.106 have been shown to contribute to cell aggregation and *in vitro* biofilm formation and colonization of the mouse GIT. *In vitro* experiments on isogenic mutants revealed that GtfA was essential for the sucrose-dependent autoaggregation of *L. reuteri* TMW1.106 cells under acidic conditions, while inactivation of Inu slowed the formation of cell aggregates. The deletion of EPS synthetic genes impaired both colonisation and competition [46].

CONCLUSIONS

The survival of the probiotic bacteria during transit to the site of colonisation is an important issue. This study investigated a possible relation between the EPS production and the resistance of intestinal *L. reuteri* within the GIT. A relationship could be seen between the quantity of EPS production and resistance to the artificial GIT conditions. *L. reuteri* L26 Bioce-nol™ demonstrated the highest production of the EPS and exhibited the highest survival rate when challenged with gastric and intestinal juices in *in vitro* conditions. In our previous study it was found, that this strain showed in *in vitro* conditions inhibitory activ-

ity against pathogenic strains, sensitivity to antibiotics, biofilm formation, production of acids and produced an α -D-glucan-type branched polysaccharide with (1→3) and (1→6) glycosidic linkages with molecular weight 8.2×10^5 Da [38, 24]. Further studies on establishing the role of EPS in survival and colonisation of strain *L. reuteri* L26 Bioce-nol™ in *in vivo* conditions of the GIT are needed.

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