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ANTIADHESIVE EFFECTS OF EXOPOLYSACCHARIDES ISOLATED FROM *LIMOSILACTOBACILLUS REUTERI*

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ABSTRACT

Probiotics with their biological properties and products contribute to optimal functioning of organ systems and form a natural barrier against the growth of undesirable microorganisms. Exopolysaccharides (EPS) isolated from selected strains of Lactobacilli are tested for their antiadhesive, anti-biofilm and other beneficial effects. EPS isolated from the SL16 strain of *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) significantly reduced adhesion of *Staphylococcus aureus* CCM 3953 and *Proteus mirabilis* CCM 7188, and this effect was directly proportional to EPS concentration and stronger against *S. aureus* CCM 3953. Achieved results indicated an anti-biofilm potential of EPS obtained from intestinal Lactobacilli, probably attributable to antiadhesive activity of the tested EPS. Further studies will be required in order to clarify a mechanism of the anti-biofilm effect at the molecular level. Within the research into innovative anti-biofilm agents (biodegradable and non-toxic), the results obtained may facilitate the development of more efficient procedures or preparations that will be used in pharmacotherapy.

Keywords: *Lactobacillus reuteri*, *Staphylococcus aureus*, *Proteus mirabilis*, lactic acid bacteria, exopolysaccharide, antiadhesive effect

INTRODUCTION

Probiotic bacteria are extremely active producers of biologically active substances, including those that are currently being paid great attention bacteriocins, biosurfactants, K2 vitamin and exopolysaccharides for their significant multiple effects on a macroorganisms **(Fedorová *et al.*, 2018, 2019)**. In addition to their technological benefits, such as improving the properties of fermented foods (viscosity and flexibility), natural thickeners, emulsifiers, gel-forming agents, physical stabilisers etc., exopolysaccharides (EPS) synthesised by lactic acid bacteria (LAB) also have beneficial physiological effects on health, primarily the antitumour, antiulcer, immunomodulatory, antiviral and antioxidant activities, as well as their ability to reduce cholesterol **levels (Zhang *et al.*, 2011; Kongo *et al.*, 2013; Zawistowska-Rojek *et al.*, 2016; Oleksy *et al.*, 2018; Nishimura, 2020; Jurášková *et al.*, 2022)**.

The *Limosilactobacillus* genus (formerly *Lactobacillus*) is the largest genus of LAB, including *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) which is currently an intensively studied probiotic bacterium **(Slížová *et al.*, 2015; Nemcová *et al.*, 2017; Fedorová *et al.*, 2018; Dailin *et al.*, 2022; Ragan *et al.*, 2022; Schwaiger *et al.*, 2023; Vitale *et al.*, 2023; Ananda *et al.*, 2024)**.

Study of **Luo *et al.* (2023)** summarizes the effects of *L. reuteri* on intestinal flora and immune regulation, and discusses the feasibility of its application in atopic dermatitis, asthma, necrotizing enterocolitis, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, and provides insights for the prevention and treatment of immune-related diseases.

Most bacterial species form biofilms during their growth in the environment. A biofilm is one of the factors of bacterial virulence; it contributes not only to adhesion of bacteria inducing infections on multiple types of surfaces, but also to resistance of bacteria to antimicrobial and antiseptic agents. Biofilm formation represents a significant challenge in many aspects, including industrial corrosion and biological contamination, as well as chronic and nosocomial infections. Inhibitors of bacterial biofilm formation should therefore be beneficial in prevention of infections **(Francolini and Donelli, 2010; López *et al.*, 2010; Høiby *et al.*, 2011; Suzuki *et al.*, 2015)**. It has been known that persistence of staphylococcal infections associated with foreign objects is caused by biofilm formation. Bacteria in biofilms communicate via molecules, which activate some genes responsible for production of virulence factors and, to some extent, for a biofilm structure **(Høiby *et al.*, 2011)**. At presents, biofilms are recognised to participate in more than 80% of chronic infections caused by bacteria, including various forms of otitis, endocarditis, urinary and pulmonary infections and infections in patients with cystic fibrosis **(Hall-Stoodley and Stoodley, 2009; García *et al.*, 2014)**.

60%–85% of all microbial infections are caused by bacteria that form biofilms on natural tissues (skin, mucosa, endothelium, epithelium, teeth, bones) or artificial materials (venous, peritoneal and urinary catheters, dental materials, heart valves, intrauterine contraceptives, contact lenses and various types of implants) **(Francolini and Donelli, 2010)**.

Bacterial EPS mediate the interactions that are necessary for the formation, integrity and stabilisation of bacterial biofilms. Many studies have shown that some bacterial polysaccharides inhibit formation of biofilms in a wide range of bacteria and fungi, *in vitro* as well as *in vivo* **(Kim *et al.*, 2009; Lutay *et al.*, 2011; Rendueles *et al.*, 2013)**. It has been proved that bacterial interference mediated by signalling molecules and surfactants has an antagonistic effect on biofilm formation; this indicates that non-antibiotic molecules produced during competitive interactions between bacteria might be used to reduce biofilm formation **(Valle *et al.*, 2006)**. Antibacterial and anti-biofilm activity of *L. reuteri* EPS against some oral bacteria, such as *Staphylococcus aureus* and others, was investigated in a study by **Abed *et al.* (2022)**, who also pointed out a difference in the EPS structure, which plays a key role in their efficacy. Exopolysaccharides of *L. reuteri* SJ-47 strain protect human skin fibroblasts under UVA radiation; EPS can increase the antioxidant capacity of cells, decrease the amount of reactive-oxygen species and malondialdehyde, while improve the expression of antioxidant enzymes **(Zhao *et al.*, 2022)**. The probiotic strain *L. reuteri* L26 and its exopolysaccharide affect porcine intestinal-epithelial cells IPEC-J2 infected with *Salmonella* Typhimurium **(Kiššová *et al.*, 2022)**.

S. aureus is a Gram-positive bacterium responsible for most hospital-acquired (nosocomial) and community-acquired infections worldwide. **Pan *et al.* (2023)**

describe systematic screening and evaluation of protective effects of engineered *Lactobacillus* against *S. aureus* infection in terms of different delivery vehicle strains and *S. aureus* antigens and in localized and systemic infection models.
The emergence of biofilm-forming, multi-drug-resistant *Proteus mirabilis* infections is a serious threat that necessitates non-antibiotic therapies. *L. reuteri* exhibited antimicrobial and antibiofilm activities and could be utilized to combat Proteus-associated urinary tract infections **(Shaaban *et al.*, 2020)**.
The purpose of this study was to investigate the antiadhesive activity of EPS isolated from *L. reuteri* SL16 against two microbial strains (*S. aureus* CCM 3953 and *P. mirabilis* CCM 7188).

MATERIALS AND METHODS

Lactobacillus strain *L. reuteri* SL16 obtained from hen faeces was tested. *S. aureus* CCM 3953 and *P. mirabilis* CCM 7188 were used as potentially pathogenic, and pathogenic (indicator) strains. Compositions of the used culture media are presented in Table 1.

Table 1 Compositions of the used culture media

Culture Media	Components
PYG broth (pH 6.9)	Bacteriological Peptone 5 g; Enzymatic Casein Hydrolysate 5 g; Yeast Extract 10 g; Glucose 10 g; 1000 ml Distilled Water
SDM (semi-defined medium) enriched with 10% Saccharose (pH 7)	Bacteriological Peptone 10 g (Roth, Germany); Yeast Nitrogen Base 5 g (Sigma-Aldrich, USA); Tween 80 (Acros, USA) 4 ml; Trisodium Citrate (Merck, Germany) 2 g; Acetic Acid Sodium Salt (Merck, Germany) 5 g; $MgSO_4.H_2O$ (Roth, Nemecko) 0.1 g; $MnSO_4.H_2O$ (Roth, Germany) 0.05 g; K_2HPO_4 2 g (Sigma-Aldrich, USA); 100 g Saccharose (Centralchem, Slovakia); 1000 ml Distilled Water

Isolating EPS produced by *L. reuteri* strains

EPS were isolated by applying a modified method of extraction and precipitation proposed by Sims et al. (2011) from the *Lactobacillus* strain *L. reuteri* SL16. EPS were isolated using a semi-defined medium (SDM) with low amounts of equivalent EPS components **(Kimmel *et al.*, 1998)** enriched with 10% saccharose (a carbon source) with a pH value of 7. Following the inoculation, *Lactobacillus* strains were statically incubated in the above mentioned medium for 48 hours at 37°C.
Prior to EPS extraction, *Lactobacillus* cultures were heated for 15 minutes at 100°C to ensure denaturation of enzymes degrading EPS **(Bergmaier *et al.*, 2001)**. Subsequently, the cultures were cooled on ice and centrifuged (Rotina 420R, Eurolab LAMBDA, Germany) for 40 minutes at 4,500 rpm at 4°C (elimination of bacterial cells and debris). The obtained supernatant was then subjected to precipitation of peptides using 14% trichloroacetic acid (Sigma-Aldrich, USA) for 1 hour on ice, and subsequently centrifuged for 50 minutes at 7,500 rpm at 4°C. The procedure for isolating EPS from the supernatant comprised the addition of 2 volumes of cooled 96% ethanol (Roth, Germany) to one volume of supernatant. The mixture was then mixed and left at 4°C. On the next day, the precipitated EPS were separated by centrifugation for 30 minutes at 4,500 rpm at 4°C. The EPS sediment was resuspended in distilled water, and EPS were precipitated again by adding two volumes of 96% ethanol; this was followed by incubation overnight at 4°C and centrifugation for 30 minutes at 4,500 rpm at 4°C. In the final stage, EPS were resuspended in distilled water and the dissolved EPS were subjected to a dialysis (12,000–14,000 mw cutoff; 4°C; 72 h; D-Tube™ Dialyzer, Merck). The supernatant was then subjected to a dialysis against water (4°C; 72 hours) in order to remove salts and other components with the aim of obtaining pure EPS. At the end of the procedure, the samples were lyophilised and weighed. The same isolation procedure was applied to a non-inoculated SDM. Sterility of samples was verified by cultivating them on blood agar. The result of test was evidence of no microbial growth after incubation. A total EPS concentration (expressed in mg/l) in each tested sample was identified by spectrophotometry (SP-870 PLUS spectrophotometer, Metertech, Germany) applying the phenol-sulphuric acid method proposed by **Dubois *et al.* (1956)**. Absorbance of samples was measured at OD_{490} while glucose solution was used as a standard. Results are expressed as a mean of three independent experiments ± standard deviation. EPS production by *L. reuteri* strain SL16 in SDM medium containing 100 g/l saccharose was 168.56 ± 1.20 mg/l.

Testing antiadhesive activity of isolated EPS SL16

Antiadhesive activity of EPS isolated from *L. reuteri* SL16 against two microbial strains (*S. aureus* CCM 3953 and *P. mirabilis* CCM 7188) was measured by applying a modified method proposed by Gudiña et al. (2010). The isolated SL16 EPS were dissolved in a PBS buffer solution (pH=7.4) to prepare 10 ml of a sample of SL16 EPS with a concentration of 5 mg/ml (designated as SL16/5) and 10 ml of a sample with a concentration of 10 mg/ml (designated as SL16/10). The pH values of the prepared samples were 7.15 (SL16/5) and 7.09 (SL16/10). The samples were filtered using a microbiology filter with a pore size of 0.22 µm (Minisart®, Biotech).
Pathogenic (indicator) bacteria *S. aureus* CCM 3953 and *P. mirabilis* CCM 7188 were dissolved in 5 ml of saline solution, and an OD value was adjusted to 1.0 McFarland. Following the vortexing step, 100 µl of the suspension were added to 10 ml of filtered PYG broth and then vortexed again. 200 µl doses of both EPS samples (SL16/5 and SL16/10), representing a surface treated by exopolysaccharide, were pipetted across 96-well micro-titration plates with a flat bottom. PBS buffer solution was used as a control (a surface untreated with EPS). The filled micro-titration plates were incubated at 4°C for 18 hours. After they were taken out, the contents of the strips were poured out and twice washed with PBS buffer. Subsequently, 200 µl of standardised indicator strains, or filtered PYG broth, were added and incubated for 4 hours at 4°C. The contents of the strips were poured out, and unattached microorganisms were removed by two washes in PBS buffer. Non-adhered microorganisms were fixed using 200 µl of methanol (Sigma-Aldrich, USA) for 15 minutes at a room temperature. Subsequently, the contents of the strips were poured out and left to dry at a room temperature. This was followed by staining using 0.1% crystal violet.
The antiadhesive properties of EPS isolated from *L. reuteri* SL16 were evaluated by two-way analysis in the statistical program Statistica 7.0. StatSoft with supplementary Tukey's test.

RESULTS

Experiments were aimed at examining the effect of EPS isolated from the *L. reuteri* SL16 strain with concentrations of 5 mg/ml and 10 mg/ml on adherence of *S. aureus* CCM 3953 and *P. mirabilis* CCM 7188.

Effect of SL16 EPS with a concentration of 5 mg/ml

Results of a statistical analysis showed that antiadhesive properties of EPS isolated from *L. reuteri* SL16 with a low concentration (5 mg/ml) correlated with a particular application thereof ($F_{1.42} = 182.899$; $p < 0.00001$), the particular selected pathogenic strain subjected to examination ($F_{1.42} = 33.235$; $p < 0.00001$) and with their effects: application x pathogenic strain ($F_{1.42} = 5.527$; $p = 0.02349$). A statistically significant effect of a 4-hour exposure to EPS with a concentration of 5 mg/ml on the surface of the plates manifested itself as an impairment of their adhesive properties ($p < 0.001$).
P. mirabilis exhibited statistically significantly higher adhesion to the surface of the plates than *S. aureus* ($p < 0.001$).

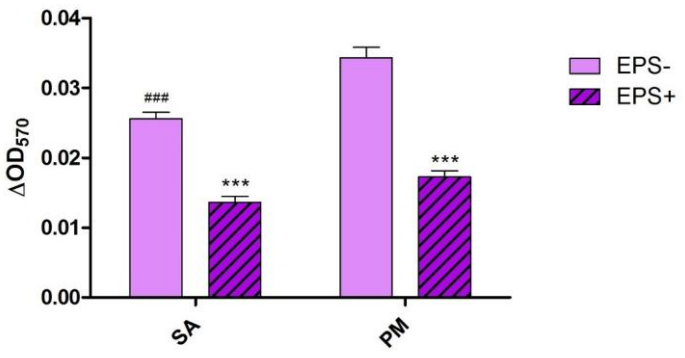

Figure 1 Adhesion after exposure to EPS isolated from *L. reuteri* SL16 with a concentration of 5 mg/ml on *S. aureus* CCM 3953 and *P. mirabilis* CCM 7188

SA – *S. aureus* CCM 3953; PM – *P. mirabilis* CCM 7188; EPS- – plates without EPS; EPS+ – plates with EPS; ΔOD_{570} – difference of values OD_{570} compared to equally exposed controls.
EPS x pathogenic strain: $p = 0.02349$
*** $p < 0.001$ within the same strain
$p < 0.001$ within the same EPS

Statistically significant effects were observed for the EPS activity on the surface of the plates and the subsequently used pathogenic strain. Exposure of the plates to EPS significantly reduced adhesion to the material for both pathogenic strains

($p < 0.001$ vs a respective pathogenic set not exposed to EPS, Figure 1). Results of a statistical analysis also showed that adhesion of *P. miralilis*, when compared to adhesion of *S. aureus*, to the material not exposed to EPS was statistically significantly higher ($p < 0.001$ vs the SA set not exposed to EPS, Figure 1), and the EPS activity had a stronger antiadhesive effect on *P. mirabilis* than on *S. aureus* ($p > 0.05$ vs the SA set exposed to EPS, Figure 1).

Effect of EPS isolated from SL16 with a concentration of 10 mg/ml

Results of a statistical analysis showed that antiadhesive properties of EPS isolated from SL16 with a high concentration (10 mg/ml) correlated with a particular application thereof ($F_{1,46} = 540.3542$; $p < 0.000001$) and the effects between a previous EPS application and a pathogenic strain ($F_{1,46} = 16.2911$; $p = 0.000203$). Post-hoc analysis revealed a statistically significant effect of activity of EPS isolated from SL16 with a concentration of 10 mg/ml on adhesive properties of the plate surfaces while a 4-hour exposure of the material to EPS significantly impaired adhesive properties of the used laboratory material ($p < 0.001$).
As for the mutual effects of the EPS activity on the surface of the plates and the subsequently used pathogenic strain, statistically significant effects were observed at several levels. Exposure of the plates to EPS significantly reduced adhesion to the material for both pathogenic strains ($p < 0.001$ vs a respective pathogenic set not exposed to EPS, Figure 2). Results of a statistical analysis also showed that EPS activity had a significantly weaker effect on adhesion of *P. mirabilis* than on adhesion of *S. aureus* ($p < 0.001$ vs the SA set exposed to EPS, Figure 2).

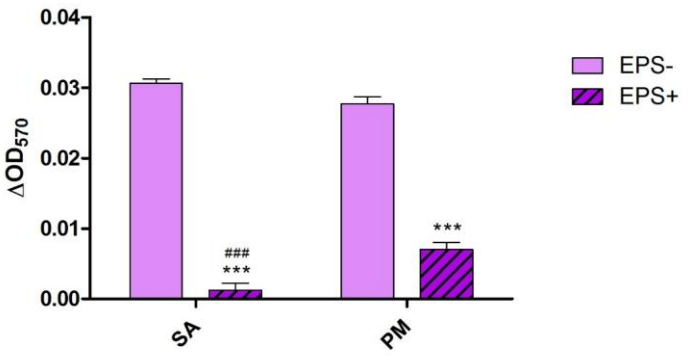

Figure 2 Adhesion after exposure to EPS isolated from *L. reuteri* SL16 with a concentration of 10 mg/ml on *S. aureus* CCM 3953 and *P. mirabilis* CCM 7188

SA – *S. aureus* CCM 3953; PM – *P. mirabilis* CCM 7188; EPS- – plates without EPS; EPS+ – plates with EPS; ΔOD_{570} – difference of values OD_{570} compared to equally exposed controls.
EPS x pathogenic strain: $p = 0.000203$
*** $p < 0.001$ within the same strain
$p < 0.001$ within the same EPS

DISCUSSION

Antibacterial activity against pathogenic microorganisms is indisputable in several *Lactobacillus* strains **(Davoodabadi *et al.*, 2015; Halder *et al.*, 2017; Mu *et al.*, 2018; Werning *et al.*, 2022; Yu *et al.*, 2023)**. The representatives of the *L. reuteri* strain in particular, out of 13 *Lactobacillus* representatives, exhibited the most significant antiadhesive and antiproliferative activity against several enteropathogenic microorganisms **(Hasannejad Bibalan *et al.*, 2017)**. This is largely facilitated by the adhesion ability of individual representatives of *L. reuteri*, which has been confirmed to correlate with their immunomodulation activity in *in vivo* models **(Gao *et al.*, 2016)**. It has also been proved that antiadhesive properties of *L. reuteri* within *Lactobacillus* strains are largely attributable to the production of biosurfactants **(Larsen *et al.*, 2007; Zakaria Gomaa, 2013)**, although one of relatively important factors is their ability to co-aggregate with pathogenic microorganisms, including *S. aureus* NCTC 7447 or *P. vulgaris* ATCC 27974 **(Zakaria Gomaa, 2013)**.
A comparison of efficacy of EPS isolated from *L. reuteri* revealed higher sensitivity to EPS isolated from *L. reuteri* SL16, manifested by lower formation of biofilms in pathogenic microorganisms (*S. aureus* CCM 3953 as well as *P. mirabilis* CCM 7188). An interesting finding was that at a lower concentration, the EPS isolated from *L. reuteri SL16* exhibited stronger antiadhesive activity against *P. mirabilis* CCM 7188, whereas at a higher concentration it was stronger against *S. aureus* CCM 3953. However, as has already been proved, *P. mirabilis*, when compared to the *S. aureus* strain, is capable of forming a biofilm to a much larger extent **(Mihai *et al.*, 2014)**; on the other hand, however, it is much more sensitive to antiadhesive activity of some *Lactobacillus* strains **(Osset *et al.*, 2001)**. According **Fatheree *et al.* (2017)** study, the 42-day application of *L. reuteri* BG exhibited a significant clinical effect in infants, manifested by lower counts of Gram-negative bacteria (*Klebsiella*, *Proteus*, *Veillonella*) in their stools and by changes in counts of neutrophils in serum. The results obtained in this study give rise to an assumption that the application of EPS isolated from *L. reuteri* SL16 might have a significant potential for further clinical studies.
Even though multiple studies have been published on immunomodulation properties of lactic acid bacteria **(Paubert-Braquet *et al.*, 1995; Donnet-Hughes *et al.*, 1999; Chiang *et al.*, 2000; Strompfová *et al.*, 2014)**, the effect of purified EPS on the immune response has not yet been sufficiently investigated. However, it is well-known that it depends on a structure of EPS, i.e. on the chain length and branching, a composition and orientation of saccharides, and on differences between saccharides in their glycosidic bonds, as these properties are decisive for a structure of antigenic epitopes on polysaccharides **(Mazmanian and Kasper, 2006; Kšonžeková *et al.*, 2016)**. A diametrically opposed effect of EPS on the immune system was indicated by the results of previous experiments, while EPS of pathogenic bacteria exhibited a strong inhibitory effect, whereas EPS of some *Lactobacillus* strains exhibited an immunostimulatory effect. As has already been proved, glucan-type EPS of individual *Lactobacillus* strains have different immunomodulatory properties, depending on their spatial structure. While, for example, a relatively linear chain of EPS isolated from *L. reuteri* BG, containing 1-4 and 1-6 glycosidic bonds, increases expression of IL-1β, an EPS chain with more branches with 1-3 and 1-6 glycosidic bonds isolated from *L. reuteri* L26 increases expression of NF-κB, TNF-α or IL-6 **(Kšonžeková *et al.*, 2016)**. Even though according to our initial results this newly isolated EPS from *L. reuteri* SL16 is also a glucan-type EPS, a detailed analysis of its structure is still in process.

CONCLUSION

The genus *Lactobacillus* is the largest from genera of lactic acid bacteria, from which is now intensively studied probiotic bacterium *L. reuteri*. The results confirmed the effectiveness of *L. reuteri* strain. EPS/*L. reuteri* SL16 also significantly reduced adhesivity of *S. aureus* CCM 3953 a *P. mirabilis* CCM 7188 depending on the concentration. Further studies will require clarification of the antiadhesive and moreover anti-biofilm effects mechanism with a focus on assessing changes in the genes expression responsible for synthesis of bacterial factors involved in the biofilms formation process.

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