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MICROBIOLOGY

Exopolysaccharides of lactic acid bacteria isolated from honeybee gut and effects of their antibiofilm activity against *Streptococcus mutans*

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Abstract: *Streptococcus mutans* is one of the main factors in formation of cariogenic biofilms. New strategies need to be developed to reduce the formation of cariogenic biofilms. For this purpose, bacterial exopolysaccharides (EPS) could be considered as new agents against biofilms. Therefore, cell-bound (b-EPS) and released exopolysaccharides (r-EPS) were extracted from the strains *Apilactobacillus kunkeei* K1.10 and *Latilactobacillus curvatus* Kar.9b isolated from the microbiota of honeybees, and their antibiofilm effects on *S. mutans* biofilm formation were determined. The highest reduction in biofilm formation was achieved by r-EPS of *L. curvatus* Kar.9b and *A. kunkeei* K1.10. Scanning electron micrographs (SEM) showed that r-EPS inhibited biofilm formation by reducing adhesion of *S. mutans*. To increase the production of r-EPS from *A. kunkeei* K1.10, the effects of different incubation conditions were also analyzed. The highest EPS production was obtained during 48 h-incubation at 37ºC in a medium containing 1% fructose. r-EPS can be used as a raw material to inhibit cariogenic biofilms. Further studies revealing the detailed structural analysis of r-EPS and the mechanism of action of its antibiofilm effect could be beneficial. Finally, b-EPS and r-EPS from lactic acid bacteria were found to have very different properties in terms of their antibiofilm properties.

Key words: antibiofilm, exopolysaccharides, lactic acid bacteria, *Streptococcus mutans*.

INTRODUCTION

Lactic acid bacteria (LAB) are the subject of numerous research studies due to their diverse biological properties and their ability to produce various metabolites. LAB are generally recognized as safe (GRAS) and their functional metabolites such as organic acids, antimicrobial peptides, fatty acids and exopolysaccharides may have wide-ranging industrial and clinical applications (Oleksy & Klewicka 2018). Basically, polysaccharides can be divided into two categories: Storage polysaccharides and structural polysaccharides. Structural polysaccharides are also released into the extracellular environment (exopolysaccharides, EPS) and also play a role in the assembly of various cell structures (Bernal & Llamas 2012). EPS produced during growth of LAB in the culture medium are both released into the extracellular environment and bound to structures such as the cell wall as capsular polysaccharides (Sutherland 1972).

Bacterial EPS, of which LAB are also an important source, exhibit a wide range of structural and physical properties that create areas of application in industrial, pharmaceutical, and biotechnological fields (Vu et al. 2009). The versatile properties of bacterial EPS include antimicrobial and antibiofilm activities (Kanmani et al. 2013). Today, intensive work is being done to develop alternative strategies to

combat biofilms. LAB and their products are also an important resource for the discovery of new products with antibiofilm activity (Barzegari et al. 2020).

Dental caries is an infectious disease caused by biofilm-forming oral bacteria on tooth surfaces (Selwitz et al. 2007). Early colonization some oral commensals creates the necessary conditions for the growth and mature biofilm formation of *Streptococcus mutans* (Socransky 2002). *S. mutans*, one of many members of the oral microbiome and also a LAB species, is one of the most common species in the oral environment due to its metabolic flexibility (Daneo-Moore et al. 1975, Dewhirst et al. 2010, Jijakli & Jensen 2019). *S. mutans* is capable of fermenting a variety of dietary sugars, and lactic acid fermentation by *S. mutans* can lead to the development of dental caries on tooth surfaces (Quivey et al. 1995).

Some LAB strains can influence microbial ecology by inhibiting the growth of pathogens in their environment. LAB can inhibit pathogen growth primarily by competing for surface adhesion in the oral cavity and co-aggregating with pathogens, but also by lowering the pH of their environment, secreting antimicrobial substances and competing for nutrients. The most important factor determining the intercellular and surface adhesive functions of LAB are EPS (Barzegari et al. 2020). There is increasing evidence that EPS of LAB reduce or prevent biofilm production. EPS of LAB can prevent biofilm production of pathogenic microorganisms by modifying cell surfaces, which has important effects on adhesion, by delaying aggregation, or by acting as a signaling molecule that suppresses the expression of genes responsible for biofilm production (Abdalla et al. 2021).

The bacterial microbiota of honeybees is particularly diverse (Elzeini et al. 2021). Although studies on honeybee LAB have mostly focused on characterization of isolated species, novel functional properties of isolated LAB have also been the subject of research in recent years. In this study, the antibiofilm activity of EPS produced by LAB isolated from the gut microbiota of honeybees was investigated against *S. mutans*. The biofilm activities of the released EPS of the tested LAB strains, both bound to the cell surface and released into the extracellular environment, were compared.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions

The strains of *L. curvatus* Kar.9b and *A. kunkeei* K1.10 (nucleotide database accession numbers, MW829532.1 and MW600523.1, respectively) used in this study were isolated from the gut microbiota of honeybees (*Apis mellifera*) in previous studies (Kiran et al. 2022). These strains were selected for their high EPS production. *S. mutans* ATCC 25175 was preferred for the biofilm experiments. The LAB strains were grown in MRS agar (De Man, Rogosa and Sharpe Agar, Merck, Germany) at 37ºC for 24 h under static conditions. *S. mutans* ATCC 25175 was grown in an anaerobic plate carrier (Thermo Scientific™ Oxoid™, UK) with an AnaeroGen paper sachet (Thermo Scientific™ Oxoid™, UK) for 24 h at 37ºC on BHI agar (Brain Heart Infusion, Merck, Germany). All bacterial strains were stored in 50% glycerol at -86ºC for further analysis.

r-EPS and b-EPS extraction

In this study, released EPS (r-EPS) and cell-bound EPS (b-EPS) were extracted by different methods. For r-EPS extraction, LAB strains previously activated overnight in 5 mL of MRS medium were inoculated into MRS medium (200 mL) at a rate of 1%. The cultures were then incubated at 37ºC

for 24 h. At the end of incubation, the cultures were centrifuged at 25,000 g for 15 min. The supernatants obtained were filtered to remove the remaining bacterial cells (membrane filter with 0.22 um pore size, Sartorious, France). The filtered supernatants were treated with trichloroacetic acid (TCA, Merck, Germany) at a final concentration of 20% (chemical deproteinization). The treated supernatants were incubated at 4ºC for 2 h. After incubation, they were centrifuged at 25,000 g for 20 min at 4ºC. After centrifugation, a double volume of 95% ethanol (Merck, can be omitted, Germany) was added to each supernatant obtained and the supernatants were incubated overnight at 4ºC. The samples were then centrifuged at 4,000 g for 30 min at 4ºC. The pellets were subjected to a freeze-drying procedure. Finally, the amounts of EPS were determined and dissolved in 2 mL of dH_2O (Tallon et al. 2003).

The same inoculation and incubation procedure as described above was used for b-EPS extraction. At the end of incubation, the cultures were centrifuged at 25,000 g for 15 min. The supernatant was removed and the obtained pellets were rinsed once in 5 mL of physiological saline (0.9% NaCl). The rinsed pellets were resuspended in 5 mL of 1 M NaCl solution. The suspensions were sonicated on ice with a 1 mm diameter probe at 120 W (60 amplitude), 20 kHz (0.8 cycles) for 3 min (Dr Hielscher-UP200H, Germany). After this sonication step, the cell-bound EPS were released into the supernatant. The sonicated samples were then centrifuged at 4,000 g for 30 min at 4ºC to remove the bacterial cells. Whether the bacteria were damaged by the sonication process was checked by counting before and after sonication. After centrifugation, a double volume of absolute ethanol (Merck, Darmstadt, Germany) was added to each supernatant. The samples were incubated overnight at 4ºC. Samples were then centrifuged at 4,000 g for 30 min at 4ºC. The pellets were subjected to a freeze-drying procedure. Finally, the amounts of EPS were determined and dissolved in 2 mL of dH_2O (Tallon et al. 2003).

Total carbohydrate determination

EPS concentrations were determined using the phenol-sulfuric acid method (Dubois et al. 1956). In each case 500 µL phenol and 5 mL sulfuric acid were added to 500 µL EPS suspensions and incubated for 10 min at room temperature. After incubation, the samples were thoroughly mixed and incubated at 30ºC for 20 min. The optical densities of the samples were measured using a spectrophotometer set at 490 nm (UV-1601, Shimadzu, Japan). Concentration values were calculated based on the results of glucose standards prepared at different concentrations.

Optimization of EPS production

Effects of different carbohydrate sources

The strain *A. kunkeei* K1.10, which has strong antibiofilm properties with its r-EPS, was investigated in studies to optimize EPS production. For this purpose, 5 mL each of a glucose-free Tryptone Glucose Extract medium [TGE; 5 g/L tryptone (Merck, Germany), 3 g/mL yeast extract (Merck, Darmstadt, Germany)] containing 1% fructose, sucrose and lactose was prepared. EPS extraction and quantification were performed from an *A. kunkeei* K1.10 culture grown for 24 h at 37ºC in medium containing the indicated carbon sources. Finally, the ideal carbohydrate source was determined.

Effects of incubation temperature

The strain *A. kunkeei* K1.10 was incubated for 24 h at 25, 37 and 40ºC in the medium containing the ideal carbohydrate source. At the end of incubation, EPS extraction and quantification

were performed and the ideal incubation temperature was determined.

Effects of incubation time

The strain *A. kunkeei* K1.10 was incubated for 12, 24, 48, and 72 h under ideal conditions (ideal carbohydrate source and incubation temperature). At the end of incubation, EPS extraction and quantification were performed. Finally, the ideal incubation time for EPS production was determined.

Antibiofilm activity of EPS

The antibiofilm activities of both r-EPS and b-EPS were evaluated using different approaches at different concentrations (1, 10 and 100 µg/mL). Biofilm samples were prepared in 96-well polystyrene microtiter plates with U-bottom (LP Italiana, Italy). Quantification of biofilm was performed using a partially modified crystal violet binding assay (Stepanovic et al. 2000). In this context, the plate wells were rinsed three times with sterile saline (0.9% NaCl) at the end of incubation. 200 µL of 95% methanol (Merck, Germany) was added to each well and the plates were fixed at room temperature (RT) for 10 min. After fixation, the wells were emptied and 200 µL of a 0.1% crystal violet solution was added to each well. After incubation with crystal violet solution at RT for 30 min, the plates were washed with distilled water to remove the unbound dye. Finally, 200 µL of a 33% glacial acetic acid solution was added to each well to dissolve the bound dye, and the plates were incubated for at RT 15 min. The dissolved dye was measured in an ELISA reader (µQuant™, BioTek, USA) set to a wavelength of 595 nm. Wells containing the medium and different concentrations of exopolysaccharide served as negative controls, wells containing the medium and inoculum served as positive controls, wells containing different concentrations of exopolysaccharide, medium and inoculum served as test groups. The potential reduction in biofilm amount was calculated using the formula [(C-B)-(T-B)]/[(C-B)] × 100 (C: positive control; B: negative control; T: test groups).

All biofilm studies were performed in Brain Heart Infusion (BHI, Merck, Germany) medium and under incubation conditions ideal for biofilm formation of the *S. mutans* ATCC 25175 strain (BHI supplemented with 1% sucrose, anaerobic conditions, 37° C, 24 h) (OmerOglou et al. 2022). An overnight active culture of *S. mutans* ATCC 25175 in BHI broth was used as inoculum for all biofilm assays. Biofilm samples were prepared in each well by adding 15 µL of the overnight culture to 135 µL of the appropriate medium (medium only or medium adjusted with different EPS concentrations).

pre-treatment

150 µL of the b-EPS and r-EPS suspensions at different concentrations (1, 10 and 100 µg/mL) were added to the wells. The plates were incubated at 37ºC for 24 h and then the wells were emptied. Wells that were not pre-treated with EPS but contained the medium and inoculum served as positive controls. Wells that were both pre-treated and contained medium and untreated but contained only medium served as negative controls. 135 µL of BHI medium was added to each well and the wells were inoculated with 15 µL of the activated culture. After the plates were incubated under ideal conditions, the crystal violet binding assay was performed.

co-treatment

Biofilm sampling was performed under the influence of media containing b-EPS and r-EPS at different concentrations (1, 10 and 100 µg/mL). At the end of incubation, the crystal violet binding assay was performed.

post-treatment

In this context, biofilm production was first performed. After incubation, the wells were emptied and filled with 150 µL of b-EPS and r-EPS suspensions at different concentrations (1, 10 and 100 µg/mL). Some wells in the positive control group were also filled with distilled water (used as a solvent for EPS) and some wells in the positive control group were simply left empty. The plates were then incubated under appropriate conditions. At the end of incubation, the crystal violet binding assay was performed.

Scanning electron microscope analysis

The antibiofilm activity of r-EPS of strain *A. kunkeei* K1.10 at a concentration of 1000 ug/mL was analyzed using SEM. Glass surfaces (coverslip, R: 10 mm) in 6-well tissue culture plates were used for this purpose. 2 mL of a 1% sucrose-enriched BHI medium adjusted to 1000 ug/mL r-EPS was added to the wells containing the coverslip, and the plate was incubated under appropriate conditions. Wells without r-EPS, with medium, coverslip, and inoculum were considered positive controls. After incubation, the coverslips were carefully washed with PBS (phosphate buffer saline) and fixed with 2.5% glutaraldehyde solution for 1 h at RT. After this step, the surfaces were washed 3 times with PBS and treated with 2% osmium tetraoxide for 30 min. After this step, the surfaces were washed again with PBS and dehydrated with serial ethanol concentrations (30, 50, 70, 80, 90, 100%) (Onbas et al. 2018). The air-dried surfaces were coated with gold and examined in a scanning electron microscope (ZEISS, EVO 40, Germany).

Statistical analysis

All phases of the study were performed with 3 replicates. The SPSS 15.0 analysis program (SPSS, USA) was used for statistical analyzes. Data were analyzed using one-way analysis of variance (ANOVA), Tukey's test, and the independent t test.

RESULTS

r-EPS and b-EPS extraction

The amounts of EPS were determined by the phenol-sulfuric acid method based on the glucose standard curve (Figure 1a). According to the data obtained, the highest amount of EPS was obtained from the EPS released into the extracellular environment (r-EPS). There was no significant difference between the r-EPS and b-EPS production amounts of strains *A. kunkeei* K1.10 and *L. curvatus* Kar.9b (Figure 1b).

Optimization of EPS production

The strain *A. kunkeei* K1.10 was preferred for EPS production optimization and the production conditions for its r-EPS were optimized. When the results were evaluated for the effects of different carbon sources on the production of r-EPS by *A. kunkeei* K1.10, it was found that the highest production was obtained when 1% fructose and 1% glucose were used. When fructose and glucose were compared as carbon sources, it was found that fructose was more efficient in EPS production by the strain (Figure 2a).

When r-EPS production was determined after incubation at 25, 37 and 40ºC in 1% medium with fructose, it was found that the highest production was obtained at 37ºC (Figure 2b). Finally, when the effect of incubation time on the r-EPS production capacity of the strain was examined, it was found that the production results at 24 and 48 h were close, but that significantly more r-EPS was produced at 48 h. After 48 h, r-EPS production decreased (Figure 2c).

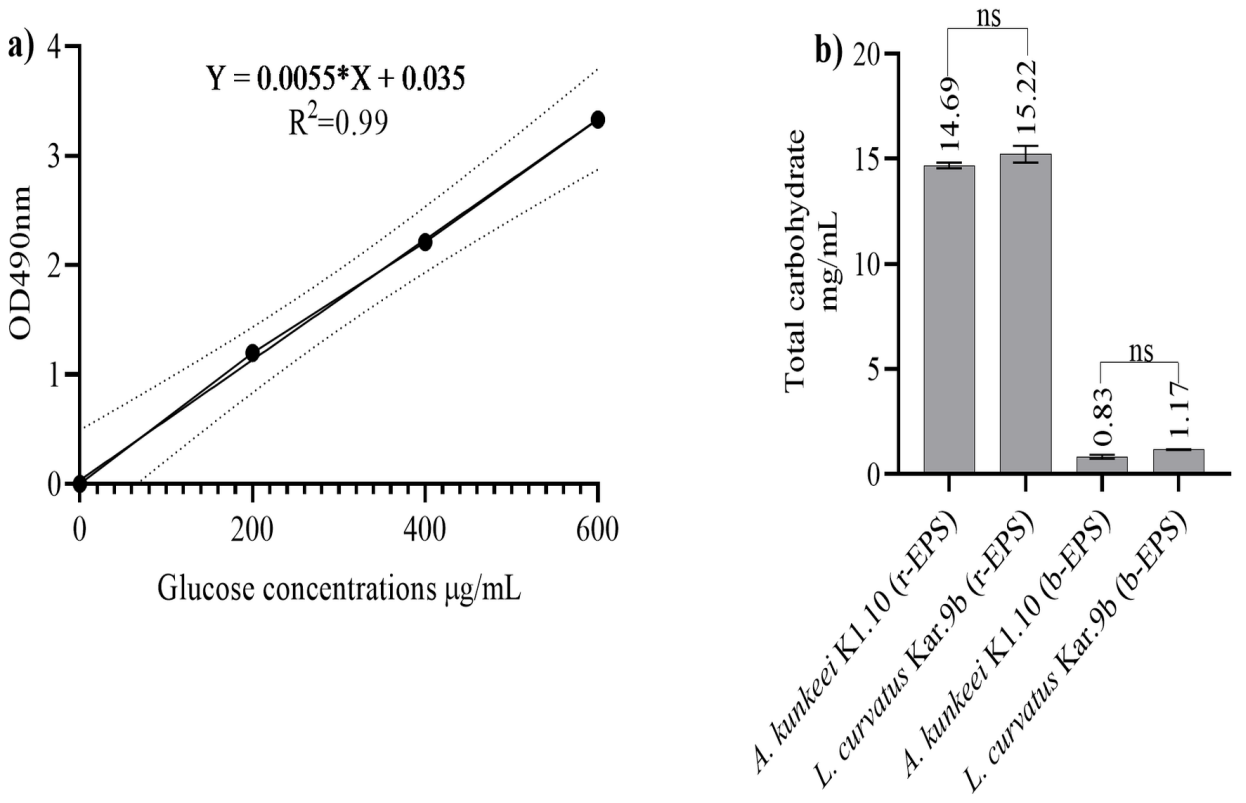

Figure 1. Estimation of b-EPS and r-EPS production (a) Calibration curve of glucose standards (b) EPS production.

Antibiofilm activity of EPS

Different results were obtained in all three approaches (pre-treatment, co-treatment and post-treatment) in which the antibiofilm activities of EPS were evaluated. As a result of pre-treatment and co-treatment, an increase in biofilm production by *S. mutans* was observed in b-EPS of *L. curvatus* Kar.9b at increasing concentrations. This increase was significant at concentrations of 100 and 1000 µg/mL b-EPS of *L. curvatus* Kar.9b (Figure 3a-b, Table I).

When the antibiofilm activities of EPS were evaluated using the co-treatment approach, a steady decrease in biofilm production was observed in both strains with increasing concentrations of r-EPS. For both strains, b-EPS exhibited antibiofilm properties only at the lowest concentration (1 µg/mL), whereas stimulation of biofilm production was observed at increasing concentrations (Figure 3b, Table I). The r-EPS content of both strains was superior to b-EPS in terms of dispersing established biofilms, and the r-EPS content showed a dramatic inhibitory effect on mature biofilms (Figure 3c, Table I). Based on the cumulative analysis of the results, it was clear that the antibiofilm potential of EPS was provided by r-EPS.

Scanning electron microscope analysis

The reduction in biofilm production observed when *S. mutans* ATCC 25175 was co-incubated with r-EPS produced by *A. kunkeei* K1.10 was also confirmed by SEM analysis. This is because EPS contents that do not show antimicrobial activity even at the highest concentrations (data not shown) may exhibit antibiofilm properties. As shown in Figure 4a, *S. mutans* biofilm accumulated very densely in the control sample, while the amount of biofilm production on the coverslip decreased under the influence of r-EPS

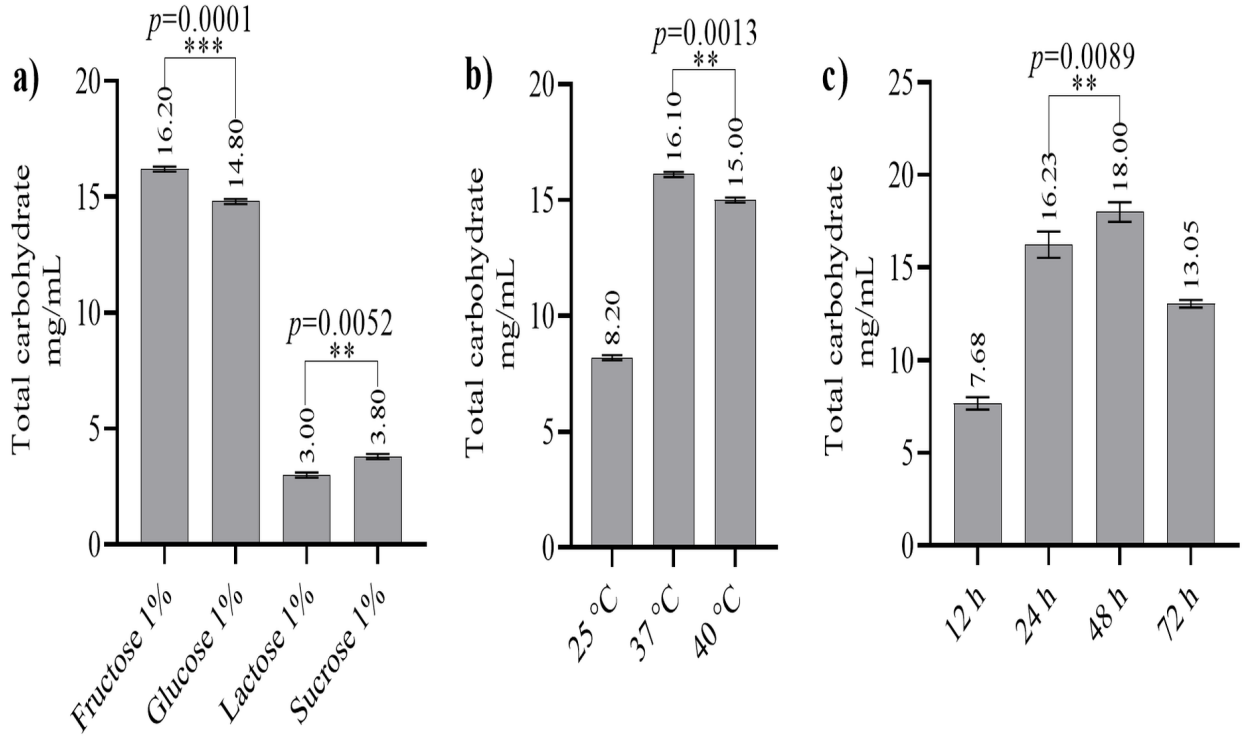

Figure 2. Effects of culture media and incubation conditions on r-EPS production of *A. kunkeei* K1.10 strain (a) Effects of different carbohydrate sources (b) Effects of incubation time (c) Effects of incubation temperatures.

(Figure 4b). In this context, it can be seen that the content of *A. kunkeei* K1.10 r-EPS prevents the adhesion of *S. mutans* to the glass surface.

DISCUSSION

LAB have been used for many years for a variety of applications in the pharmaceutical, food and chemical industries. The ability of the strains of LAB, to secrete EPS is also at the forefront of their applications. These EPS have great commercial value due to their useful physicochemical properties. Since the structure and amount of EPS can be influenced by many factors such as the carbohydrate and nitrogen sources of the culture medium and the incubation conditions (temperature, pH, time), new microbial strains that synthesize industrially important products (dextran, xanthan gum, gellan gum and alginates) are currently being developed. The studies conducted to select and define their culture conditions have gained momentum. Therefore, the discovery of EPS suitable for specific applications is very important (Looijesteijn et al. 2000, Tallon et al. 2003, Abdalla et al. 2021).

EPS produced by LAB strains and their derivatives, oligosaccharides, have great potential for use in the food, pharmaceutical and medical industries. For clinical and commercial use of EPS, they should be obtained as pure and high quality as possible. Considering the various uses and benefits of EPS produced by LAB strains, it is very important to increase and improve the efficiency of EPS production. To increase the efficiency of EPS production, several parameters affecting the fermentation capacity of producer strains should be optimized. One of the main factors limiting the use of EPS from an industrial point of view is the low yield during fermentation. For many EPS-producing microorganisms, the carbon sources determine the quantity and quality of the product (Audy

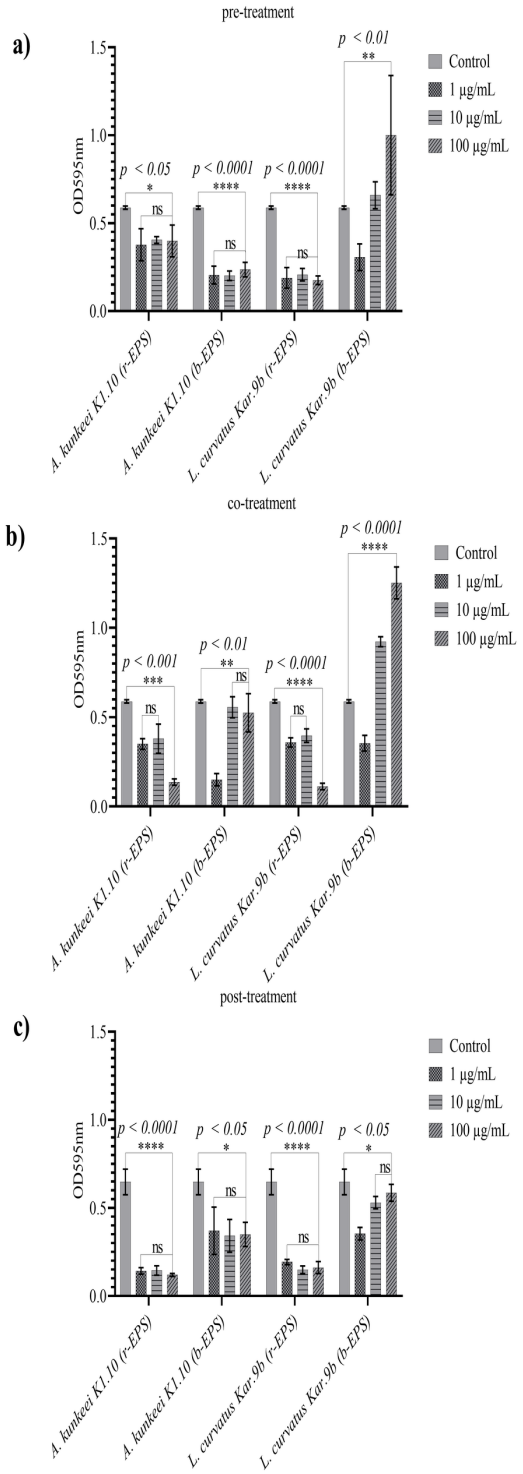

Figure 3. Antibiofilm effects of tested b-EPS and r-EPS against *S. mutans* ATCC 25175 (a) pre-treatment (b) co-treatment (c) post-treatment. One-way ANOVA was performed within each group. Tukey's Test was performed between subgroups. *; *p < 0.05*, **; *p < 0.01*, *; *p < 0.001*, ****; *p < 0.0001*.**

et al. 2010). Many studies have shown that the amount of EPS production by bacteria varies from strain to strain and is directly dependent on the composition of the culture medium and growth conditions (Page 1992, Pal et al. 1999). For example, *Lactobacillus rhamnosus* RW 9595 has been reported to have EPS amounts ranging from 931 to 1275 mg/L depending on the use of glucose or lactose as a carbon source (Van Calsteren et al. 2002). In this context, our study investigated the effects of carbon source on bacterial growth and EPS production. It was found that the highest EPS production was obtained when fructose was used as carbon source. Similar results were reported by Kanmani et al. (2011) and Ismail and Nampoothiri (2010). In a study by Grobben et al. (1996), it was suggested that the regulation of the biosynthetic pathway of EPS produced by strain *Lactobacillus bulgaricus* NCFB 2772 may be dependent on the carbohydrate source (fructose). In another study, growth of strain *Lactobacillus sakei* 0-1 in fructose caused inhibition of key enzymes involved in EPS synthesis (Degeest et al. 2021).

Incubation temperature is also critical for EPS production. In the study, EPS production amounts were compared at three different incubation temperatures and the highest EPS amount was obtained when incubated at 37ºC. Tallon et al. (2003) found that there was an inverse relationship between EPS production and growth temperature of strain *Lactobacillus plantarum* EP56. It was observed that the amount of EPS production decreased with increasing incubation temperature. In order to obtain a higher amount of EPS in our study, a fixed temperature and a fixed carbon source were used in the next stages of the experiment. In the continuation of the study, the effects of different incubation times on the amount of EPS production were determined and the maximum EPS production was obtained after 48 h of

Table I. Biofilm reduction (%) rates as results of pre-, co- and post-treatment of b-EPS and r-EPS from LAB strains.

	pre-treatment		
	1 µg/mL	**10 µg/mL**	**100 µg/mL**
A. kunkeei K1.10 (r-EPS)	36.02 ± 15.77	31.12 ± 7.18	31.10 ± 13.88
A. kunkeei K1.10 (b-EPS)	65.14 ±14.77	65.82 ± 7.91	59.86 ±12.15
L. curvatus Kar.9b (r-EPS)	67.91 ± 17.18	64.80 ± 10.51	70.24 ± 7.22
L. curvatus Kar.9b (b-EPS)	48.29 ± 11.16	*a*	*b*
	co-treatment		
	1 µg/mL	10 µg/mL	100 µg/mL
A. kunkeei K1.10 (r-EPS)	40.65 ± 7.42	34.99 ± 15.98	76.70 ± 5.21
A. kunkeei K1.10 (b-EPS)	74.49 ± 10.02	*a*	*a*
L. curvatus Kar.9b (r-EPS)	38.96 ± 7.80	32.64 ± 10.41	80.90 ± 5.36
L. curvatus Kar.9b (b-EPS)	39.59 ± 14.37	*b*	*b*
	post-treatment		
	1 µg/mL	10 µg/mL	100 µg/mL
A. kunkeei K1.10 (r-EPS)	78.03 ± 4.94	77.62 ± 7.08	81.53 ± 2.24
A. kunkeei K1.10 (b-EPS)	37.05 ± 18.95	42.34 ± 16.11	40.85 ± 18.75
L. curvatus Kar.9b (r-EPS)	70.22 ± 4.01	77.06 ± 5.82	75.10 ± 9.19
L. curvatus Kar.9b (b-EPS)	45.37 ± 4.01	*a*	*a*

Legends: *a*; no significant reduction, *b*; stimulates biofilm formation.

incubation. The data obtained in this study are in agreement with some existing results in the literature (De Vuyst et al. 1998, Tallon et al. 2003). Finally, the EPS (r-EPS) produced by the strain *A. kunkeei* K1.10 isolated from the bee microbiota showed antibiofilm activity against *S. mutans*, which is effective in the formation of dental plaque. The highest amount of EPS was obtained by incubating the strain *A. kunkeei* K1.10 at 37 ºC for 48 h in culture medium containing 1% fructose.

LAB bacteria with potential benefits can be isolated from dairy and non-dairy products and from a variety of organisms. The gut of honeybees is also an important source for isolation of beneficial LAB bacteria. *A. kunkeei* can commonly be isolated from honeybee guts and honeybee products and has high probiotic activity for honeybees. Due to the widespread consumption of honeybee products, strains of *A. kunkeei* also have high probiotic potential for human use. In addition, studies to date have not provided clinical evidence that *A. kunkeei* or its products are harmful to humans (Vergalito et al. 2020). The strain A. *kunkeei* K1.10 was preferred for the studies to optimize EPS production because the existing literature supports its probiotic potential and high antibiofilm activity against *S. mutans*.

Data from the literature show that EPS produced by various LAB species can prevent biofilm formation of pathogenic bacteria, and EPS obtained from LAB can be evaluated as an agent against biofilm (Bernal & Llamas 2012, Song et al. 2020). The biofilm activities of LAB species are due to the biosurfactants, EPS, some metabolites found in the cell-free extract and bacteriocin-like inhibitory components

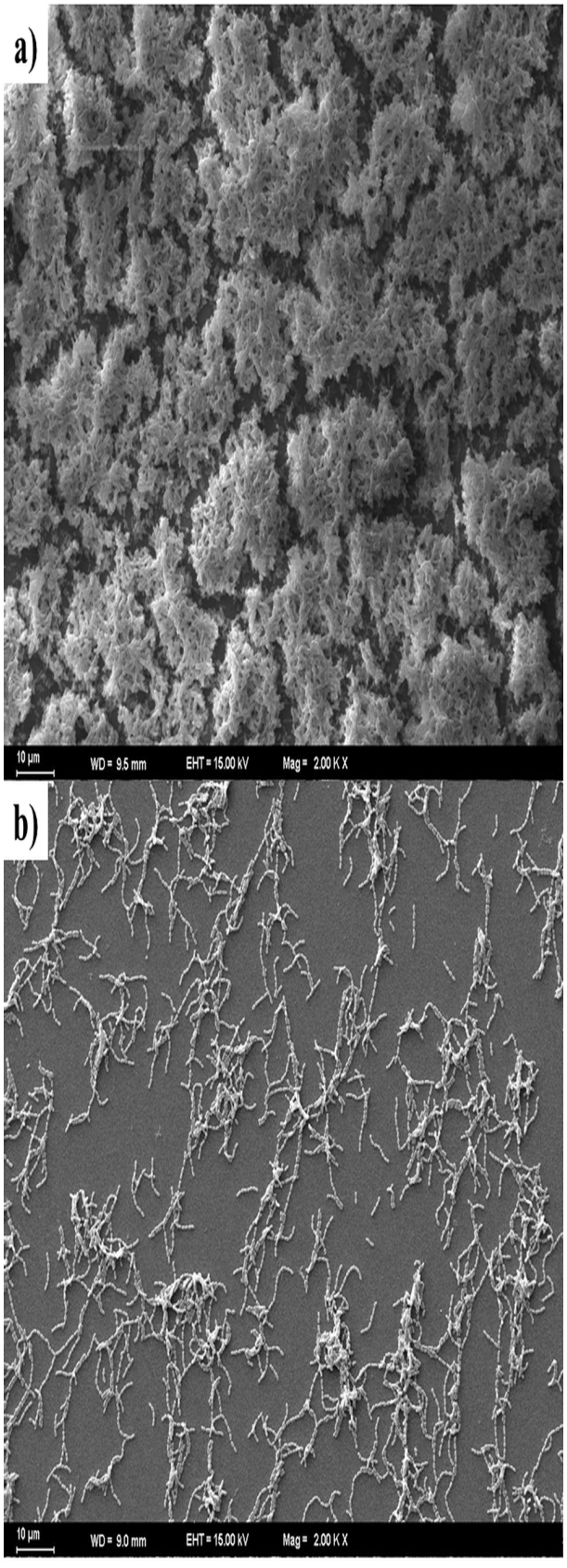

Figure 4. SEM images of *S. mutans* ATCC 25175 biofilms on glass surfaces (a) Untreated control group (2,000×) (b) *S. mutans* biofilm formation on glass surface under the effect of 1000 µg/mL r-EPS from *A. kunkeei* K1.10 strain.

(Walencka et al. 2008, Fracchia et al. 2010, Ramos et al. 2012). The fact that EPS prevent biofilm formation or destabilize biofilm structure makes these biomaterials important as potential biofilm agents (Xu et al. 2020). The presumed antibiofilm mechanisms of action of EPS are related to (i) their ability to act as surfactants, (ii) their stimulatory effects as signaling molecules, (iii) their interactions with other adhesive molecules (Bernal & Llamas 2012). *S. mutans* is an important etiological factor in plaque formation. It is argued that EPS can easily penetrate the biofilm of *S. mutans* and are perceived as natural metabolites and can easily exert their activities. Although bacterial EPS appear to act as agents against biofilms, the mechanisms by which they do so are still unclear. In addition to antimicrobial activity, EPS from LAB may also have direct antibiofilm activity (Xu et al. 2020). The inhibitory effect of r-EPS and b-EPS on *S. mutans* biofilm formation was investigated with pre- and co-treatment assays. A post-treatment assay was also performed to investigate the potential dispersing effect of EPS on established *S. mutans* biofilms. The r-EPS and b-EPS of the two LAB strains examined in this study showed no antimicrobial activity against *S. mutans* (data not shown). In this context, the fact that r-EPS effectively inhibits *S. mutans* biofilm formation suggests that it prevents the initial adhesion to surfaces or aggregation between cells, which is important for biofilm formation. The EPS may serve as emulsifiers agents that mask the hydrophobic sites on the cell surfaces of bacteria (Daba et al. 2021). According to the results of this study, it was a handicap that b-EPS (cell surface-bound EPS) of strain *L. curvatus* Kar.9b

stimulated biofilm production of *S. mutans* at increasing concentrations. Strong aggregation was observed in *S. mutans* cells cultured with b-EPS of this strain. This shows that a bacterial EPS can have a different nature, both promoting and preventing biofilm formation. The very different effects of b-EPS and r-EPS in this strain suggest that their structural diversity should be clarified by further studies.

LAB species naturally occurring in the gut of honeybees have a protective effect against a variety of pathogens. Some metabolites (culture supernatant) of an *A. kunkeei* strain isolated and identified from honeybee gut show antibiofilm properties against various pathogens (Leska et al. 2022). In addition to its broad spectrum of antimicrobial components, *A. kunkeei* also exhibits inhibitory effects against pathogens of wound infections and honeybee pathogens with the proteins it produces as part of its own biofilm production (Butler et al. 2016, Olofsson et al. 2016). *A. kunkeei* strains are also known to exhibit effective antibiofilm properties in in vitro and insect infection models generated by virulent *Pseudomonas aeruginosa* strains (Berríos et al. 2018). Although there are some studies in the literature on the antibiofilm and antimicrobial properties of *A. kunkeei*, this study was the first to show that EPS of *A. kunkeei* has antibiofilm properties especially against *S. mutans*. The r-EPS of strain *A. kunkeei* K1.10 can be evaluated as a raw material for plaque removal products with further studies. In addition, the obtained results could allow the development of new approaches by using EPS, which has a potential to remove biofilms, in various pharmaceutical products. It seems likely that r-EPS produced by strain *A. kunkeei* K1.10 can also be evaluated in different product concepts (gel, dressing, etc.).

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