Archives of Razi Institute, Vol. 77, No. 6 (2022) 2215-2221

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DOI: 10.22092/ARI.2022.358341.2203

Original Article

Comparison between Antimicrobial and Antibiofilm Activity of Exopolysaccharides (EPS) Extracted from *Lactobacillus reuteri* and *Streptococcus mitis* against Oral Bacteria

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Received 2 April 2022; Accepted 22 April 2022
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Abstract

Due to the increased resistance to antibiotics and chemical biocides, the use of bacterial exopolysaccharides has been considered. The objective of the current study was to investigate the strength of the antibacterial and antibiofilm activity of EPS extracted from *Lactobacillus reuteri* and *Streptococcus miti*s because previous studies showed there were structural differences between EPS, during this study, EPS extracted from *Lactobacillus reuteri* and *Streptococcus miti* by ethanol precipitation method and estimated antibacterial and antibiofilm activity against several Oral Bacteria (*Staphylococcus aureus, Staphylococcus hominis, Acinetobacter baumannii, Raoultella ornithinolytica, Streptococcus thoraltensis* in different concentration as (100,150,200,250,300 mg/ml), the results showed carbohydrates rate in extracted EPS from *L. reuteri* and *S. mitis* were recorded was 85, 80 % respectively.The concentration 100 and 150 mg/ml for EPS from *L. reuteri* and *S. mitis*, there was no inhibitory effect, except in *S. aureu*s (1.1±0.10) and *S. hominis* (1.0±0.10) at 100 mg/ml, 3.1±2.01, 2.1±0.54 mg/ml respectively at 150 mg/ml concentration but no significant differences ($P \leq 0.05$). However, the antibacterial effect of that EPSs started at the concentration of 200 and upwards, where different results were recorded between the concentrations of both EPSs against all bacteria isolated ($P \leq 0.05$), On the other hand, the effect of EPS from *L. reuteri* and *S. mitis* was clear against the formation of biofilm compared with the control, worth mentioning that EPS from *L. reuteri* was more effective compared with EPS from *S. mitis* in all isolates ($P \leq 0.05$) except for *S. thoraltensis* where it was noted that the EPS from *S. mitis* is more effective than EPS from *L. reuteri*. Through the results obtained in this study, it was noted that the difference in the structural nature of EPS has an important role in its effectiveness as an antibacterial and anti-biofilm formation and, as it was found that the EPS from *L. reuteri* showed more effectiveness than EPS from *S. mitis* and thus the mechanism of preventing and inhibiting bacteria depending of the structural nature of EPS.

Keywords: EPS, *Lactobacillus reuteri, Streptococcus Mitis*, Antibacterial, Antibiofilm

1. Introduction

Microbial polysaccharides have various properties, and they are used as drug delivery agents, bio-absorbents and bio-flocculants in the food, pharmaceutical industries and cosmetics (1, 2). Polysaccharides are employed in green manufacturing of silver nanoparticles because they are more beneficial than synthetic polymers (3).

The interaction of metal ions with hydroxyl groups of EPS significantly impacts the form and size of nanoparticles (4) and found that EPS is biodegradable, non-toxic, biocompatible, and plentiful in natural sources. *Enterococcus sp., Bifidobacterium sp., Lactococcus sp., Pediococcus sp., Leuconostoc sp., Streptococcus sp., Lactobacillus sp.* and *Weissella sp.* are the most common EPS producers (5). Among the

many microorganisms consumed by humans, Probiotic bacteria survive in bile presence, gastric secretions, and low pH and colonize the gastrointestinal tract (6, 7). Antibiotic use decreases the antagonistic activity of normal microbial flora against pathogenic germs (8).

Probiotics can counteract antibiotics, which can cause immunological suppression if consumed regularly. They strengthen the immune system to suppress diseases caused by pathogen microorganisms (9). As a result, probiotic Exopolysaccharides (EPS) are used to treat autoimmune diseases, inflammatory gastrointestinal diseases, colon cancer, gastric ulcers, cardiovascular diseases, and obesity in humans (10).

Biological roles and functional features of bacterial EPSare influenced by their chemical structures, which are diverse and complex. They are classified as hemopolysaccharides (HoPS) or heteropolysaccharides (HePS), which contain two or more monosaccharides. Most of the EPS produced by LAB is HePS, which is produced intracellularly, but other LAB species/strains created EPS as (HoPS) via external enzymes (11). HoPS generated by LAB can be classed as fructans, glucans, or galactans; they contain D-galactose, D-fructose D-glucose (12).

Hemopolysaccharides have a more complex structure as they are composed of several repeating units of sugars, such as hexose, pentose, uronic acids or N-acetylated monosaccharides and may be branched or unbranched (13). The present study investigated the antimicrobial and antibiotic activity of exopolysaccharides (EPS) extracted from *Lactobacillus ruteri* and *Streptococcus mitis* against oral bacteria.

2. Materials and Methods

2.1. Bacteria Isolated from the Oral Cavity.

The isolated (*S. aureus, S. hominis, A. baumannii, R. ornithinolytica* and *S. thoraltensis*) obtained from the department of Microbiology and Parasitology, college of veterinary medicine, which was isolated from the oral cavity of some animals such as camels and cows and confirmed with Vitek system.

2.2. Purification and Extraction EPSs.

EPSs are extracted using ethanol precipitation (14). *L. reuteri* was grown in MRS broth for 24 hours, whereas *S. Mitis* was cultivated in brain heart broth for 24 hours. After centrifuging at 8,000 g for 20 min at 40^{C}, the supernatant was collected and denatured with a trichloroacetic acid (final concentration of 14 %). The supernatant was added to absolute cold ethanol (two-fold volume of supernatant) and kept at $4C^{0}$ for 24 hours before being centrifuged at 8000g for 20 minutes. The supernatant was removed after centrifuging the precipitated exopolysaccharide for 15 minutes at $4C^{0}$. Finally, the precipitate was dissolved in deionized water and dialyzed for 24 to 48 hours using a dialysis membrane. The precipitated exopolysaccharides dried at $30C^{0}$ to remove the ethanol before being concentrated, and carbohydrate content was determined using the phenol-sulfuric-acid technique.

2.3. Determination MIC of EPSs

In Eppendorf tubes, 100 µl of bacterial suspension (adjusted to 0.5 McFarland standard) was added, followed by 50 µl of EPSs, and the tubes were incubated for 24 hr. at 37 C^{0}. The tubes were then checked for turbidity, with the tubes that exhibited turbidity being excluded and those that did not show turbidity being recognized as the minimal inhibitory concentration (15).

2.4. Determination of the Antibiotic Activity of EPSs

Isolates were activated in nutrient broth (18 to 24 h) and then corrected to a McFarland standard even to 1.5 X 10^{8} CFU/ml) then subculture on Mueller-Hinton agar followed by creating equal-sized (6 mm diameter) to fill up with 0.1 ml of prepared concentration of EPS (15).

2.5. MIC Effects of EPSs on Preformed Biofilm

The wells were filled with 100 µl of tryptic soy broth (TSB) cultured bacteria (96-well plate), then incubated at 37°C for 24 h biofilm formation; the medium was generously aspirated while formed biofilm was washed three times with PBS to remove non-adherent cells; finally, 200 µl of EPS in TSB was added to the well

and incubated for 24 hr. at 37C^0, and the experiment was performed without the MIC of EPS as a control (16).

2.6. Statistical Analysis

All data collected using Microsoft Excel 2016 and SPSS version 18 to assess the significance of the observed differences between the treatments used were subjected to analysis of variance (ANOVA), and Fisher's least significant difference (LSD) (P<0.05) was measured statistically significant.

3. Results and Discussion

The results of the determination of carbohydrates rate in purified EPS from *L. reuteri* and *S. mitis* were recorded the carbohydrate content was 85, and 80%, respectively, by using the phenol-sulfuric-acid-method while the 15-20% other components, such as acetyl group, Ketal Linked pyruvate, group, Hexominase and uronic acids (17). TCA was used to denature protein components during the extraction process (14), which according to the study of Dilna, Surya (18), in the extraction of exopolysaccharides from long-chain molecules consisting of sugar and/or sugar derivatives, but rhamnose, galactose, glucose were obtained (19). EPS showed the highest proportion of carbohydrates.

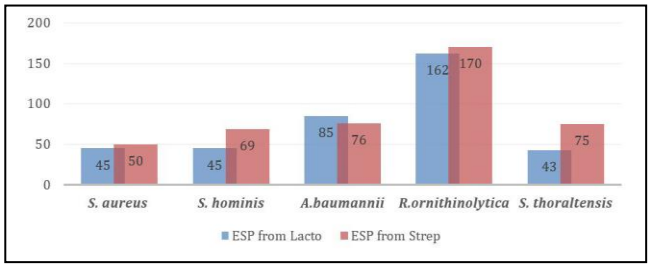

Figure 1. MIC activity of EPS of *L. reuteri* and *S. mitis* against bacteria isolated from the oral cavity

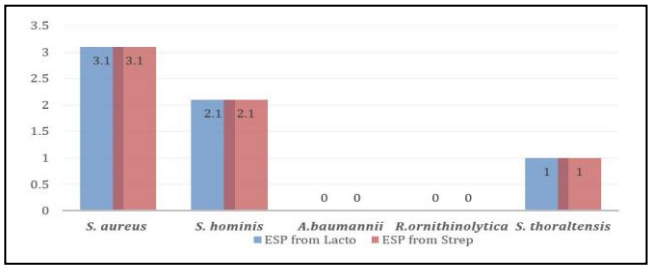

Figure 3. Antibacterial activity of EPSs in concentration 150 mg/ml

The results of the MIC activity recorded that EPS from *L. reuteri* at 50 mg and *S. mitis* at 60 mg was able to inhibit the growth of *S. aureus*, but the study recorded that high concentrations of EPS from *L. reuteri* and *S. mitis* at 85 mg, 76 mg respectively required to inhibit *R. ornithinolytica* as well as 162 mg, 170 mg respectively required to inhibit *S. thoraltensis* as in figure 1.

When using the concentrations 100 and 150 mg/ml for EPS from *L. reuteri* and *S. mitis*, there was no inhibitory effect on the isolates used in the study, except in *S. aureus* and *S. hominis* which both EPS from *L. reuteri* and *S. mitis* recorded 1.1±0.10 against *S. aureus* and 1.0±0.10 against *S. hominis* at 100 mg/ml while at 150 mg/ml against S. *aureus* 3.1±2.01, *S. hominis* 2.1±0.54 mg/ml but no significant differences between concentrations of EPS (P≤0.05), however, the antibacterial effect of that EPSs started at the concentration of 200 and upwards, where different results were recorded between the concentrations of both EPSs against all isolate with the presence of significant differences (P≤0.05), in addition, the study showed that the antibacterial effect of EPS from *L. reuteri* was higher compared to the antibacterial effect of EPS from *S. mitis* depending on the area of inhibition as in the figures 2, 3, 4, 5 and 6 and table 1.

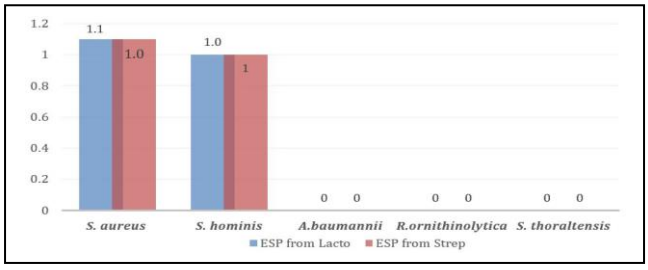

Figure 2. Antibacterial activity of EPSs in concentration 100 mg/ml

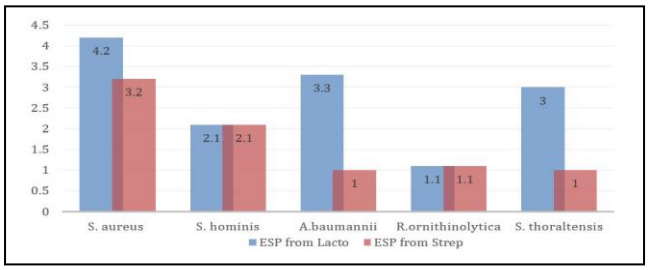

Figure 4. The antibacterial activity of EPSs in a concentration of 200 mg/ml

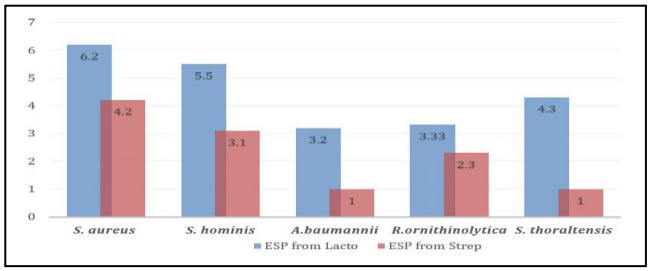

Figure 5. Antibacterial activity of EPSs in concentration 250 mg/ml

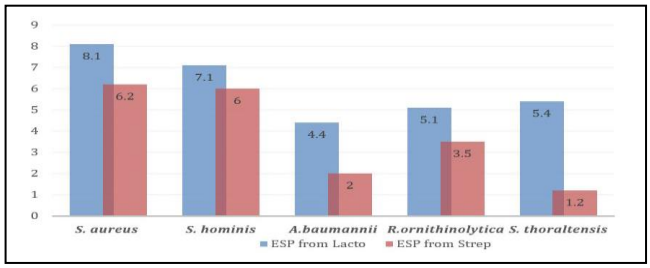

Figure 6. Antibacterial activity of EPSs in concentration 300 mg/ml

Table 1. Comparison of antibacterial activity of EPSs in different concentration (mg/ml) depending inhibition diameter zone (mm) by using T test (Mean±S.D)

Isolates	EPS	100 mg/ml	150 mg/ml	200 mg/ml	250 mg/ml	300 mg/ml
S. aureus	EPS 1	1.1±0.10[a]	3.1±2.01[a]	4.2±0.62[a]	6.2±0.76[a]	8.1±0.11[a]
	EPS 2	1±0.02[a]	2.1±0.02[b]	3.2±0.12[b]	4.2±1.02[b]	6.2±0.02[b]
S. hominis	EPS 1	1.0±0.00[a]	2.1±0.54[a]	3.3±0.32[a]	5.5±0.80[a]	7.1±0.02[a]
	EPS 2	0±0.00[a]	1±0.02[a]	2.1±0.41[b]	3.1±0.52[b]	6.0±0.00[b]
A. baumannii	EPS 1	0±0.00[a]	0±0.00[a]	1.1±0.23[a]	3.2±0.92[a]	4.4±0.32[a]
	EPS 2	0±0.00[a]	0±0.00[a]	1.0±0.25[a]	1.0±0.00[b]	2.0±0.00[b]
R. ornithinolytica	EPS 1	0±0.00[a]	0±0.00[a]	1.2±1.02[a]	3.33±1.92[a]	5.1±0.12[a]
	EPS 2	0±0.00[a]	0±0.00[a]	1.1±0.20[a]	2.3±2.32[b]	3.5±0.30[b]
S. thoraltensis	EPS 1	0±0.00[a]	1±0.00[a]	3.0±1.20[a]	4.3±2.82[a]	5.4±0.58[a]
	EPS 2	0±0.00[a]	0±0.00[a]	1.0±0.00[b]	1.2±1.12[b]	3.0±0.00[b]

EPS1: EPS from *L.reuteri*, EPS2: EPS from *S. mitis*. The different letter means there was a significant difference between $P \leq 0.05$, the similar letters mean there was no significant difference between $P \leq 0.05$

The antibacterial activity of EPS in the current results are consistent with various studies (20-22); EPS obtained in LAB has a significant antibacterial effect. Gram-negative and positive bacteria. The EPS generated by *L. rhamnosus* showed significant antibacterial activity against the pathogens *E. Coli* and *S. enterica* (23).

Bacillus cereus, V. *parahaemolyticus*, *S. Typhimurium*, *S. aureus* and *B. longum* were affected by EPS produced by Bifidobacterium longum (24). EPS from *L. gasseri was* found to have antibacterial action against various pathogens, including *Listeria monocytogenes*, which was significantly suppressed in another study (25). When tested against *E. coli* and *S. aureus*, the EPS-C70 generated by *L. Plantarum* C70 isolated from camel milk resulted in a 2–3 log reduction in viability (26).

Although there is currently no clear mechanism for the antibacterial activity of EPS LAB against gram-positive and gram-negative bacteria, research is ongoing to establish an acceptable mechanism (s) for the antibacterial activity found. Since EPS has the potential to change the structure of bacterial cell coat, especially the peptidoglycan layer, it has been proposed as a suitable inhibitory mechanism (27).

The capacity of EPSs such as kefiran that interacts with the bacterial or eukaryotic cells led to the theory that kefiran acts as masking or decoy (28), this action may disrupt Gram-negative bacteria's outer membrane receptors or channels. Based on the study of Salachna, Mizielinska (29), the formation of secondary metabolites in the growth medium may be aided by EPS, which can damage bacteria. The antibacterial action of EPS is attributed to the functional groups in

EPS structure interacting with bacterial cell envelopes (30). On the other hand, the effect of EPS from *L. reuteri* and *S. mitis* was clear against the formation of biofilm compared with the control; worth mentioning that EPS from *L. reuteri* was more effective compared with EPS from *S. mitis* in all isolates with the presence of significant differences except for *S. thoraltensis* isolates where it was noted that the EPS from *S. mitis* is more effective than EPS from *L. reuteri* 0.119±1.38, 0.150±0.40 respectively as in figure 7 and table 2.

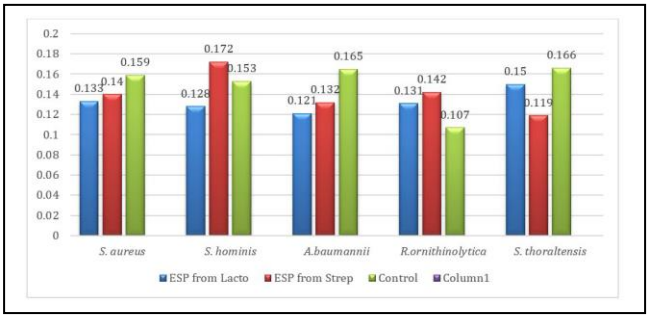

Figure 6. Antibiofilm activity of EPSs EPSsagainst bacteria isolated from the oral cavit

Table 2. Comparison of Antibiofilm activity of EPSs against bacteria isolated from the oral cavity

Isolates	Control	EPS from *Lac*	EPS from *Strep*
S. aureus	0.159±2.12^{a}	0.133±0.02^{c}	0.140±1.02^{b}
S. hominis	0.153±1.02^{a}	0.128±0.11^{c}	0.172±0.98^{b}
Abaumannii	0.165±0.32^{a}	0.121±0.87^{c}	0.132±0.90^{b}
Rornithinolytica	0.107±0.44^{a}	0.103±0.42^{b}	0.104±0.35^{b}
S.thoraltensis	0.166±0.65^{a}	0.150±0.40^{b}	0.119±1.38^{c}

The different letter means there was a significant difference between $P \leq 0.05$ and the similar letters mean there was no significant difference between $P \leq 0.05$

The antibiofilm results were consistent with (31-33). LAB's polysaccharides have anti-biofilm, immunostimulatory, and antioxidant properties (34). The findings revealed that the ability to remove biofilms is linked to the concentration of EPS (35). It is not understood how probiotics reduce biofilm development. Furthermore, EPS produced by *L. acidophilus* significantly reduced the expression of genes involved in chemotaxis and curli formation in *E. coli*, inhibiting biofilm development (36). These probiotics affect the expression of various *C. albicans* genes, such as ALS3, HWP1, EFG1 and SAP5. According to the findings, these probiotics were found to be efficient in suppressing biofilm formation and eliminating preformed biofilms of *C. albicans* (37). Because of the chemical structure of EPS, there are differences in antibacterial activity between EPS from *L. reuteri* and *S. mitis.* Exopolysaccharides (EPSs) are carbohydrate polymers with a high molecular weight that are released into the extracellular matrix by various bacteria. Based on their monosaccharide compositions, EPSs are divided into two groups homo-exopolysaccharides, made up of only one type of sugar with repeating subunits. Hetero-exopolysaccharides, are made up of two or more types of monosaccharide subunits (23). Medium ingredients and cultural circumstances all influence their structure and qualities. Furthermore, because of their molecular weights and functional groups' glycosidic linkages, their functional and rheological properties vary among industries (38).

4. Conclusion

The results obtained in this study was noted that the difference in the structural nature of EPS has a vital role in its effectiveness as an antibacterial and anti-biofilm formation, as it was found that the EPS from *L. reuteri* showed more effective than EPS from *S. mitis* and thus the mechanism of preventing and inhibiting bacteria depending of the structural nature of EPS, at the same time the results showed that some bacteria were resistant to both extracts depending on the radius of the inhibition zone at EPSs concentration lower than 200mg/ml but the antibacterial activity of both EPS was apparent at a concentration higher than 200 mg/ml.

Authors' Contribution

Study concept and design: S. M. A. and H. J. J.
Acquisition of data: H. J. J.
Analysis and interpretation of data: G. H. H.
Drafting of the manuscript: M. M. M. A.

Critical revision of the manuscript for important intellectual content: S. M. A. and H. J. J.
Statistical analysis: S. M. A.
Administrative, technical, and material support: H. J. J.

Ethics

In the present study, all ethical standards were approved by the Sawa University, Samawah, Iraq ethics committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

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