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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Determination of Antioxidant and Antimicrobial Activities of Cell-Free Supernatant (CFS_{KC27L}) and Exopolysaccharide (EPS_{KC27L}) obtained from *Ligilactobacillus salivarius* KC27L

Ligilactobacillus salivarius KC27L'den Elde Edilen Hücresiz Süpernatant (CFS_{KC27L}) ve Ekzopolisakkaritin (EPS_{KC27L}) Antioksidan ve Antimikrobiyal Aktivitelerinin Belirlenmesi

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Abstract

Twenty-six lactic acid bacteria were obtained from poultry feces sampled located in the Ankara area (Türkiye) and belong to the *Lactobacillus* genus according to the results obtained by biochemical methods. This study screened these isolates for exopolysaccharides (EPS) production. EPS production was detected in these isolates, varying from 8 mg L^{-1} to 353 mg L^{-1}. The highest EPS-producing isolate (KC27L) was selected for further studies. The isolate was identified as *Ligilactobacillus salivarius* by 16S rRNA analysis. Furthermore, the anti-biofilm and antioxidant abilities of the cell-free supernatant (CFS_{KC27L}) and different concentrations (0.5 mg L^{-1} and 1 mg L^{-1}) of EPS belonging to the KC27L strain (EPS_{KC27L}) that exhibited high EPS production were determined. CFS_{KC27} and different concentrations (0.5 mg L^{-1} and 1 mg mL^{-1}) of EPS_{KC27L} determined the anti-biofilm impact on *Escherichia coli* ATCC 11229, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* EB-1. The highest anti-biofilm effect in 1 mg mL^{-1} EPS_{KC27L} was detected at *E. coli* ATCC 11229 with 87 % inhibition. Three different methods (1.1-Diphenyl-2-picrylhydrazyl radical (DPPH) removal impact, Fe^{2+} ion chelating and superoxide anion radical scavenging activity) designated antioxidant activity. The highest 1.1-Diphenyl-2-picrylhydrazyl radical (DPPH) removal impact, Fe^{2+} ion chelating, and superoxide anion radical scavenging activity were found in 1 mg mL^{-1} EPS_{KC27L} (79.6%, 24.9%, and 61.6%, respectively). Both anti-biofilm and antioxidant activities of 1 mg mL^{-1} EPS_{KC27L} were higher than postbiotic. Finally, its molecular characterization was done following the partial purification of the EPS_{KC27L}. The EPS_{KC27L} has two fractions with molecular weights of $1.6x10^3$ and $6.4 x10^4$ Da. Monosaccharide components of EPS_{KC27L} were found to be glucose (53.1%), fructose (18.5%), arabinose (14.6%) and mannose (13.8%). CFS_{KC27L} and EPS_{KC27L} obtained from *L. salivarius* can be antioxidants and anti-biofilm agents.

Keywords: *Ligilactobacillus salivarius*, Cell-free supernatant, Exopolysaccharide, Anti-biofilm, Antioxidant

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Öz

Ankara bölgesinden (Türkiye) alınan kanatlı dışkılarından biyokimyasal yöntemlerle elde edilen sonuçlara göre *Lactobacillus* cinsine ait 26 laktik asit bakterisi izole edilmiştir. Bu çalışma, bu izolatları ekzopolisakkarit (EPS) üretimi açısından taramıştır. Bu izolatlarda 8 mg L^{-1} ila 353 mg L^{-1} arasında değişen EPS üretimi tespit edilmiştir. Bundan sonra yapılacak çalışmalar için en yüksek EPS üreten izolat (KC27L) seçilmiştir. İzolat 16S rRNA analizi ile *Ligilactobacillus salivarius* olarak tanımlanmıştır. Ayrıca, yüksek EPS üretimi sergileyen KC27L suşuna (EPS_{KC27L}) ait hücresiz süpernatantın (CFS_{KC27L}) ve farklı konsantrasyonlardaki (0.5 mg L^{-1} ve 1 mg L^{-1}) EPS'nin anti-biyofilm ve antioksidan yetenekleri belirlenmiştir. CFS_{KC27} ve farklı konsantrasyonlarda (0.5 ve 1 mg mL^{-1}) EPS_{KC27L}, *Escherichia coli* ATCC 11229, *Enterococcus faecalis* ATCC 29212 ve *Staphylococcus aureus* EB-1 üzerinde anti-biyofilm etkisini belirlemiştir. 1 mg mL^{-1} EPS_{KC27L}'de en yüksek anti-biyofilm etkisi % 87 inhibisyon ile *E. coli* ATCC 11229'da tespit edilmiştir. Üç farklı yöntem ile (1.1-Difenil-2-pikrilhidrazil radikali (DPPH) giderim etkisi, Fe^{2+} iyon şelatlama aktivitesi ve süperoksit anyon radikal temizleme aktivitesi) antioksidan aktivite belirlenmiştir. En yüksek 1.1-Difenil-2-pikrilhidrazil radikali (DPPH) giderim etkisi, Fe^{2+} iyon şelatlama aktivitesi ve süperoksit anyon radikal temizleme aktivitesi 1 mg mL^{-1} EPS_{KC27L}'de (sırasıyla %79.6, %24.9 ve %61.6) bulunmuştur. 1 mg mL^{-1} EPS_{KC27L}'nin hem anti-biyofilm hem de antioksidan aktiviteleri postbiyotikten daha yüksek çıkmıştır. Son olarak EPS_{KC27L}'nin kısmi saflaştırılmasının ardından moleküler karakterizasyonu yapılmıştır. EPS_{KC27L}, molekür ağırlıkları $1.6x10^3$ ve $6.4\ x10^4$ Da olan iki fraksiyona sahiptir. EPS_{KC27L}'nin monosakarit bileşenleri glikoz (%53.1), fruktoz (%18.5), arabinoz (%14.6) ve mannoz (%13.8) olarak bulunmuştur. *L. salivarius*'tan elde edilen CFS_{KC27L} ve EPS_{KC27L} antioksidan ve anti-biyofilm ajanları olabilir.

Anahtar Kelimeler: *Ligilactobacillus salivarius*, Hücre içermeyen supernatant, Ekzopolisakkarit, Anti-biyofilm, Antioksidan

1. Introduction

Lactic acid bacteria (LAB), which commonly exist in nature, are probiotics, and it has been proven that LAB have also essential roles in poultry health (Piqué et al., 2019; Rajoka et al., 2019; Cano et al., 2021). The health benefits of probiotics come through direct or indirect interactions between cells or their released metabolites (Aguilar-Toalá et al., 2018; Ke et al., 2021). The soluble products or metabolites, comprised of the cell-free supernatant (CFS) composed of bacteriocins, organic acids, H_2O_2, short-chain fatty acids, carbohydrates, enzymes, vitamins, cofactors, and complex agents, excreted by probiotics that have health benefits for the host. These are postbiotics (Nataraj and Mallappa, 2020; Salminen et al., 2021). Since the precise compositions and mechanisms of postbiotics have yet to be fully understood, and there needs to be more information about their methods of preparation and analysis, there are fewer studies about postbiotics than probiotics (Cicenia et al., 2014; Hossain et al., 2021). Because of the food matrices' physicochemical properties and food processing, it isn't easy to directly use live starter/probiotic cultures on food. Hence, postbiotics could be used as an alternative to living microorganism to benefit from their wide-spectrum antimicrobial activity and avoid the negative interactions among primary and secondary starters and food (Arıcı, 2005; Pujato et al., 2014; Moradi et al., 2021). Currently, postbiotics have drawn interest because of their valuable impacts on the host, directly or indirectly (Żółkiewicz et al., 2020). Postbiotics, which are rich in high and low molecular weight biologically active metabolites, have drawn a great deal of attention from multiple researchers due to the convenience of use and hiding, persistence in a vast range of pH and temperature, and broad spectrum of antimicrobial activity (Campana et al., 2019; Jamwal et al., 2019). Also, the obtained goods of diverse signaling molecules of various regulative functions like anti-inflammatory, immunomodulatory, anti-proliferative, and antioxidant activities are remarkable for researchers (Barros et al., 2020; Moradi et al., 2021).

Polysaccharides classified into capsule polysaccharides, lipopolysaccharides, and exopolysaccharides (EPSs) concerning their composition are common in multiple organisms, including microorganisms, plants, and animals (Min et al., 2019). Generally, EPS can either be a homopolysaccharide in which one type of monosaccharide repeating units is included or a heteropolysaccharide in which different monosaccharide repeating units are included (Abarquero et al., 2021). Some lactic acid bacteria (LAB) strains produce EPS during their normal metabolic processes (Min et al., 2019), thus affecting foods' rheological, textural, functional, and sensory features. Also, they have protective functions in the natural environment against antibiotics or toxic compounds (Ayyash et al., 2018). The general properties of any EPS depend on its monosaccharide composition, molecular weight, connection type, and degrees of branching, and these contribute to determining its biological activity (Zhou et al., 2019).

Oxidative stress plays a role in many diseases, including heart disease, comprising atherosclerosis, diabetes, Parkinson's and Alzheimer's disease, inflammation, and cancer (Hajam et al., 2022). Numerous molecules, such as EPS and beta-glucan, are considered antioxidant agents (Sengül et al., 2011). In food products, due to the negative effects and toxicity of synthetic antioxidants on human health, harmless and nontoxic antioxidants obtained from natural sources for safety in humans are preferred (Choi et al., 2021; Albaş et al., 2022). EPSs are considered important candidates for functional foods as a natural source of antioxidants (Zhang et al., 2016).

In many industries, biofilms are one of the significant sources of contamination. The conventional cleaning and disinfection regimes cause inadequate control of biofilms and the spreading of resistance. Many methods have been developed to control biofilms and biofilm-associated infections; however, new, and effective strategies are still needed worldwide to prevent biofilms formed by pathogens, especially by multidrug-resistant bacterial strains (Raftis et al., 2011; Sarikaya et al., 2017). Since the ability to form biofilm increases the survival and permanence of pathogenic bacteria, chronic and repeated infections, and rising antibiotic resistance, it causes safety concerns in the Feedstuffs (Prete et al., 2021). LAB-derived EPSs are a promising alternative anti-biofilm agent to prevent biofilm formation (Abarquero et al., 2021; Prete et al., 2021). Although the biological properties of EPSs produced by LAB (e.g., antioxidant, anti-biofilm, antimicrobial, anticancer, immunomodulatory) have been investigated (Sharma et al., 2018; Min et al., 2019; Rajoka et al., 2019; Tukenmez et al., 2019; Bikric et al., 2022), non-lactic acid bacterial EPSs have been used commercially in poultry food additives. Therefore, there is a need to investigate LAB-derived EPSs that can be used as food additives for poultry. The purpose of this study is 1) to screen the EPS generation of lactobacilli isolated from chicken feces, 2) to investigate the anti-biofilm and antioxidant abilities of cell-free

supernatant (CFS_{KC27L}) and different concentrations (0.5 and 1 mg mL^{-1}) of exopolysaccharide (EPS_{KC27L}), for characterizing the lyophilized EPS of *L. salivarius* KC27L.

2. Materials and Methods

2.1. Isolation of Lactobacilli

The fecal samples of free-range chickens were collected from seven different districts of Ankara/Türkiye. Five g of fresh feces collected from chickens at different intervals was homogenized in 50 mL of phosphate-buffered saline (PBS, pH 7.2) with a mixer for 1 min to count and isolate the lactobacilli. After homogenization, 0.1 mL of proper dilutions of the homogenates spread in MRS agar (Man-Rogosa and Sharp, pH 6.2±0.2, Merck, Darmstadt, Germany) plates. All plates were incubated aerobically for 48–72 h at 37°C. Dominant slimy colonies were selected and purified by sequential streaking using the same medium. A total of 26 lactobacilli isolates were initially examined with Gram stain, and their cell morphology, motility, and catalase activity were determined (Li et al., 2021).

2.2. Screening of high EPS-producing lactobacilli, extraction, and partial purification of the EPS

The bacterial cultures were kept at 100°C for 15 min and were removed by centrifugation at 4000 g for 20 min. Trichloroacetic acid (TCA, Merck, Darmstadt, Germany) was added to 1.7 µL of the supernatant at a final concentration of 85% (w/v), stored at 4 °C for two h, and the precipitated proteins were removed by centrifugation at 12000 g for 30 min at 4 °C. The clear supernatant was gathered, and ethanol was put into the supernatant at a 3:1 (v/v) ratio and held overnight at 4 °C. The pellet, including EPS, was centrifuged at 12000 g for 30 min at 4 °C. The EPS was treated with cold pure ethanol, without vortexing, and alcohol was flushed at 40 °C (Li et al., 2016). The residue was dissolved in ultrapure water and stored at −40 °C for further analysis. Using glucose as a standard, the phenol-sulfuric acid method determined the total quantity carbohydrates in the EPS (Dubois et al., 1956). The protein ingredient of EPS was designated with the Bradford method (Takakuwa et al., 2023).

2.3. Molecular identification of the selected isolate

Lactobacillus sp. KC27L was selected among the 26 isolates because of the highest EPS production capacity. Molecular identification performed for *Lactobacillus* sp. KC27L. DNA was extracted using a Genomic DNA purification kit (Thermo Scientific, Waltham, Massachusetts, USA). The purity and amount of genomic DNA was specified in the ELISA (Epoch) ($OD_{260}/_{280}$).

The amplification of 16S rDNA was carried out using universal primers (dos Santos et al., 2019). PCR analysis practised at RefGen Gene Research and Biotechnology Company. The sequence information obtained was analyzed with FinchTV (Version 1.5.0, United States) software, and the similarity with which microorganism sequence was determined by comparing it with other databases of known sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) program.

2.4. Preparation of CFS_{KC27L} and EPS_{KC27L}

For the cell-free supernatant (CFS_{KC27L}) preparation, *L. salivarius* KC27L density was arranged to be 10^9 CFU mL^{-1}. The culture was inoculated in MRS broth and incubated at 37 °C for 24 h. The homogeneous suspension was centrifuged at 10,000 × g, 4 °C for 15 min. The CFS_{KC27L} was gathered by filtration owing to a cellulose acetate membrane according to the Loh et al. (2009) method and stored at -20 ºC for later use.

For the partial purification of EPS_{KC27L}, extracted EPS_{KC27L} was dialyzed through a ten kDa membrane against distilled water at 4 °C for 72 h to eliminate low molecular weight impurities, and the remnant was lyophilized overnight. Lyophilized EPS (EPS_{KC27}) was prepared from the 10 mg/mL stock solution to a final volume of 0.5 or 1 mg mL^{-1}.

2.5 Anti-biofilm activity

Three strains (*Escherichia coli* ATCC 11229, *Enterococcus feacalis* 29212, and *Staphylococcus aureus* EB1) that previously exhibited the highest biofilm capacity (cut-off value: 1.052<2.073) in our laboratory (Bikric et al., 2022) selected for anti-biofilm experiments. The assessment of CFS_{KC27L} and 0.5 and 1 mg mL^{-1} EPS_{KC27L} showed their potential to prevent biofilm formation of three reference pathogen strains by adapting the microplate protocol described by Chaieb et al. (2011). Inulin (Sigma, St. Louis, Missouri, USA) (0.5 and 1.0 mg mL^{-1}), a plant-sourced prebiotic, was used as a control. Wells containing only medium were utilized as a negative control, and wells containing pathogen

bacteria were utilized as a positive control. The density of the bacteria was adjusted to 1.0 $x10^8$ CFU mL^{-1}, and then pathogens were added (180 µL) to the plate well together with 20 µL CFS_{KC27L} / EPS_{KC27L}. After incubating at 37 °C, 100 rpm for 24 h, the plate well was treated with distilled water. According to crystal violet method anti-biofilm activity was determined.

The anti-biofilm activity was determined using the below formula:

$$\text{Anti} - \text{biofilm } \% = [a/(a + b)] \text{ x } 100 \quad \text{(Eq.1)}$$

a: biofilm OD_{570} nm, and

b: planktonic OD_{570} nm.

2.6 Antioxidant activity

Three different methods designated to detect antioxidant activity. CFS_{KC27L} and different concentrations (0.5 and 1.0 mg mL^{-1}) EPS_{KC27L}, inulin, and ascorbic acid (control) were used as samples.

DPPH scavenging activities of the samples designated utilizing the method declared by Li et al. (2014). Water is utilized as a blank, and DPPH in methanol is regarded as a control. The percentage of free radical scavenging activity detected from the following equation:

$$\% \text{ Scavenging } [A0 - (A1 - A2)]/A0 \text{ x } 100 \quad \text{(Eq.2)}$$

A_0: the absorbance of the control (water)

A_1: the absorbance of the sample

A_2: the absorbance of the sample under identical conditions as A_1 with water instead of DPPH solution.

The metal chelating ability of the samples was detected according to the method declared by Qiao et al. (2009). Sterile distilled water and EDTA-Na (Merck, Darmstadt, Germany) were used as the blank and positive control, respectively.

Results are given as the percentage chelating ability of ferrous ions by applying the following formula:

$$\text{The chelating ability on ferrous ion } (\%) = [(\text{Ablank} - (\text{A sample} - A0)/\text{Ablank})] \times 100 \quad \text{(Eq.3)}$$

A_0: The absorbance of the sample under identical conditions

A_{sample}, with water instead of $FeCl_2$ solution.

In the superoxide radical scavenging activity, 50 mM phosphate buffer (pH 8.34) and samples were mixed and stored for 20 min at 25°C. 3 mM pyrogallol (Merck, Darmstadt, Germany) was incorporated. The absorbance value was measured at 325 nm (Wang et al., 2015). Results are given as the percentage of superoxide radical scavenging activity applying the formula below:

$$\text{Superoxide radical scavenging activity } (\%) = [(A0 - A1)/A0] \times 100 \quad \text{(Eq.4)}$$

A_1: The absorbance value of the samples

A_0: The absorbance values of the blank.

2.6 Characterization of EPS_{KC27L}

The molecular mass (Mw) of EPS_{KC27L} designated by size-exclusion chromatography (SEC) with an Agilent 1200 series system fitted out with a PL aqua gel-OH MIXED-H column and a refractive index detector at a National Nanotechnology Research Center (UNAM) at Bilkent University according to the method of Boymirzaev et al. (2013). First, 1.0 mg mL^{-1} EPS_{KC27L} was dissolved with ultra-pure water and filtered by a 0.45 µm filter (Millipore). Then, ten µL of EPS_{KC27L} was injected and eluted with 0.2–0.8 M $NaNO_3$ (PubChem, Bethesda, USA) at a flow rate of 0.6 mL min. Pullulan (Merck, Darmstadt, Germany) is used as standard.

Analysis of monosaccharide composition, EPS_{KC27L} was solved with ultra-pure water. Hydrolysis of EPS_{KC27L} (10 mg mL^{-1}) was performed with 4 M HCl (Merck, Darmstadt, Germany) at 90 °C for three h and neutralized with 1 M

NaOH (pH 7.0) (Ledezma et al., 2016). After the hydrolysate was dried under vacuum, aliquots of 0.5 mL of methanol (Merck, Darmstadt, Germany) were put to remove the remnant. Monosaccharides determined by HPLC (AGILENT 1260 series) with Metacarp 67 °C (300 mm x 6.5 mm) column using water as mobile phase at a flow rate of 0.5 mL min^{-1}. The elution was performed with a detector (VARIAN 350 RI) at the Middle East Technical University Central Laboratory.

Nuclear Magnetic Resonance (NMR) spectroscopy was analyzed at Çankırı Karatekin University Research Center to determine the sugar residues. ^{1}H-NMR spectra of EPS_{KC27L} recorded at 25 °C on an Agilent, 600 MHz, 14.1 Tesla Premium Compact NMR instrument with the chemical shifts (δ) informed in parts per million (ppm). Lastly, 30 mg of EPS_{KC27L} sample dissolved in 500 µL deuterium oxide (D_2O).

2.7 Statistics

SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was utilized for the analyzing data. The statistical analysis was performed to calculate variance analysis (ANOVA). All tests are carried out in triplicate. In the anti-biofilm study, the Tukey test was used to designate the significant differences between pathogenic bacteria. The variances were equally distributed in the antioxidant activity study; the Tukey test was applied to determine significant differences between the methods.

3. Results

3.1. Screening of high EPS-producing lactobacilli and bacteria identification

In the current research, a total of 26 isolates from free-range chicken fecal samples were used. A total of 26 Gram-positive and catalase-negative isolates were isolated from MRS agar and considered lactobacilli. Following the primary characterization, the isolates were examined for their capacity to produce exopolysaccharides. Different amounts of exopolysaccharide production were determined for each isolate (*Table 1*). The maximum efficiency of EPS production was 353 mg L^{-1} for the KC27L isolate, while the minimum yield of EPS was found at 8 mg L^{-1} for the KC21L isolate. In further studies, the highest exopolysaccharides-producing isolate (KC27L) was used. BLAST screening was performed on both "forward" and "reverse" sequences in high-EPS production isolate. The "forward" sequences and the "reverse" sequence with the "reverse complement" were concatenated in Bioedit (Version 7.2, United States) software, and the consensus sequences obtained after this concatenation were compared with known sequences in the NCBI database using BLAST. The highest EPS-producing isolate (KC27L) is defined as *Ligilactobacillus salivarius* based on the 16S rRNA gene sequence (Accession No: SAMN24016084).

Table 1. Exopolysaccharides (EPS) production by isolates of lactobacilli

Isolate No	EPS (mg L^{-1})	Isolate No	EPS (mg L^{-1})
KC11L	78±7	KC46L	38±7
KC21L	8±0	KC56L	338±0
KC12L	19±0	KC66L	350±0
KC23L	32±0	KC76L	15±3
KC33L	58±0	KC86L	237±4
KC43L	117±0	KC96L	280±0
KC15L	213±0	KC116L	256±0
KC25L	189±0	KC126L	52±0
KC35L	119±0	KC136L	296±9
KC45L	226±0	KC17L	64±2
KC65L	277±6	KC27L	353±6
KC16L	45±1	KC37L	77±2
KC36L	161±0	KC47L	276±6

3.2. Anti-biofilm effect

Biofilm prevention capacity of the CFS_{KC27L}, EPS_{KC27L}, and inulin (control) tested on three pathogen test bacteria, which demonstrated a robust biofilm formation (cut-off value: 1.052<2.073). The results obtained were expressed as inhibition percentages of biofilm development and presented in *Table 2*. In cell-free supernatant, the highest antibiofilm effect was 47% at *E. coli* ATCC 11229. The EPS_{KC27L} (1 mg mL^{-1}) reduced 87% biofilm on *E. coli* ATCC 11229. While inulin (1 mg mL^{-1}) inhibited 72% of biofilm formation by *E. coli* ATCC 11229.. The amount of biofilm inhibition enhanced as the exopolysaccharide concentration increased. According to the statistical analysis, a significant difference of 0.05 level was found between the biofilm inhibition of the pathogenic bacteria used. Also, a statistically significant difference of 0.05 level was found between CFS_{KC27L}, EPS_{KC27L}, and inulin (1 mg mL^{-1}) applications to prevent biofilm formation of pathogenic bacteria, depending on the type of microorganism used (*Table 2*). Also, biofilm removal percentages were lower at CFS compared to both concentrations of EPS.

Table 2. Antibiofilm activities of postbiotic, prebiotic (EPSKC27L), and inulin

Test Bacteria	Inhibition of biofilm (%)				
	Postbiotic [a,b,c]	Prebiotic (EPS_{KC27L}) [a,b,c]		Inulin [a,b,c]	
		0.5 mg mL^{-1}	1 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}
S. aureus EB1 [a]	28±4	67±5	76±5	53±1	67±4
E. coli ATCC 11229 [b]	47±2	79±4	87±2	68±4	72±3
E. faecalis ATCC 29212 [c]	46±1	66±2	82±1	51±5	59±6

The mean difference in [a,b,c] is significant at the 0.05 level.

3.3. Antioxidant effect

In the current research, the antioxidant activity of CFS_{KC27L}, different concentrations (0.5-1 mg mL^{-1}) of EPS_{KC27L}, 0.5-1 mg mL^{-1} inulin (control), and 0.5-1.0 mg mL^{-1} ascorbic acid (control) evaluated by diverse methods: DPPH, superoxide anion scavenging, and metal ion chelating. Among the three methods, the highest activity was determined as 79.6% for 1 mg mL^{-1} EPS_{KC27L} in DPPH radical scavenging activity, while the lowest activity was 18.6% for 0.5 mg mL^{-1} $EPS_{KC27}L$ in Fe^{+2} chelating activity (*Table 3*). Inulin, used as a control at a concentration of 0.5 mg mL^{-1}, exhibited no antioxidant activity. According to the statistical analysis, a significant difference of 0.05 level was detected between the methods used. Also, a statistically significant difference of 0.05 level was found between the applications used (CFS_{KC27L}, 1 mg mL^{-1} EPS_{KC27L}, 1 mg mL^{-1} inulin, and 1 mg mL^{-1} ascorbic acid).

Table 3. Antioxidant activities of postbiotic, prebiotic (EPSKC27L), and inulin

Methods	Antioxidant activity (%)						
	Postbiotic [a,b,c]	Prebiotic (EPS_{KC27L}) [a,b,c]		Inulin [a,b,c]		Ascorbic acid [a,b]	
		0.5 mg mL^{-1}	1 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}	0.5 mg mL^{-1}	1 mg mL^{-1}
DPPH radical [a]	75.7±1.0	73.4±1.9	79.6±4.7	-	73.2±2.7	84.3±0.5	93.2±0.2
Fe^{2+} Chelating activity [b]	19.3±0.0	18.6±3.0	24.9±1.2	-	23.2±1.3	78.6±0.4	89.3±0.2
Superoxide anion radical [c]	61.3±1.9	44.8±2.5	61.6±3.3	-	32.3±1.1	82.4±0.4	91.4±0.1

-: No activity
The mean difference in [a,b,c] is significant at the 0.05 level.

3.4 Characterization of EPS_{KC27L}

In characterization studies, size-exclusion chromatography is used to forecast the Molecular weight (Mw) of EPS_{KC27L}. The Mw of EPS_{KC27L} was determined as $1.6x10^3$ and $6.4x10^4$ Da, consisting of two fractions.

HPLC designates the monosaccharide composition of EPSKC27L. The monosaccharide composition of EPS_{KC27L} indicated that the ratio of each sugar varied in percentages, such as galactose (0%), mannose (13.8%), and arabinose (14.6%), fructose (18.5%), and glucose (53.1%).

1H NMR was mainly used to designate polysaccharide molecules of glycosidic bond configuration. The 1H NMR spectrum of a polysaccharide could usually separate into three major regions: The anomeric region (5.5–4.5 ppm), the ring proton region (3.0–4.5 ppm), and the alkyl region (1.2–2.3 ppm).

4. Discussions

Microbial exopolysaccharides, a multifunctional compound with many applications in the pharmaceutical and food industries, are essential for human, animal, and poultry health (Rajoka et al., 2019). The exopolysaccharides generated by LAB have been declared to have several physiological functional properties, including antioxidant, antitumor, immunomodulatory, anti-inflammatory, anti-biofilm, and cholesterol-lowering potential (Wang et al., 2020). In the current study, the isolates showed EPS production capacity in the 8-353 mg L^{-1} (*Table 1*). Previous studies reported the quantity of EPS generation as 151 mg L^{-1} for *L. salivarius* ZNY9 (Yuksekdag et al., 2014), 24 mg L^{-1} for *L. paracasei* HCT (Xu et al., 2010), and 430 mg L^{-1} for *L. salivarius* E2 strain (Mercan et al., 2015). In this study, the amounts of EPS production by *Lactobacillus* isolates showed similar results to those reported in the literature. The EPS production amounts of the LAB vary and depend on the species, type of strain, isolation sources, and growth conditions (medium, temperature, pH, carbon source, concentration, incubation time, and inoculum size) (Ren et al., 2016).

It determined that the isolate with the highest EPS capacity (353 mg L^{-1}) selected for other studies was *Ligilactobacillus salivarius* because of molecular identification. There is minimal information regarding the topological differences between lactobacilli in the chicken gastrointestinal system and feces. *Lactobacilli* are the most widespread species of chicken, fattening pigs, beef cattle, and humans. *L. salivarius* strains are important members of the animal microbiota in the gastrointestinal tract (Raftis et al., 2011).

Three pathogenic bacteria that exhibited a robust biofilm formation (1.052<2.073) were selected to be used in anti-biofilm studies among 12 pathogenic bacteria whose ability for biofilm formation was previously determined by Bikric et al. (2022). In the current study, biofilm prevention capacity was detected by three pathogenic bacteria (*E. coli* ATCC 11229, *E. feacalis* 29212, and *S. aureus* EB1). Using biofilms produced by three pathogenic bacteria, the inhibition activity of CFS_{KC27L} at two different concentrations (0.5 and 1 mg mL^{-1}) of EPS_{KC27L} and inulin was determined (*Table 2*). In all conditions, the highest biofilm inhibition was realized by *E. coli* ATCC 11229. The highest biofilm inhibition at CFS_{KC27L} was detected as 47% by *E. coli* ATCC 11229. Sevin et al. (2021) determined the anti-biofilm activity of postbiotics against ruminant mastitis-causing pathogens (*Staphylococcus aureus* (MRSA) ATCC 43300, *Streptococcus agalactiae* ATCC 27956, and *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957). The highest inhibition was determined at 25 mg mL^{-1} (96.2% by MRSA 43300, 95.10% by *S. agalactiae* ATCC 27956), and 17.5 mg mL^{-1} (92.65% *S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957). On the other hand, in cell-free supernatants in this study, the biofilm inhibition value was lower compared to Sevin et al.'s (2021) results. Probiotics' primary mechanism of action on pathogens depends on the production and number of bioactive compounds produced during metabolism (Ohshima et al., 2019). This could be due to the different strain resources, metabolite differences, and their amount in cell-free supernatant. This study did not investigate the chemical composition of CFS_{KC27L}, limiting identification of the specific metabolite(s) responsible for the observed biofilm inhibition. The EPS_{KC27L} has different rates of antibiofilm effect against the pathogen bacteria tested, and the highest inhibition was found in 1 mg mL^{-1} as 87% by *E. coli* ATCC 11229. Biofilm activity in three pathogen bacteria decreased by more than 0.5 mg mL^{-1} in 1 mg mL^{-1} in inulin and EPS_{KC27L}. Biofilm removal was higher in EPS_{KC27L} than inulin (*Table 2*). The results showed that EPS_{KC27L} has a broad anti-biofilm activity against biofilm-forming bacteria. Therefore, EPS_{KC27L} could be used as an alternative to inulin to inhibit the growth of biofilm-forming bacteria. The anti-biofilm effect against biofilm-forming bacteria was higher in both EPS_{KC27L} concentrations than in CFS_{KC27L}, whose metabolites and their concentrations are unknown. While EPS (a single metabolite) acts directly, all metabolites (CFS) may interact, and biofilm inhibition may be reduced. EPS may exert its anti-biofilm activity through several mechanisms. EPS could exert its anti-biofilm activity by altering bacterial cell surfaces (Rendueles et al., 2013), Preventing bacterial cells from adhering to surfaces (Valle et al., 2006), or also acting as signaling molecules to suppress gene expression involved in biofilm formation (Kim et al., 2009; Wang et al., 2015).

Because of their potential for scavenging free radicals and binding transition metal ion catalysts, besides their reducing activity and capability to prohibit chain initiation, natural antioxidants play an essential role against various diseases and aging processes (Wang et al., 2015). The DPPH free radical has an unpaired valence electron at one atom of its nitrogen bridge, and this causes a significant decrease in exposure to the proton radical scavenger. Also, it has a powerful, damaging effect on body organs (Li et al., 2014).

This research carried out antioxidant activities of CFS_{KC27L}, EPS_{KC27L}, inulin, and ascorbic acid (positive control). DPPH radical antioxidant activity of CFS_{KC27L} was determined as 75.7%. Izuddin et al. (2020) reported cell-free

supernatant produced from *L. plantarum* RG14, RG11, and TL1 used for antioxidant activity of DPPH, and values obtained as TL1 67%, RG11 72%, and RG14 74%. Our study and other results (Izuddin et al., 2020) are close. The scavenging activities of different concentrations (0.5 and 1 mg mL^{-1}) of EPS_{KC27L} on DPPH radicals changed depending on the concentration. Maximum scavenging activity was observed at 1 mg mL^{-1} at EPS_{KC27L} (79.6%). Similarly, the DPPH radical scavenging activity of the commercial prebiotic inulin was found to be 73.2%. Our results demonstrated that all applications had a higher scavenging activity of DPPH and were much better than previously reported results for lactobacilli themselves at 1 mg mL^{-1}: 78.7% for 4 mg mL^{-1} EPS (Sevin et al., 2021), 75.98% for 4 mg mL^{-1} EPS (Rani et al., 2018), 53.63% for 5 mg mL^{-1} EPS (Trabelsi et al., 2017).

Metal chelating activity, one of the antioxidant mechanisms, decreases the concentration of the catalyzing transition metal in lipid peroxidation. Iron is an essential lipid oxidation pro-oxidant among transition metals because it shows high reactivity (Min et al., 2019; Rajoka et al., 2019). In the current research, Fe^{2+} chelating activity of CFS_{KC27L} was 19.3%. The chelating activity was higher than in 1 mg mL^{-1} EPS_{KC27L} (32.3%) than inulin (23.2%). Until now, ferrous ion chelating activity has rarely been utilized to evaluate cell-free supernatant's antioxidant capacity and LAB-derived EPS. Min et al. (2019) reported that the ferrous ion chelating activities of EPS103 rose to the maximum value of 69.7% when its concentrations arrived at 10 mg mL^{-1}. In another study, EPS111 exhibited a much higher ferrous ion chelating impact at 4 mg mL^{-1} concentration (72.8%) (Rajoka et al., 2019). The low activity in our study could be due to the low concentrations used.

The superoxide anion is a free radical and is evaluated as a reactive oxygen species (ROS) interfering with biological macromolecules, resulting in tissue damage. It supports the generation of other ROS, such as hydroxyl radical, hydrogen peroxide, and singlet oxygen (Rajoka et al., 2019). Therefore, in this study investigated the superoxide radicals scavenging capacity of CFS_{KC27L} and different concentrations of EPS_{KC27L}. The superoxide anion scavenging activity observed in CFS_{KC27L} was 61.3%. The superoxide anion scavenging activity was higher than in 1 mg mL^{-1} EPS_{KC27L} (61.6%) compared to 1 mg mL-^{1} inulin (32.3%). Researchers reported the superoxide anion scavenging activity of some lactobacilli EPS; *L. plantarum* EPS (18%) (Zhang et al., 2016), *L. rhamnosus* EPS115 (5.9%), and *L. rhamnosus* EPS 111 (29.4%) (Rajoka et al., 2019). In our study, the superoxide anion scavenging activity was higher at both 0.5 and 1 mg mL^{-1} concentrations compared to these studies. The scavenging capacity is mainly attributed to the direct binding of the EPS molecules with superoxide radicals to form steady radicals, which could end the radical chain reaction (Rajoka et al., 2019). However, our results in three different antioxidant determination methods are higher and/or similar to other studies, with lower values observed than the ascorbic acid used as the positive control group. Since antioxidants harm the body (Wang et al., 2023), new safe and green antioxidants must be investigated. CFS_{KC27L} and EPS_{KC27L}, whose antioxidant activities are examined in this study, may be natural antioxidant agents. As free radical scavenging activity is utilized for a preliminary evaluation, *in vitro* and *in vivo* experiments sometimes give different results. Therefore, a definitive judgment can be made after more comprehensive research on the *in vivo* antioxidant activity of CFS_{KC27L} and EPS_{KC27L}.

The Mw of EPS_{KC27L} produced by LAB is a crucial characteristic influencing its bioactivities. Since the low molecular weight of EPS could easily pass through multiple cell membrane barriers, it exhibits better bioactivity (Li et al., 2016). In the current study, the molecular weight of EPS_{KC27L} was determined as $1.6x10^3$ and $6.4 x10^4$ Da consisting of two fractions. Similarly, Tukenmez et al. (2019) detected the molecular weight of the EPSs, which was isolated from *L. plantarum* GD2, *L. rhamnosus* E9, and *L. delbrueckii* ssp. *bulgaricus* B3 consisting of two fractions ranging from 10^2 to 10^4 Da. However, some studies on the structure analysis of LAB-derived EPS reported that the EPS had a one fraction with a molecular weight of $1.86x10^5$ for *L. gasseri* FR4 (Izuddin et al., 2020) and 3.32×10^5 Da for *Streptococcus thermophilus* EPS333 (Ren et al., 2016). In contrast, some researches declared that the EPS had three fractions (Li et al., 2014). Bikric et al. (2022) declared that the Mw EPS_{BIS312} obtained from the *L. salivarius* BIS312 strain was determined as 9.0×10^3, 2.8×10^4, 7.2×10^5, and 6.8×10^6, while the Mw of the EPS_{BIS722} obtained from *L. salivarius* BIS722 strain was designated to be 1.3×10^4, 5.4×10^4, 8.9×10^5, and 6.9×10^6 consisting of four fractions. Diverse bacterial resources, culture conditions, and heritable characters could cause differences in the molecular weight of EPS (Min et al., 2019).

LAB-derived EPS mainly comprises glucose, galactose, and mannose with different molar ratios (Wang et al., 2010; Li et al., 2014). In this research, EPS_{KC27L} contained different monosaccharides (glucose, fructose, arabinose, and mannose) and showed varying percentages (53.1%, 18.5%, 14.6%, and 13.8%, respectively). Similarly, Rani et al.

(2018) noticed that the EPS generated by *L. gasseri* FR4 obtained from the native chicken was primarily composed of glucose (65.31%), mannose (16.51%), galactose (8.45%), rhamnose (6.55%), and a small fraction of fucose (3.18%). As announced by Bikric et al. (2022), the EPS_{BIS312} and EPS_{BIS722} involved glucose (34.53, 35.31%), mannose (26.41, 27.60%), rhamnose (13.58, 11.28%), and galactose (25.47, 25.82%), but the other monosaccharides (fructose and arabinose) were not determined. The difference in the monosaccharide composition of LAB-derived EPS could be related to different strain types, growth conditions, and carbon-resourced media content (Wang et al., 2010).

The anomeric region NMR signals, which can be divided into two categories, are frequently used to distinguish the anomeric protons of sugar residual in polysaccharides (5.5–4.9 ppm for α-anomers and 4.9–4.5 ppm for β-anomers) (Zhao et al., 2020). In the anomeric region of the 1H NMR spectrum of EPS_{KC27L} (Data not shown), three proton signals occurred at 5.2, 5.0, and 4.9 ppm, indicating that EPS_{KC27L} was mainly composed of three types of sugar residues. The peaks at 5.2 ppm correspond to the anomeric proton of α-D-mannose, 5.0 ppm to α-D-glucose and 4.9 ppm to β-D-glucose indicating that EPS_{KC27L} contained α and β configurations (Ismail et al., 2013; Ai et al., 2016). In general, α- and β-configurations are simultaneously present in hetero-polysaccharides from LAB (Ai et al., 2016). In addition, the strong signal at 4.7 ppm is presented in the spectrum attributed to the solvent hydrogen-deuterium oxide HOD (Shu et al., 2020). The signals procured in the spectrum between 4.4 and 3.2 ppm were owing to the ring protons bonded to C2–C6 and insufficiently eliminated because of the chemical shifts of the complex and heterogeneous nature of EPS_{KC27L} (Ayyash et al., 2018).

5. Conclusions

Although using different species of lactobacilli as probiotics in chickens has shown beneficial effects, there still needs to be more effective data to select promising lactobacilli and their exopolysaccharides and cell-free supernatant. This study determined that cell-free supernatant (CFS_{KC27L}), EPS obtained from *L. salivarius* KC27L (EPS_{KC27L}), had anti-biofilm and antioxidant activity. The study of Yildiz et al. (2023) determined that EPS_{KC27L} (12.50, 25.00, 50.00, and 100.00 μg mL^{-1}) did not cause a remarkable genotoxic impact in chromosome aberration (CA), sister chromatid exchange (SCE), micronucleus (MN), and comet assays. EPS_{KC27L} significantly reduced the CA, SCE, and MN frequency induced by mitomycin-C (0.20 μg mL^{-1}) methyl methanesulfonate (5.00 μg mL^{-1}). EPS_{KC27L} also significantly decreased DNA damage caused by hydrogen peroxide (100 μM). Utilizing naturally derived EPS as a poultry feed additive holds promise, potentially offering superior or comparable results to commercially available prebiotics like inulin. However, more studies should be conducted to recommend EPS obtained from strains as an additive in poultry feeding, and animal trials should also support research. With their antioxidant and anti-biofilm activities, the EPS_{KC27L}, regarded as a functional food ingredient candidate, could add value to the food and reduce the use of artificial additives.

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Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Authors Contribution Statement

Concept: Yuksekdag, Z., Design: Celik, K., Data Collection or Processing: Celik, K., Yuksekdag, Z., Cinar Acar, B., Literature Search: Celik, K., Cinar Acar, B., Writing, Review and Editing: Celik, K., Yuksekdag, Z., Cinar Acar, B., Kara, F.

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