



Gene Expression, Structural Characterization, and Functional Properties of Exopolysaccharide Produced from Potential Probiotic *Enterococcus faecalis* NOC219 Strain

Nilgün Özdemir¹

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Abstract

This study aimed to reveal the structural characterization and functional properties of microbial EPS-NOC219 material produced by the *Enterococcus faecalis* NOC219 strain with high EPS yield isolated from yogurt, with simultaneously, demonstrating the potential of this EPS for future industrial applications. According to the results of the analyses made for this aim, it was determined that the NOC219 strain contains the *epsB*, *p-gtf-epsEFG*, and *p-gtf-P1* genes. In addition, it was also revealed that the EPS-NOC219 structure is expressed by the *epsB*, *p-gtf-epsEFG*, and *p-gtf-P1* genes and has a heteropolymeric feature consisting of glucose, galactose, and fructose units. According to the results of the analyses made for this aim, it was determined that the EPS-NOC219 structure, which was produced from the NOC219 strain containing the *epsB*, *p-gtf-epsEFG*, and *p-gtf-P1* genes, had a heteropolymeric structure consisting of glucose, galactose, and fructose units. On the other hand, it was shown that this structure had a thickener property, high heat stability exhibited a pseudoplastic flow behavior, and had a high melting point. This showed that the EPS-NOC219 had high heat stability and could be used as a thickener in heat treatment processes. In addition, it was revealed that it is suitable for plasticized biofilm production. On the other hand, the bioavailability of this structure was demonstrated with its high antioxidant activity (55.84%) against DPPH radicals and high antibiofilm activity against *Escherichia coli* (77.83%) and *Listeria monocytogenes* (72.14%) pathogens. These results suggest that the EPS-NOC219 structure may be an alternative natural resource for many industries as it has strong physicochemical properties and a healthy food-grade adjunct.

Keywords *Enterococcus faecalis* NOC219 · Exopolysaccharide · Biopolymer · Bioactivity · Structural properties

Highlights

- *E. faecium* NOC219 produce EPS harbors *epsB*, *p-gtf-epsEFG*, and *p-gtf-P1* genes.
- *E. faecium* NOC219 produce EPS-NOC219 has probiotic potential.
- EPS-NOC219 structure has a high melting point and a pseudoplastic flow behavior.
- EPS-NOC219 has high heat stability and can use as a thickener in heat treatment processes.
- EPS-NOC219 has high antioxidant activity and high antibiofilm activity.

✉ Nilgün Özdemir
nilgun.ozdemir@omu.edu.tr

¹ Department of Food Engineering, Ondokuz Mayıs University, Engineering Faculty, 55139 Samsun, Turkey

Introduction

In recent years, one of the most striking issues in the food industry is the microbial metabolites produced by microorganisms in the microbiota of fermented foods [1]. One of these microbial metabolites is exopolysaccharide (EPS), which has a high-molecular-weight natural-carbohydrate polymer structure [2]. EPS structures are used for different functions in many areas such as textiles, food, medicine, and pharmaceuticals. It is known that especially when EPS is used in the food industry, it can affect a food's technological and functional properties [3]. For example, some EPS structures have physicochemical properties such as thickener, emulsifier, and stabilizer [4, 5]. On the other hand, some EPSs have bioactive effects such as antibacterial, antiviral, antidiabetic, antitumor, antioxidant, anti-inflammatory, anti-hypoglycemic, hypocholesterolemic antihypertensive, cholesterol-lowering, gastro-protective, and prebiotic properties [6–8]. EPS structures even affect the sensory properties of food [3]. EPS-producing microorganisms in the food industry commonly belong to the lactic acid bacteria (LAB) species [9]. LAB-derived EPSs are natural, non-toxic, bio-products with diverse chemical structures and biological activities [3]. Therefore, although LAB-EPS structures are a very popular research topic, research on them is still insufficient.

Bacteria of the genus *Enterococcus* belong to the LAB group and are generally known as low-grade pathogens. However, their role as primary pathogens remains in question [10]. Some strains of this genus, especially some of the *Enterococcus faecalis* strains, do not show pathogenicity [11]. They have even been found to have potential health-promoting benefits such as antidiabetic, antimicrobial, antioxidant, and proteolytic activities, along with probiotic characteristics [2, 12]. One of these properties is EPS production. In the literature, the discoveries of a few types of EPS produced by some *Enterococcus faecalis* strains have been documented [4]. However, the number of those gaining industrial importance with significant market appeal is limited, especially regarding their use as biomaterials. In addition, the high EPS production yield of some *Enterococcus faecalis* strains reveals the necessity of bringing the EPSs produced by these strains to the industry.

This study aimed to reveal the structure and biological properties of microbial EPS material produced by an *E. faecalis* strain with high EPS yield isolated from “yogurt,” simultaneously, demonstrating the potential of this EPS for future industrial applications.

Materials and Methods

Materials

In this study, an isolate that codes NOC-219 was used as material. This isolate was isolated from 15 artisanal yogurt samples produced and consumed by the local people living in Samsun province of Turkey. The NOC-219 isolate was selected as the highest-yielding EPS-producing strain among the 325 indigenous LAB isolates from the traditional yogurt in Ondokuz Mayıs University, Department of Food Engineering, Biotechnology Laboratory. The other materials were purchased the analytical grade chemicals and reagents from Sigma-Aldrich Co. (USA) and Supelco Co. (USA), and the microbial growth mediums from Merck Co. (Germany).

Selection and Identification of EPS-Producing Isolate

The 325 indigenous LAB isolates were isolated using the previously described methodology [13] from the yogurt samples. Colonies with mucoid phenotype were selectively picked and subcultured in modified MRS-sucrose agar, which contains 40 g/L sucrose instead of glucose 20 g/L [14]. Among LAB isolates producing EPS, the NOC219 isolate, which produced the maximum amount of EPS, was chosen for further analysis.

The selected isolate was identified using 16S rRNA gene sequencing. For this, primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1491R (5'-ACGGCTACCTTGTTACGACTT-3') were used to amplify the 1.5 kb 16S rRNA gene of the NOC219 isolate. PCR reaction was performed with the following program: 95 °C for 15 min, followed by 35 cycles 95 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min, and 72 °C for 10 min final extension. Subsequently, the amplicons were sequenced by MacroGen Inc. (Amsterdam, Netherlands). The similarity was determined with the NCBI database (97–100%).

Determination of EPS Genes of NOC219 Strain

The *eps* genes in the genome of this isolate were identified with PCR using specific primer pairs. The target gene regions were *epsA* (putative transcriptional regulator), *lev* (levansucrase) and *gtf* (glucansucrase), *epsB* (putative polymerization and chain length determination protein gene), and *p-gtf* (putative priming glycosyltransferase gene), representing genes required for the production of homopolymeric and heteropolymeric EPS, respectively. Table 1 shows the primers, the target amplicon lengths [13, 15], and the PCR conditions.

Determination of Potential Probiotic Properties of NOC219 Strain

Resistance to Simulated Gastric Medium and Intestinal Medium Tests

First, 18-h active cell culture of the NOC219 strain was centrifuged. Subsequently, the cell pellet was collected and washed. The viable pellet cells were re-suspended in the simulated gastric medium (PBS solution containing 0.3% pepsin pH 2.0, Sigma-Aldrich) by filtration (filter membrane 0.22 µm), which contained about 8 log CFU/mL. On the other hand, for the resistance test to the simulated intestinal medium, a similarly prepared pellet of the NOC219 strain was re-suspended in the simulated gastric medium (PBS solution containing 0.5% bile salt (w/v), 0.2% pancreatin (Sigma-Aldrich) (w/v), 0.1% trypsin (w/v) pH 8.0, Sigma-Aldrich) by filtration (0.22 µm) [16, 17].

The media were incubated at 37 °C at 100 rpm. For the cell viability test in gastric medium, samples were taken at 0, 60, and 120 min. For cell viability tests in medium intestinal resistance, tests were taken at 0, 120, and 240 min for cell viability. MRS agar was seeded, and after 48 h of incubation at 37 °C, the results were determined as CFU/mL.

Hemolytic Activity and Antibiotic Susceptibility Tests

The NOC219 strain was streaked in Columbia Blood agar plates (Merck, Germany), which contained 5% defibrinated sheep blood. After incubation (48 h, 37 °C), the hemolytic

Table 1 Primers used for the detection of eps genes

Primer	Sequences (5'-3')	Gene target	Expected amplicon (bp)	PCR conditions
epsA-F	TAGTGACAACGGTTGTACTG	epsA	800	35 cycles of 95 °C (30 s), 49 °C (45 s), 72 °C (1 min)
epsA-R	GATCATTATGGACTGTAC			
epsB-F	CGTAGGATTCGTACGACCAT	epsB	1150	35 cycles of 95 °C (45 s), 58 °C (1 min), 72 °C (1 min)
epsB-R	TGACCAGTGACACTTGAAGC			
epsD/E-F	TCATTTTATTCGTA AAAACCTCAATTGAYGARYTNCC	p-gtf	189	5 cycles of 95 °C (30 s), 67 °C (30 s), 72 °C (30 s); 40 cycles of 95 °C (30 s), 60 °C (45 s), 72 °C (30 s)
epsD/E-R	AATAATTAITACGACCTSWNAYYTGCCA			
DexreuV	GTGAAAGGTA ACTATGTTG	Dexreu-gtf	600	35 cycles of 95 °C (30 s), 50 °C (45 s), 72 °C (1 min)
DexreuR	ATCCGCATTAAAGAATGG			
epsEFG-F	GAYGARVTNCCNARYTNWKAAYGT	p-gtf	1600	35 cycles of 95 °C (45 s), 49 °C (45 s), 72 °C (1 min)
epsEFG-R	TGCAGCYTCWGCCACATG			
G-Lr-Bact-F	ATGAGTTTGGTTGGACCAAGACTCC	P1-p-gtf	276	10 cycles of 95 °C (30 s), 66.5 °C (40 s), 72 °C (30 s); 35 cycles of 95 °C (30 s), 55.2 °C (40 s), 72 °C (30 s)
G-Lr-Bact-R	TTTAATAGGCTCCAGTTGGA			
LevV-F	GAYGNTGGGAYWSNTGGC	Lev	800	35 cycles of 95 °C (30 s), 42 °C (45 s), 72 °C (1 min)
LevV-R	TCNTYYTCRCNSWNRMCAT			
Gtf-F	GAYGNTGGGAYWSNTGGC	gtf	660	35 cycles of 95 °C (30 s), 48 °C (45 s), 72 °C (1 min)
Gtf-R	TCNTYYTCRCNSWNRMCAT			

Y = C or T; R = A or G; W = A or T; K = G or T; S = C or G; M = A or C; V = A, C, or G; N = A, C, G, or T; I = inosine

activity was determined by observation of a clear zone of hydrolysis around the colonies (β -hemolysis), partial hydrolysis, and greenish zone (α -hemolysis), or no reaction (γ -hemolysis).

Broth culture of the NOC219 strain (18 h, 37 °C, adjusted to 0.5 McFarland (8 log CFU/mL)) was cultivated on an MRS agar sterile petri dish with the spread plate method and allowed to dry for 15 min. Afterward, commercially available different antibiotics (metronidazole, ampicillin, erythromycin, amoxicillin, vancomycin, and tetracycline) disks (Bio-analyse, Turkey) were placed upwards and pressed on the agar plates. After incubation at 37 °C for 24 h, the presence of a zone of inhibition around the disks was noted as resistance [18].

Production and Purification of EPS from NOC219 Strain

EPS production and purification processes were carried out by minor modifying the previously reported method [19]. Briefly, the *E. faecalis* NOC219 was grown in a modified MRS-sucrose broth medium in 1 L final volume on a shaker with a speed of 150 rpm at 37 °C for 48 h aerobically. The fermentation medium was adjusted to pH 6 every 6 h using 3 M NaOH. Subsequently, the fermented broth was centrifuged at 10,000×g at 4 °C for 30 min, and the pellet and supernatant parts were separated. The cell-free supernatant was subjected to purification. For the purification, an equal volume of cold ethanol was added to this supernatant and kept at 4 °C overnight for EPS to precipitate. Afterward, the precipitated EPS was collected, some distilled water was added to it, and dissolved. This solution was added to two times the volume of cold ethanol and kept at 4 °C overnight. Subsequently, EPS was separated from the supernatant by re-centrifugation. For the removal of compounds that may be found other than the crude EPS obtained. It was dissolved in pure water and dialyzed (12–14 kDa) for 3 days, and then 10% trichloroacetic acid solution was added and kept at 4 °C overnight. Finally, pure EPS precipitated by centrifugation was obtained. It was lyophilized and stored at –80 °C for further studies.

Determination of Monosaccharide Composition of EPS

The freeze-dried EPS solution prepared at a concentration of 20 mg/mL was taken from 1000 μ L EPS solution, 800 μ L was taken, and 218 μ L of 72% formic acid was added, and kept in a water bath at 95 °C for 2 h. It was then neutralized by adding 500 μ L of 5 M KOH, centrifuged at 12,000 ×g for 5 min, and filtered through a filter (0.45 μ m), was determined by HPLC analysis using a CARBOsep CHO-682 Pb Column (Chrom-tech, 7.8 mm ID × 300 mm, USA) and RID-10 (a refractive index detector) with a mobile phase of H₂O, using glucose, galactose, fructose, and rhamnose standards. The flow rate is 0.7 mL/min and the column temperature is at 25 °C [1].

Determination of Functional Groups of EPS by FTIR Analysis

The major structural groups of the EPS were detected using Fourier transform infrared spectroscopy (ATR-FTIR, Perkin Elmer, Spectrum-Two, USA). For this, 1 mg of lyophilized exopolysaccharide was taken and mixed homogeneously with 100 mg of KBr powder. A disk was pressed into a pellet by using a hydraulic press, and the resulting disk was subjected to FTIR spectral measurement in the range of 4000–400 cm⁻¹ wavelength.

The obtained peaks were evaluated [7, 20]. FTIR spectrum was determined in transmission mode, and the number of scans was 32. The infrared spectral resolution was 4 cm^{-1} .

Differential Scanning Calorimeter (DSC) Analysis of EPS

The thermal property of the EPS was determined using differential scanning calorimetry (DSC 8000, PerkinElmer, Shelton, CT, USA). It was weighed 5 mg from the EPS to aluminum pans and sealed. Then, the sample was analyzed by applying a heating rate of $10\text{ }^{\circ}\text{C}/\text{min}$ in the temperature range of $10\text{--}450\text{ }^{\circ}\text{C}$ in a nitrogen gas atmosphere and using an empty pan as a reference [9].

Rheological Analysis of EPS

An aqueous solution of EPS (4%) was prepared at a pH value of 6.0. Rheological measurements of this solution were carried out using an air-controlled rheometer (Anton Paar, MCR-302, Germany). The cone plate (C35/2 $^{\circ}$ Ti L; 17.5 mm diameter, 2° cone angle, 0.1 mm gap) with plate-controlled temperature ($4\text{ }^{\circ}\text{C}$) was employed to perform the following test.

Determination of Steady Shear Flow Behavior Property of EPS

A shear rate range ($\dot{\gamma}$) of 0.1 1/s to 100 1/s was used in steady shear property measurement. Herschel-Bulkley's model was employed to describe the flow behavior of EPS. The value at a shear rate of 50 1/s was shown as an apparent viscosity value (η_{50}) [21].

Herschel-Bulkley model:

$$\tau = \tau_0 + K(\dot{\gamma})^n \quad (1)$$

- τ_0 shear stress (Pa)
- K consistency coefficient (Pa sn)
- $\dot{\gamma}$ shear rate (s^{-1})
- n flow behavior index

Temperature-Dependent Flow Behavior of EPS

The change in viscosity of EPS (η) was analyzed as a function of temperature from 10 to $80\text{ }^{\circ}\text{C}$. The temperature ramp rate was $3\text{ }^{\circ}\text{C}/\text{min}$ at a constant shear rate of 20 1/s. The activation energy of flow was calculated according to the Arrhenius equation:

Arrhenius model:

$$\eta = \eta_0 e^{\frac{E_a}{RT}} \quad (2)$$

- η shear stress (Pa/s)
- η_0^e frequency factor
- E_a activation energy (J/mol)
- R universal gas constant
- T temperature ($^{\circ}\text{K}$)

Determination of Dynamic Shear Flow Behavior Property of EPS

Amplitude sweep test was applied at the frequency (0.1 Hz) at 4 °C, at between 0.001 and 10 Pa. Frequency sweep test was performed at 4 °C for 120 s at various frequencies ranging from 0.1 to 100 Hz (0.628–628.3 rad/s, 0.1 Pa) to evaluate viscoelastic behavior of EPS at constant strain within the linear viscoelastic region (less than 1%). The obtained elastic or storage modulus (G') and viscous or loss modulus (G'') values were calculated as a function of frequency (Hz).

Scanning Electron Microscopy (SEM) Analysis of EPS

The microstructure of the EPS sample was determined using a scanning electron microscope (SEM; JEOL, Tokyo, Japan). The sample was fixed to the SEM stubs with double-sided tape and coated with a layer of gold-palladium coating ~10 nm thick. Then, the samples were observed at 500, 1000, and 2500 magnitudes at 10 kV [22, 23].

Antibiofilm and Antioxidant Activities of EPS

This test was performed against *Bacillus subtilis* (NRRL-B209), *Escherichia coli* (ATCC-25922), *Listeria monocytogenes* (ATCC-7644), and *Staphylococcus aureus* (ATCC-33862) pathogenic bacteria. Firstly, active cultures (at 37 °C 18 h) of pathogenic microorganisms were prepared. One hundred eighty microliters of nutrient broth, 10 μ L of active pathogenic culture, and 10 μ L of the EPS solution (0.4 mg/mL) were added to the wells of a microtiter plate, and it was incubated for 18 h at 37 °C [24]. After the plate was gently washed with PBS buffer (pH 7.2), crystal violet (0.2 %) was added to it, and it was incubated for 15 min. After washing the plate with distilled water, 100 μ L of acetic acid (30 %, v/v) was added to each well. The absorbance was taken at 570 nm, and biofilm inhibition was measured by the equation: Antibiofilm activity (%) = $(OD_{\text{control}} - OD_{\text{sample}}/OD_{\text{control}}) \times 100$ [25].

Antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH \bullet) radical [26]. Briefly, 2 mL of EPS solution (4 mg/mL) was mixed with 4 mL 0.1 mM DPPH \bullet solution. Then the mixture was placed for 30 min, and the absorbance was determined at 517 nm. The result was calculated by the equation as follows: Scavenging activity (%) = $(1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}) \times 100$.

Statistical Analysis

All analyses were measured at least three times. One-way ANOVA was performed to determine the significance ($P < 0.05$) of differences between the data with Duncan's multiple comparison test.

Results and Discussions

Identification and Potential Probiotic Properties of NOC219 Strain

The taxonomic position of the NOC219 strain selected for secreting the most EPS was identified by biochemical and 16S rRNA gene sequencing analysis. The strain showed positive for gram staining, glucose utilization, and Voges-Proskauer tests and negative for methyl red, and gelatin hydrolysis, hemolytic activity (γ -hemolytic). On the other hand, the

16S rRNA gene sequence of this strain NOC219 showed a high degree (99.79%) of similarity with the type strain *E. faecalis* ATCC 19433^T (Fig. 1) and was named *Enterococcus faecalis* NOC219. Also, its gene sequence was deposited to GenBank (OM982617).

When examined in terms of potential probiotic properties, after 2 h of incubation in the prepared simulated gastric medium, the *E. faecalis* NOC219 strain decreased from 9.02 ± 0.23 to 7.48 ± 0.52 log CFU/mL, showing a 1.54 log CFU/mL reduction. As for tolerance to the simulated intestinal medium, it decreased from 8.96 ± 0.34 to 7.73 ± 0.14 log CFU/mL, showing a 1.18 log CFU/mL reduction in the simulated intestinal medium (Table 2A). Although the decrease in the viability of the strain is significant in both gastric and intestinal environments, it was determined that the strain had good tolerance to both environments and had probiotic potential, since the numbers of these environments were 6 logs and above after the applied treatment [27]. In some studies in the literature [12, 28], it was determined that some *E. faecalis* strains with potential probiotic properties showed similar tolerance. Furthermore, as another probiotic property condition, it was determined that the *E. faecalis* NOC219 strain showed high susceptibility to the ampicillin, erythromycin, amoxicillin, and tetracycline antibiotics with disk diameters of 47.02 ± 0.26 , 41.34 ± 0.27 ($P > 0.05$), 30.25 ± 0.09 , and 24.17 ± 0.13 mm ($P < 0.05$), respectively.

In accordance with these results, the *E. faecalis* NOC219 strain was determined to have potential probiotic properties such as high antimicrobial activity, no antibiotic resistance, and resistance to gastric and intestinal environments (resistances to low pH, bile salts, and digestive enzymes).

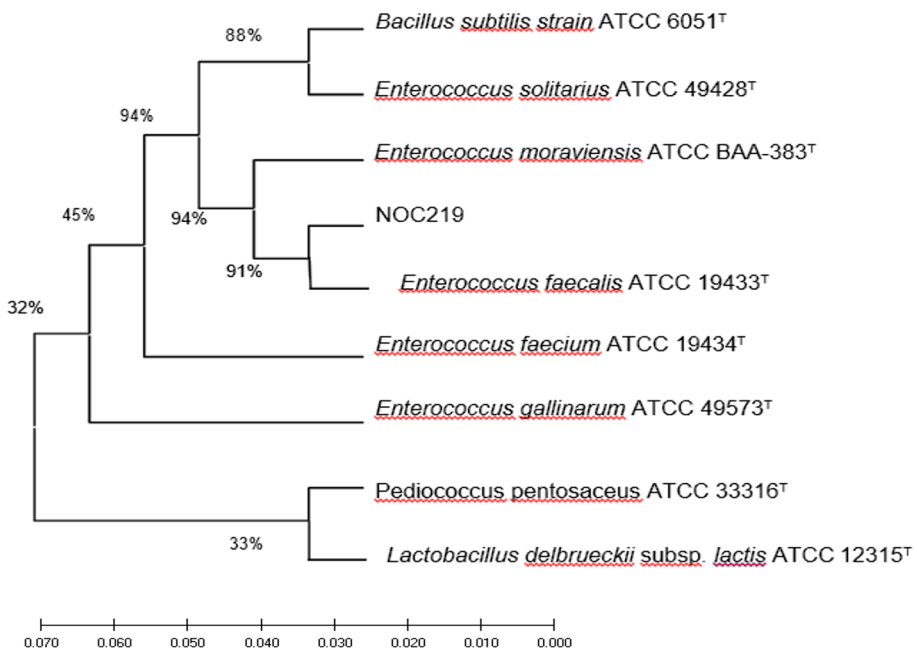


Fig. 1 Phylogenetic tree showing the relative positions of the isolates as inferred by the neighbor-joining method of complete 16S rRNA gene (S) sequences

Table 2 Resistances to simulated gastric and intestinal medium of NOC219 strain (A); genes in EPS production (B)

(A) Resistance to simulated gastric and intestinal medium			
For gastric medium (log CFU/mL; period of simulated medium)	0 h	1 h	2 h
	9.26 ± 0.23 ^a	8.12 ± 0.18 ^b	7.48 ± 0.52 ^c
For intestinal medium (log CFU/mL; period of simulated medium)	0 h	2 h	4 h
	8.96 ± 0.34 ^a	8.22 ± 0.27 ^{ab}	7.73 ± 0.14 ^b
(B) Screening of genes involved in homopolymeric EPS and heteropolymeric EPS production			
Gene regions		Gene regions	Gene regions
epsA	–	p-gtf-epsEFG	+ Gtf-Dexreu
epsB	+	p-gtf-P1	+ Gtf
p-gtf-epsD/E	–	Lev-LevV	–

+ presence of the corresponding gene, – no detection of the corresponding gene

a–c Different lowercase (superscript) letters indicate significant differences between the time periods in each of the simulated environments

Molecular Detection of EPS Genes in NOC219 Strain

The EPS production characteristic properties of yogurt isolates at strain levels of different species were determined with the previously described methodology [19], and strains were selected for further screening of the eps genes [13, 29]. The target genes for the detection of the eps genes were gtf (glucansucrase), lev (levansucrase) and epsA (putative transcriptional regulator), epsB (putative polymerization and chain length determination protein gene), and p-gtf (putative priming glycosyltransferase gene) representing genes required for the production of homopolymeric and heteropolymeric EPS, respectively, and PCR conditions and primers used for the detection of the target genes were described elsewhere [15].

According to the screening results of EPS genes, it was revealed that the NOC219 strain harbored the epsB, p-gtf-epsEFG, and p-gtf-P1 genes (Table 2B, Fig. 2A). For the heteropolymeric EPS production, an eps gene cluster harboring epsA, epsB, and epsD-epsE genes as well as other genes is required [30], whereas a single gene is described as gtf or ftf is responsible for homopolymeric glucan or fructan type, respectively [15]. This was a molecular indication that heteropolymeric EPS could be synthesized by the NOC219 strain. In a study [31], it was determined that the EPS-MS79 structure, which was produced from an *Enterococcus faecium* (MS79) strain, was composed of the three monosaccharides arabinose, mannose, and glucose, and therefore, it was a heteropolymeric structure. However, different strains of the same species are known to produce EPSs with different structures. For example, EPSs produced by *E. faecium* WEFA23 [32] and *E. faecium* MC13 [33] differ from each other. Moreover, the monosaccharide composition has a significant influence on the functionalities of the exopolysaccharide [34]. Therefore, EPSs produced by new strains should be examined in detail. Besides, several factors, including medium and fermentation conditions as well as genetic factors, affect the EPS production levels of a LAB strain. The monosaccharide composition of EPS examined in the present study was mentioned below.

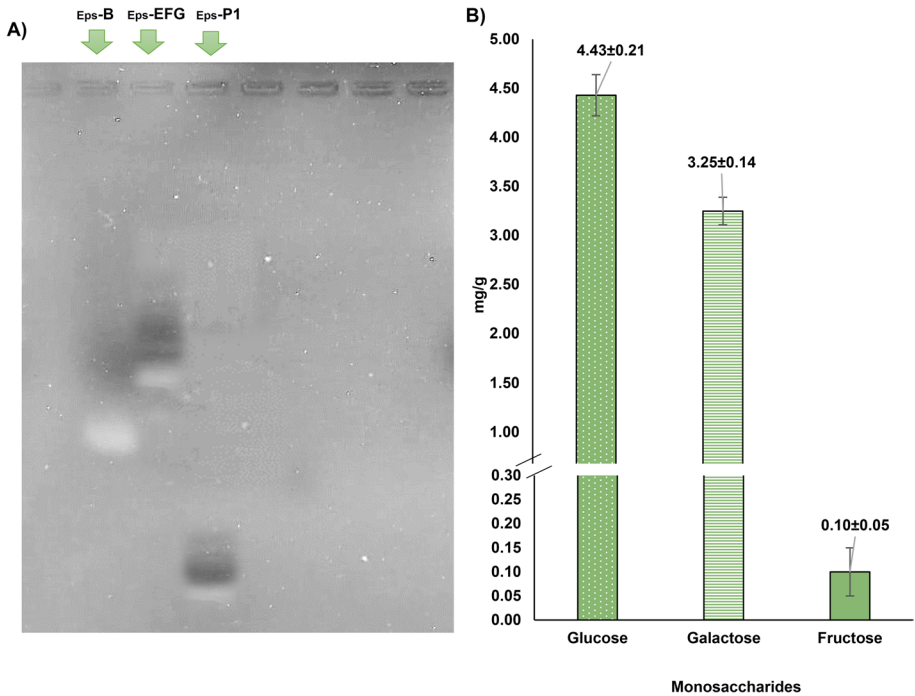


Fig. 2 Exopolysaccharide genes in *E. faecalis* NOC219 (A); monosaccharide composition of the purified EPS-NOC219 from *E. faecalis* NOC219 (B)

Production and Purification of EPS from NOC219 Strain

The *E. faecalis* NOC219 strain exhibited probiotic characteristics and produced an EPS yield of 564.62 ± 0.15 mg/L. This structure was named EPS-NOC219. Yields of EPS structures produced by LAB differ depending on the type of strain, growth conditions, carbon source, initial pH, culture medium, incubation temperature, and inoculum size [33]. In the literature, it was observed that the EPS yields of EPS-producer LAB strains varied considerably. It was reported that the EPS yields were 13.20 g/L of the EPS from *Leuconostoc mesenteroides* S81 [9], 28.82 g/L of the EPS from *Lactobacillus fermentum* CFR 2195 [33], and 7.47 g/L of the EPS from *Enterococcus faecium* MC13 [33] belonging to the same species as in this study. However, it was determined as the EPS yields were 429.4 mg/L of the EPS from *Lactobacillus plantarum* ZDY2013 [36], 130 mg/L of the EPS from *Enterococcus faecium* WEFA23 [32], and 355 mg/L of the EPS from *Enterococcus faecium* K1 [28]. According to the results, it was observed that the EPS yield of the strain in the presented study was higher than many strains belonging to the same species.

Monosaccharide Composition of EPS-NOC219

The monosaccharide components of the EPS-NOC219 carried out after acid hydrolysis of it were examined by HPLC. The results showed that the EPS-NOC219 was a

heteropolymer consisting of glucose (4.43 mg/g), galactose (3.25 mg/g), and fructose (0.10 mg/g) in comparison with respective standards (Fig. 2B). Previous studies suggested the heteropolysaccharide-EPS production from the various strains of the *E. faecium*, including *E. faecium* MS79 [31], *E. faecium* WEFA23 [32], and *E. faecium* MC13 [33]. However, homopolysaccharide-EPS produced by a different strain of the same species, such as *E. faecium* MC-5 [24], were also determined. In the study, the relative molar ratio of these sugar monomers in the EPS-NOC219 was 44.3:32.5:1. The main monosaccharides were glucose and galactose, while fructose also was present in very low concentrations. Here, it was thought that galactose was converted from glucose by the UDP-glucose 4-epimerase of the *Enterococcus faecium* NOC219 strain via the Leloir pathway [37] as a result of the use of sucrose. Besides, if this strain was grown in a medium containing lactose and sucrose together, it is also predicted that it will probably produce lactosucrose, a prebiotic [38]. It is known that the monosaccharide composition of an EPS has a significant influence on its functional properties (antioxidant antiviral and anticoagulant properties, prebiotic effect) [34]. The results supported the view that this EPS structure may be prebiotic and determined the path for further work.

Functional Groups of EPS-NOC219

The functional groups of EPS-NOC219 were determined by FTIR analysis. Figure 3A shows the FTIR spectra of EPS-NOC219. The polysaccharide showed high absorbance in the region 1200–1000 cm^{-1} , which was within the so-called fingerprint region (below 2000 cm^{-1} and spectral regions corresponding to polysaccharides are between 890 and 1175 cm^{-1}) [39]. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic bond vibration [40]. Therefore, the strongest absorption band at 1012.51 cm^{-1} has indicated that the substance is a polysaccharide and suggests that the monosaccharide in it has a pyranose ring [41]. Similarly, the absorption at 1146.76 cm^{-1} was dominated by glycosidic linkage $\nu(\text{C–O–C})$ -stretching vibration. It is known that characteristic anomeric region absorption bands in carbohydrates at about 898 cm^{-1} for β -anomer and about 845 cm^{-1} for an α -anomer form of the pyranoid ring [20, 42]. In the IR spectra, the characteristic absorption at 844.56 cm^{-1} indicated α -anomeric configuration in the EPS-NOC219. The absorption at 912.05 cm^{-1} indicated that the EPS-NOC219 had β -glycopyranosidic linkages.

As for the other important absorption bands, the few weak peaks at $\sim 1407.95 \text{ cm}^{-1}$ were characteristic of the carboxyl groups (C=O) (1444–1400 cm^{-1}) or carboxylate (COO^-), indicating that EPS-NOC219 is not an acidic polysaccharide. Besides, the peak at the 1000–1125 cm^{-1} range, which showed the presence of guluronic acid and mannuronic acid [43], was not determined in a way that supports it. Also, there was no peak around 1700–1775 cm^{-1} , suggesting that neither glucuronic acid nor diacyl ester was present [7]. As for the peak at 2902.12 cm^{-1} , it was due to C–H stretching vibration [44], and the peak observed at 2985.42 cm^{-1} between 2860 and 2990 cm^{-1} was associated with the methyl groups as well as the C–H stretching vibration. Finally, a wide peak observed at 3326.82 cm^{-1} (between 3044 and 36,127 cm^{-1}) corresponded to the hydroxyl groups of polysaccharides, suggesting that this polymer was a polysaccharide. The FTIR spectrums of EPSs produced by some *E. faecium* strains [25, 28, 45] are relatively different compared with the present EPS-NOC219. This suggests that these EPS have different characteristics.

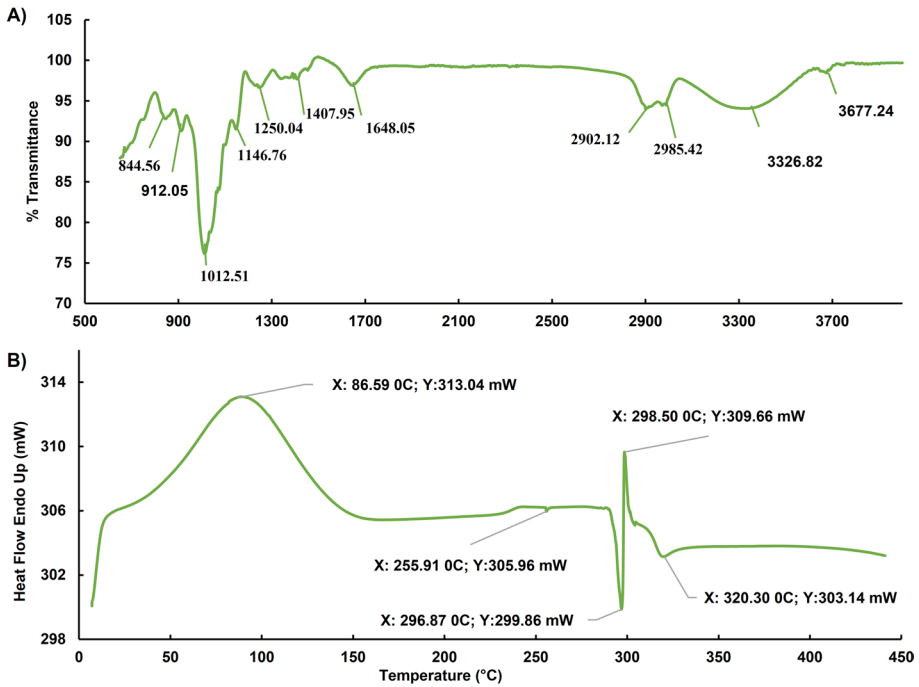


Fig. 3 FTIR spectra (A); DSC thermogram of the purified EPS-NOC219 (B) from *E. faecalis* NOC219

Thermal Property of EPS-NOC219

The thermal transitions of the EPS-NOC219 were determined by the DSC analysis. DSC thermogram of EPS exhibited distinct endothermic peaks at 255.91 °C, 296.87 °C, and 320.30 °C, respectively (Fig. 3B). The sharp and distinct peak observed at 296.87 °C has exhibited the melting point (T_m) of the EPS-NOC219. To the best of our knowledge, this is one of the highest records observed for the melting point of EPSs as previous reports showed the melting point of different EPSs ranging between 53.04 and 178.4 °C [28, 33, 43, 46]. However, high melting points were detected in a limited number of studies. In a study by [25], the melting point of the EPS-DU10 produced by *E. faecalis* DU10 was determined as 270 °C. In addition, in a study by İspirli et al. [1], the melting point of glucan-E81 produced by *Lactobacillus reuteri* E81 was determined as around 290 °C, and in another study by [47], the melting point of glucan-KX57763 produced by *Enterococcus hirae* KX57763 was determined as around 296.67 °C. It is suggested that the EPS, which had a relatively higher melting temperature, possessed a stronger ability to retain water [26]. Besides, it is known that differences in the thermal behavior of EPS may be due to the molecular configuration of the biopolymer, and the polymer degradation at higher temperatures may be associated with the higher molecular weight of EPS [24]. Therefore, this EPS structure can give important advantages to be used in different food systems, especially in thermally processed food systems.

Rheological Behaviors of EPS-NOC219

Steady Shear Property

Aqueous EPS-NOC219 solutions (4%, w/w) were loaded in the cone geometry. To the best of our knowledge, only one study has been available about rheological information on EPS produced by *E. faecium* [31]. Therefore, the rheological results of the present study are quite important. The results were shown in Fig. 4A. According to this, the aqueous EPS-NOC219 was shown a pseudoplastic flow behavior in which the viscosity decreased with increasing share rates, at a constant temperature (+4 °C), and it was described by a Herschel-Bulkley model. The flow behavior of EPS-NOC219 coincided with that previously described for the EPS structures produced from *Enterobacter* A47 [48], *Leuconostoc carnosum* CUPV411 [49], and *E. faecium* MS79, a strain of the same species as the microorganism in the present study; however, it was known that in the literature, the microbial suspensions of a strain of *Bacillus cereus* were exhibited a different flow behavior such as dilatant [50]. It is known that hydrodynamic forces generated during the shear can have led to the breakdown of the structural units and the physical networks in the chain structure of EPS structures [51]. Therefore, the shear-thinning data presented suggest that the EPS studied would be very suitable to improve the texture or palatability of food products. Besides, the apparent viscosity (η) of the EPS sample was determined as 186 mPas at a 50 s^{-1} shear rate because the 50 s^{-1} is an appropriate estimation of oral shear rate for food samples [52].

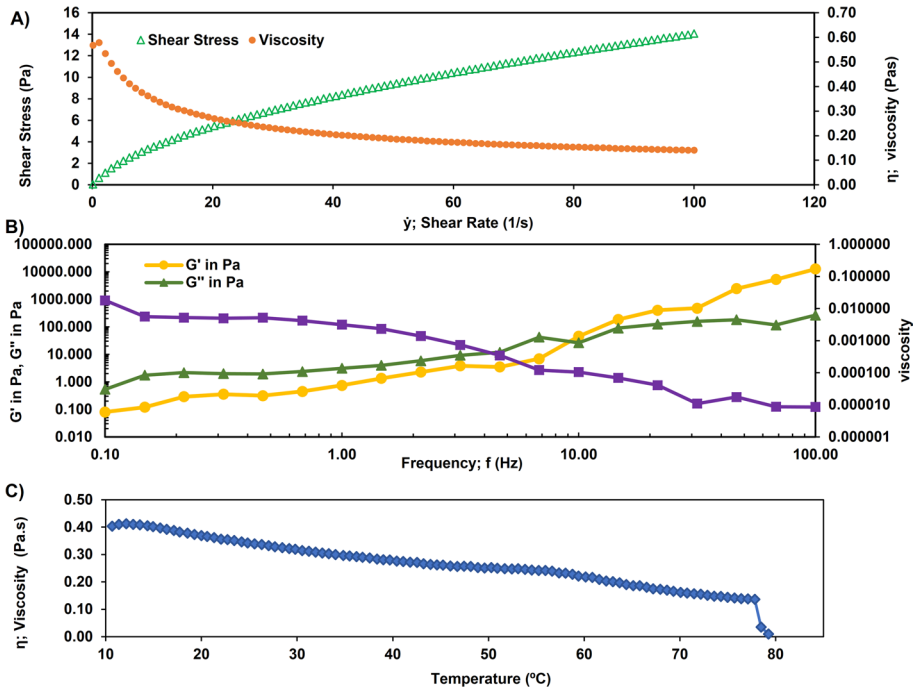


Fig. 4 Apparent viscosity (A); storage G' and loss G'' (B); temperature-dependent behavior (C) of the purified EPS-NOC219 solutions

Dynamic Shear Property

As for viscoelastic properties of the EPS-NOC219, the variations of elastic (storage modulus; G') and viscous (loss modulus; G'') moduli of the EPS sample with frequency were shown in Fig. 4B. Storage modulus represents the energy stored in the structure of the material and is a measure of its elasticity; loss modulus represents the viscous part in the material's structure where energy is lost due to deformation and frictions. Since such rheological tests are linear rheological tests, a safe linear viscoelastic region scanning was performed that would not damage the sample structure. The linear viscoelastic region of this EPS sample was determined in the range of selected as less than 1% strain.

Both moduli were seen to increase continuously with the frequency. The variation of G' and G'' modulus with frequency implied that, as the frequency increases, the viscoelastic behavior changes from being dominated by viscous properties ($G'' > G'$ at lower frequencies) to being dominated by elastic properties ($G' > G''$ at higher frequencies). The G' and G'' curves intersect ($G' = G''$) at a frequency, usually called the cross-over frequency. This frequency marks the transition from liquid-like behavior to solid-like behavior. Thus, the polymers can be characterized by the frequency at $G' = G''$, denoted as the cross-over time of the system, and the plateau value of G' at high frequencies ($\tan \delta < 1.0$) [53].

In the present study, as seen in Fig. 4B, G' was lower than G'' at the frequencies < 9.03 Hz (except at higher frequencies, where a cross-over is perceived at an angular frequency of about 9.03 Hz) for the aqueous EPS-NOC219 solutions, which means the viscous character of the aqueous EPS-NOC219 solutions was dominant rather than elastic character, and hence, this sample can be described as having a liquid-like behavior at low frequency ($\tan \delta > 1.0$). Also, this result concurs with those reported by Han et al. [54], Ayyash et al. [31], and Antunes et al. [48] who recorded higher G'' than G' of EPS-JD2, EPS-M41, and EPS-A47, respectively. This indicates that the EPS-NOC219 could be suitable as a thickener. In general, both moduli of the solution increased alongside with frequency increase; G' was greater than G'' at frequency > 9.034 Hz (ω : 56.76 rad/s). Therefore, the viscoelastic behavior of the sample changed to being dominated by elastic properties.

On the other hand, G' values of the EPS-NOC219 solution obtained were higher than that of EPS structures studied by Ayyash et al. [31, 46] and Benhouana et al. [55]. This indicates that the EPS-NOC219 more stable and homogeneous structure according to the EPS structures mentioned.

Temperature-Dependent Behavior

The temperature was an important factor affecting the rheological properties of an EPS structure. The apparent viscosity of this EPS solution was dramatically decreased with increasing temperature (Fig. 4C). This suggested that the thickening properties of the EPS solution declined with increasing temperature. This was mainly caused by the decreased molecular flexibility under lower temperatures [56]. The initial temperature of the decrease was about 20 °C (17.11 °C). This result means that the thermal energy after 20 °C is sufficient to break up the polymer structure. The finding suggests that the present EPS-NOC219 is not suitable for high thermal processes. Also, the activation energy of the EPS-NOC219 solution calculated by the Arrhenius equation was 32.58 kJ/mol. On the other hand, in a study on the EPS of a strain of the same species conducted by Ayyash et al. [31], it was detected that the EPS-M41 solution with CaCl_2 pH 6.0 among the EPS solution prepared with different salt types and pH values had the highest activation energy of 10.68 kJ/mol. Similarly, in a study by Xu et al. [56], the activation energy of EPS-3 produced from

Streptococcus thermophiles S3 was determined as 7.34 kJ/mol. This information shows that the stability of the EPS-NOC219 in the presented study against temperature increase is higher according to the EPS structure mentioned. The activation energy is related to the chain flexibility in the solution. The relatively high activation energy for EPS indicated more inter- and intra-molecular interactions between polysaccharide chains in the investigated concentration [57].

Microstructure of EPS-NOC219

Scanning electron microscopy (SEM) analysis of the macromolecules helps to correlate the known physical properties of this material with its microstructural properties. In the present study, the EPS-NOC219 was determined by the surface morphology and the microstructure of it by SEM images at 500×, 1000×, and 2500× as can be seen in SEM images (Fig. 5A). It is seen that the EPS-NOC219 has two types of structures: a layer-like, compact and closed structure and a spherical granular, compact and smooth structure. It is known that studies suggested that smooth surfaces of EPS are counted as a favorable characteristic for making plasticized biofilms of EPS, and the consistent polymeric matrix is presumed to give mechanical stability to such biofilms [58]. On the other hand, colonization of microbiota on abiotic and biotic surfaces through biofilm in the intestine may impart various health benefits. Therefore, these results may be important in food applications, as they affect the physicochemical properties and rheological properties of food.

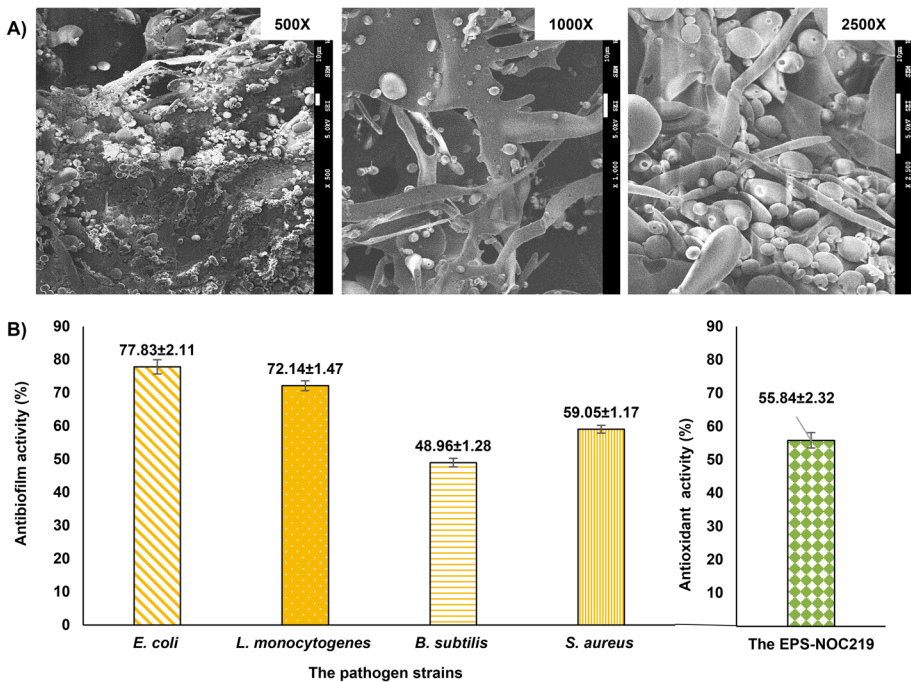


Fig. 5 Scanning electron micrograph (SEM) images of the purified EPS-NOC219 from *E. faecalis* NOC219 (A); in vitro antioxidant potential and antibiofilm potential against biofilm formed by various pathogenic bacteria (B)

Antibiofilm and Antioxidant Capacities of EPS-NOC219

Antibiofilm and antioxidant tests were carried out to examine the bioactive properties of this structure. According to the results (Fig. 5B), the EPS-NOC219 structure showed a pretty good effect with 55.84% antioxidant activity. In a study [45], the EPS produced from *Enterococcus faecium* (BDU7) strain showed similar results with the same amount of it in the present study. Also, the result is very close to the antioxidant effect (<50%) of phenolic compounds in some fruits (cantaloupe, nectarine, avocado, etc.) [59]. The EC₅₀ value of this EPS was nearly 3.5–4 mg/mL and had a lower effect than the antioxidant capacity of levan S81 from *Leuc. mesenteroides* S81 with an EC₅₀ value of 1.7 mg/mL [9].

As for the antibiofilm results, the EPS-NOC219 showed the highest activities against *E. coli* (77.83%) and *L. monocytogenes* (72.14%) ($P < 0.05$), then they were followed by *S. aureus* (59.05%) ($P < 0.05$) and *B. subtilis* (48.96%) ($P < 0.05$). EPS structures may be causing these inhibitions by competitively preventing nutrient uptakes or inhibiting cell wall reactions.

These results may expand the range of properties of the EPS structure. In other words, while improving the structural properties of a product to which EPS is added, its bioactive effect may also increase. This double effect shows that the functionality of the EPS-NOC219 material obtained is versatile.

Conclusion

In this study, the EPS-NOC219 obtained from the *E. faecium* NOC219 strain, which is a high-yield EPS producer, was investigated in terms of structural and functional properties. In addition, the expression of genes related to EPS production of this strain was also determined. The EPS-NOC219 was revealed to be a heteropolymer composed of glucose, galactose, and fructose units, and the fact that the producer strain harbors the *epsB*, *p-gtf-epsEFG*, and *p-gtf-P1* genes, required for the production of heteropolymeric-EPS. The EPS-NOC219 structure had a high melting point, exhibited a pseudoplastic flow behavior, and the viscous character of its aqueous solution was more dominant than its elastic character. This indicated that the EPS-NOC219 had high heat stability and could also be used as a thickener in heat treatment processes. Additionally, this EPS was determined to be suitable for making plasticized biofilms. On the other hand, the EPS-NOC219 has shown high antioxidant activity against DPPH radicals and high antibiofilm activity against the *E. coli* and *L. monocytogenes* pathogen strains. This situation has revealed its bioavailability. In addition, the probiotic potential of the producer strain was determined. The results have revealed that the EPS-NOC219 structure can be a good alternative natural resource that can be used in foods from a structural and functional point of view, as it has strong physico-chemical properties in addition to a healthy food grade.

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Author Contribution All steps related to the article were conducted by NO.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The author declares no competing interests.

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