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Biopolymer production by bacteria isolated from native stingless bee honey, *Scaptotrigona jujuyensis*

Biopolymer production by bacteria from *Scaptotrigona jujuyensis*

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Abstract

The products of stingless bees have been used in traditional medicine. These products have gained economic potential not only for their historical valuation but also to produce added value related to the knowledge of the qualities of their indigenous microbiota. The isolates from honey and pollen of *Scaptotrigona jujuyensis*, a stingless bee from Northern Argentina were studied. These were able to produce hydrolytic enzymes: protease, amylase, xylanase, cellulose, and lipase, and growing in bile salts.

The isolate 4A was identified as a *Bacillus* sp. and was able to produce extracellular exopolysaccharides (EPS). The carbohydrate composition of EPS consisted predominantly of fructose (44.6%). Structural characterization of EPS using Fourier-transform infrared spectroscopy (FTIR) showed high similarity with levan.

The EPS showed antimicrobial activity and the capacity to form emulsion hydrogels with omega-3 polyunsaturated fatty acids (PUFA) from ray liver oil and chia oil. The prebiotic property of *Lactobacillus casei* was evaluated with EPS and its mix with omega-3 PUFA. *L. casei*, showed better growth. Thus, an EPS with emulsifying hydrogel capacity and prebiotic activity was produced from the native microbial flora present in the honey of a stingless bee, which could be an added-value product of the *S. jujuyensis* colony used as a new nutraceutical.

Keywords: Stingless bee *Scaptotrigona jujuyensis*; *Bacillus* sp.; Exopolysaccharides Hydrogels; Prebiotic.

1. Introduction

In some industrial processes, microorganisms are used to produce different bioproducts from sugars. Among these, biopolymers are frequently used in different industrial processes. The exopolysaccharides (EPS) are extracellular polymeric substances that can be produced by microorganisms and are often formed with stress conditions (Czaczyk and Myszka, 2007).

EPS are highly soluble in water and can form three-dimensional networks that impact the texture of food products. The EPS can be used as antimicrobial compounds against important pathogens and contaminants (Yaşar Yildiz et al., 2019).

One challenge is to use microorganisms to convert different sugar into value-added bioproducts such as biopolymers. To consider the environment from the organism's perspective may help guide the determination of a pathway to convert these sugars into useful polymers.

Honey is a product used as a natural sweetener and it is used as a therapeutic agent. Several microorganisms, including bacteria and fungi, colonize honeybee species and could be present in the bee's products, such as honey, beebread, and propolis. Many of these microorganisms stimulate the immune and defense responses of honeybees against pathogens (Hroncova et al., 2015).

The products of stingless bees have been applied in traditional medicine as well as in the food area for supplementary nutrition (Bankova, 2005; Jacob et al., 2006; Ngalimat et al., 2019; Zulkhairi et al., 2018).

The stingless bees belong to the meliponids group, insects of the family Apidae (Hymenoptera: Apidae: Meliponini), such as *Scaptotrigona jujuyensis*. They are distributed in the tropical and subtropical regions of the world. In South America, ~400 species are known and studied, some of them are found in forests such as the Yungas, in Northern Argentina (Nates-Parra and Rosso-Londoño, 2013; Roig-Alsina et al., 2013).

Stingless bees honey has different characteristics compared to the honey produced by *Apis mellifera*. Their honey is less viscous which favors a greater diversity of microorganisms (Combey, 2017; Jacob et al., 2006). These microorganisms could have a relationship with stingless bees from the beginning of colony formation (Ngalimat et al., 2019).

Bacteria, yeasts, and filamentous fungi are the main microorganisms living in stingless bee's colonies. However, knowledge of this microbial biodiversity is limited (Anderson et al., 2011). There are many bacterial genera associated with bee's products. Particularly, the *Bacillus* genus has been reported among the bacteria related to the stingless bees or its products (Ngalimat et al., 2019).

Bacteria from the *Bacillus* genus are good producers of antimicrobial molecules, biosurfactants, and exopolysaccharides. These compounds have shown many advantages such as biodegradability and low toxicity (Angelini et al., 2009; Lee et al., 2019; Obakpororo et al., 2017; Reynaldi et al., 2004; Zhao et al., 2013).

The objective of this work was to isolate and characterize strains belong to the *Bacillus* genus from honey and pollen of *S. jujuyensis*. The enzymatic profiles of the *Bacillus* strains isolated were determined. A biopolymer was also produced and partially purified from the strain *Bacillus* sp. 4A. Physical-chemistry and biological properties such as antimicrobial and emulsifying activities were analyzed as well as its prebiotic properties.

2. Material and Methods

2.1. Materials

Honey and pollen samples were collected from stingless bees hives from a meliponary located in Famaillá, Tucumán, Argentina (27º 03' S, 65º 25' W, 363 meters above sea level). The collection was carried out during February 2017.

De Man, Rogosa, and Sharpe (MRS) agar, and MRS broth were purchased by Oxoid, Basingstoke, UK. Brain heart infusion broth (BHI), bovine bile salt and pepsin (porcine gastric mucosa) were obtained from Merck (Darmstadt, DE). D-Fructose and D-glucose (purity 99.5%), sucrose (purity 99.9%), cetyltrimethylammonium bromide (CTAB) and all other chemicals were acquired from Sigma Aldrich Co. (St. Louis, MO, USA). Chloroform and octanol solvents were purchased from Sintorgan Co. (Buenos Aires, BA, AR).

The strains used were *Listeria monocytogenes* Scott A, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Lactobacillus casei* ATCC 1232. The microorganisms were provided by the Faculty of Pharmaceutical Sciences - Food Research Center, University of São Paulo (São Paulo, SP, BR).

2.2. Microorganisms isolation

The mixtures of honey or pollen were made from 9 hives. The hives were sanitized using 0.5% (v/v) NaClO and transferred to a clean area for taking samples. The honey was aspirate with sterile syringes and the pollen was collected using a sterile spoon. The samples from each individual hive were placed in sterile tubes and then 3 g of each sample (honey or pollen) was mixed with the other hives, to form a composite sample. This procedure was carried out in triplicate. The collected samples (honey or pollen) were stored at 4.0 ºC until further use (for a maximum of 2 days).

For bacterial isolation, a suspension of 2.0 g of sample in 18 mL of peptone water (0.1% w/v) (Sigma Aldrich) was prepared. Then, the samples were serially diluted with peptone water and each dilution was plated on three different culture media: tryptic soy broth agar (TSA) (Sigma Aldrich); yeast extract, peptone, dextrose (YPD) (Sigma Aldrich) and MRS agar (Oxoid).

The samples were incubated at 30 ^{o}C for 2 days. Bacterial colonies were picked, incubated and purified using serial subculturing and plating. Then each isolate was stored at −80 °C in the culture medium with glycerol (20%) for a maximum of 3 months. Isolates were chosen for further morphological analysis and enzyme activity.

2.3. Bacterial identification

2.3.1. DNA extraction and PCR amplification of 16S rRNA genes

Considering the potential biotechnological properties, some isolates were selected for molecular identification using the conserved region of the 16S rRNA gene (Kullen et al., 2000).

One colony was recovered and grown in TSA at 30 °C for 18 h. After incubation, 1.0 mL of the culture was centrifuged at 6,000 × g (5804 R, Eppendorf, Hamburg, DE) at 4 °C for 10 min. The pellet was processed for DNA extraction (Murray and Thompson, 1980; Wagner et al., 1987). The pellet was dispersed in 500 uL of extraction buffer: 0.7 M NaCl, 1% CTAB, 50 mM tris-HCl, pH 8.0, 10 mM EDTA, 1% 2-mercaptoethanol and was mixed (Vortex, Vicking Co. Buenos Aires, BA, AR) for 5 min. After that, 10 µL RNase 10 mg/mL, (Promega, Madison, WI, USA) were added to the suspention and incubated in a water bath at 37 °C for 30 min. Then, 5 µL proteinase K, 20 mg/mL, (Promega) were added and incubated at 37 °C for 30 min. The extract was emulsified with an equal volumen of chloroform:octanol (24:1) and centrifuged at 13,000 × g, at room temperature (20 to 25 ºC) for 10 min. The DNA was resuspended in 50 µL of sterile double distilled water and the samples were stored at 4 °C for a maximun of 2 days.

The quality and integrity of DNA was analyzed using an agarose gel (2.0%, w/v), using TAE buffer (40 mM tris-acetic acid buffer, 0.5 M EDTA, pH 8.0). DNA was run in an electrophoresis chamber (EC 370M, Minicell, Sigma Aldrich) at 60 V. The agarose gel was stained with Gel Red (Nucleic Acid Gel Stain, Biotium, Fremont, CA, USA) at 1.0 µL/50 mL and visualized under UV illumination (Bio-Rad 2000, 240V 250x250 mm, Bio-Rad Laboratories, Co., Hercules, CA, USA).

The size of the DNA bands was estimated by comparing them with the molecular weight size markers, 117 to 8,454 bp λ-BstEII, (New England Biolabs, Ipswich, MA, USA) and a 100 bp (Promega), for PCR products.

For PCR, the Illustra Pure Taq ready-to-go PCR beads kit (GE Healthcare, Giles, UK) was used, and the oligonucleotides in the assay were 27F (5´AGAGTTTGATC (C/A) TGGCTCAG3´) and 1492R (5´TACGG (C/T) TACCTTGTTACGACTT3´), as described by Woese et al. (1980).

The PCR was done as follows: 4 min at 94 °C, 35 cycles of 1.5 min at 94 °C, 1.5 min at 55 °C, and 2.0 min at 72 °C, and 7.0 min at 72 °C. The products of the reactions were used for gel electrophoresis as described above.

2.3.2. Sequencing and sequence analyses of nucleotides

After PCR, the products were purified and sequenced by Macrogen Inc. Service (Seoul, South Korea) using the Sanger methodology based on the sequencing of DNA molecule. The arrangement of their 4 nucleotides was obtained (A, T, C, and G) (Sanger et al., 1977).

The nucleotide sequences were compared with sequences previously deposited in the Gen-Bank using the Basic Alignment Search Tool (BLAST) software system. The BLAST found regions of local similarity between the nucleotide sequences. The program compared nucleotide sequences to sequences in a database and calculated the statistical significance of the matches (http://www.ncbi.nlm.nih.gov).

2.3.3. Phylogenetic analysis

The 16S rDNA sequences were fitted into an EzTaxon-e server using the link https://www.ezbiocloud.nett. The DNA of the strains were related taxonomically with the isolates of close strains (Kim et al., 2012). The alignments of the sequences selected from the previous analysis were built using the SILVA Incremental Aligner (SINA) Service (software package ARB, Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, DE) from the SILVA database (http://www.arb-silva.de) and edited to remove gaps and ambiguous nucleotides (Pruesse et al., 2012).

Evolutionary distances were calculated using the Tamura-Nei method and phylogenetic trees were reconstructed using the neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods using the MEGA 6 (Molecular Evolutionary Genetics Analysis across computing platforms, www.megasoftware.net) (Tamura et al., 2013). The confidence values of the branches of the trees were determined using bootstrap analyses, a statistical procedure using MEGA 6 software that resamples a single dataset to create many simulated samples.

2.4. Determination of extracellular enzyme activities

A semi-quantitative assessment of the enzymatic activity was done for the bacteria colonies that showed significant hydrolysis halos. Each halo was measured in mm using a Vernier caliper of 0 - 150 mm, (with subdivisions of 0.1 mm) (Merck). The hydrolytic activities were estimated according to the method reported by Anagnostakis and Hankin (1975). The ratio between the diameter of the hydrolysis halo and the diameter of the colony was determined and express as the halo/colony diameter (h/c) ratio. The experiments were done in triplicate and data was statistically analyzed according to the Tukey’s tests ($p<0.05$).

2.4.1. Cellulase and xylanase activities

From fresh cultures, colonies were grown on the media containing 1.0% (w/v) carboxymethyl cellulose or beechwood xylan (Sigma Aldrich) as the only source of carbon. For development, the plate surface was covered with a 0.1% Congo red solution (Sigma Aldrich), (in distilled water), incubated for 15 min at room temperature, and washed with 0.1 M NaCl solution to remove the excess of Congo red. If positive, the hydrolysis halos are visualized as a lighter halo, around the colony, on a red background (Mikán and Castellanos, 2004).

2.4.2. Lipolytic activity

The fermentation solid medium was supplemented with 2.0% olive oil (AGD Co., Buenos Aires, BA, AR) and 0.001% Rhodamine B (Sigma Aldrich), (Kouker and Jaeger, 1987). Culture plates with the colonies were incubated at 30 °C and examined for 4 days. Lipolytic activity was measured using the fluorescence zone around colonies observed after the irradiation at 350 nm, an UV transilluminator (Bio-Rad 2000, 240 V, 250x250 mm).

2.4.3. Protease activity

Agar medium was prepared with 1.0% (w/v) skimmed milk, (La Serenísima, Co., Buenos Aires, BA, AR) in Petri dishes. The microorganisms were inoculated and incubated overnight at 30 °C. The clear zone around the colonies showed the proteolytic activity and this was measured (Zilda et al., 2013).

2.4.4. Amylase activity

The starch agar medium was used to determine amylase production. Each bacterium isolate was incubated overnight at 30 °C in the starch medium. The plates were then spread with iodine solution (0.3% iodine and 1.0% KI). Amylase positive strains were identified by the presence of a clear zone around the bacterial growth (Pamela et al., 2014).

2.5. Growth in bile salts

The microorganisms selected were screened for bile tolerance using MRS agar (pH = 6.4) with 3.0 g/L of bile salts added. Each microorganism was streaked onto plates of MRS agar containing bile salts and incubated at 37 °C for 48 h. MRS agar was used as control (Chou and Weimer, 1999). If growth was observed the isolate was considered to be bile tolerant.

2.6. Production of extracellular polymer matrix and EPS production

Considering the previous results, 4 strains were selected to evaluate the extracellular polymeric matrix production following the technique of Wang et al. (2015) with modification. The production of extracellular polymeric matrix was made in solid MRS medium. The extracellular polymer matrix was found as the production of mucous substance between the colonies.

The microorganism that showed the major production of extracellular polymeric matrix was selected to evaluate EPS production. This was developed in MRS broth culture with aerobic conditions, at 30 °C for 36 h. The cells were removed using centrifugation at 6,000 × g at 4.0 °C for 15 min. The supernatant was treated with ethanol 98% (1:1, v/v) at 4.0 °C overnight and the biopolymer precipitated was recovered using centrifugation (6,000 × g at 4.0 °C for 20 min).

2.7. EPS characterization

2.7.1. Physicochemical characterization

EPS samples were lyophilized and resuspended in sterile twice distilled water at a concentration of 1.0 g/L. Acid hydrolysis of the EPS was carried out for 60 min at 75 °C in a solution of 0.37 N HCl. The profile of sugars was determined following the technique described by Bogdanov et al. (1996) with some modifications, using high performance liquid chromatography (HPLC) (Waters 1525, Waters Co. Dublin, IE) coupled to a refractive index detector (Waters 2414, Waters Co.).

For the separation of the compounds, a Polyamine II column (250x4.6 mm, YMC, Waters Co.) was used. The mobile phase was acetonitrile:water 8:2 (v/v) and the flow rate was 1.0 mL/min. The system was maintained at 35 °C. The identification of the individual compounds was made by comparing the retention times of the compounds identified in the EPS with the commercial standards and the quantification was carried out using calibration curves with each of the compounds. Fructose, glucose and sucrose were identified and quantified.

For the identification of functional groups in the EPS, a Fourier transform infrared (FTIR) analysis was carried out following the techniques of Romero et al. (2018). The analysis was done using potassium bromide tablets (1 mg of sample and 100 mg of potassium bromide) in an atmospheric pressure plasma at room temperature using a Perkin Elmer 1600 FTIR (PerkinElmer Co., Waltham, MA, USA).

2.7.2. Antimicrobial Activity

The antimicrobial activity of the EPS was done according to the “spot on the lawn” described by Farias et al. (1994). The pathogenic microorganisms were grown in BHI broth (Sigma Aldrich), for 18 h at 37 °C. An aliquot of 0.2 mL was inoculated in 1.5

mL of BHI agar (0.7% agar). This inoculum was poured over solid agar plates. Aliquots of 50 µL EPS solution containing 1.0, 0.5 and 0.25 mg/mL were dripped directly over the surface of the media containing the test strains. Evidence of activity was provided by the presence of growth inhibition halos after 24 h of incubation at 37 ºC.

The pathogenic microorganisms were used to test the antimicrobial activity of EPS using 10^6 CFU/mL. Each microorganism was grown in soft agar with 10 µL of dilutions of EPS at 30 °C for 24 h. The inhibition on the lawn showed antimicrobial activity.

2.7.3. Emulsifying Activity

The evaluation of emulsifying property was done following the method of Cooper and Goldenberg (1987). For this assay, 2.0 mL EPS at a concentration of 1.0 g/L (w/v) were mixed with 3.0 mL of hydrophobic compounds: Chia (*Salvia hispánica*) oil, (Chia Vita, NYNAGRO, Yerba Buena, TUC, AR), kerosene (YPF S.A., San Miguel de Tucumán, TUC, AR) and fish (ray liver) oil, provided by the National Institute for Fisheries Research and Development, INIDEP, Mar del Plata, Buenos Aires, BA, AR, in test tubes with a flat bottom. The samples were mixed using Vortexing for 2.0 min and then left to stand for 24 h at room temperature. The emulsifying index was calculated using the equation of Velho et al. (2011):

$EI_{24} = He/Ht \times 100$. The heights were measured using the Vernier caliper.

Where:

EI_{24}: emulsifying index at 24 h

*H*e: height of the emulsified column

*H*t: total height

2.7.4. Prebiotic activity

The effect of the EPS on *L. casei* was evaluated following the technique of Szwengiel and Nkongha (2019) with some modifications. Also, the effect of the EPS in a mixture with omega-3 was evaluated. The prebiotic effect of EPS on *L. casei* was studied in MRS broth limiting the carbon source (20.0 g/L, soy peptone; 5.0 g/L, yeast extract; 1.08 g/L, Tween 80; 2.0 g/L, K_2HPO_4; 5.0 g/L, CH_3COONa; 2.0 g/L, ammonium citrate; 0.2 g/L, Mg_2SO_4 and 0.05 g/L $MnSO_4$; pH = 6.4). The substrates evaluated were added as follows: EPS (5.0% w/v), hydrolyzed EPS (5.0% w/v), omega-3 (5.0% w/v), and omega-3 plus EPS. Each condition was inoculated with the microorganism at 30 °C and the growth was monitored for 48 h measuring the OD at 600 nm (UV-Visible Spectrophotometer Zl-5000 Plus, Zeltec, Beijing, China).

2.8. *In vitro* resistance to the gastrointestinal tract (GIT) of *Bacillus* sp. 4A

An isolate capable of growing in the presence of bile salts was selected to evaluate its resistance to a simulated GIT following the technique of De Carvalho et al. (2009) with some modifications. For that, the selected strain was grown in liquid MRS medium for 2 days at 37 °C.

The isolate was centrifuged at 8,000 × g, at room temperature for 10 min, the pellet was washed (3 times) with sterile 5.0 g/L NaCl solution and then resuspended in the same solution.

Gastric juice composition: 5.0 g/L NaCl, 3.0 g/L pepsin, pH = 2.0; 2.5 and 3.0 adjusted with 1 N HCl.

Enteric juice composition: 5.0 g/L NaCl, 10 g/L of bile salts, pH = 8, adjusted with 0.1 M NaOH,

The cultures with gastric juices (pH 2.0, 2.5 and 3.0) were incubated at 37 °C for 120 min on a rotary shaker (150 rpm). Samples were removed at 0, 15, 30, 60, and 120 min. Serial dilutions were made in 8.5 g/L saline solution and the CFU/mL count was done in BHI agar after 48 h of incubation at 37 °C.

The cultures treated with gastric juice were centrifuged at 8,000 × g at room temperature for 10 min. The cells were resuspended in sterile 5.0 g/L NaCl solution and transferred to tubes containing enteric juice. They were incubated at 37 °C, 150 rpm for 24 h. Samples were withdrawn at 0, 1, 2, 4, and 24 h. Subsequently, serial dilutions were made in BHI agar to count CFU/mL at 37 °C for 48 h.

2.9. Statistical analysis

Statistical analysis was done using Minitab software version 14 for Windows (Statistical Software for Windows, Minitab, Co., 2004, State College, PA, USA). The analysis of variance (one-way ANOVA) was carried out to detect the significance among the variables. The treatment means were compared using the Tukey test at the 5.0% probability.

Results and Discussion

Stingless bees honey is different in many ways from the honey of *A. mellifera*. The main difference is the water content, generally higher than *A. mellifera* honey. This relative abundance of water in stingless bees honey allows microorganisms to survive and to be active (Sanz et al., 1995). Bacteria were found from honey and pollen collected from 9 stingless bees colonies. The microorganisms in the stingless bee's products such as the honey could come from pollen, the digestive tract of bees, air, soil, or nectar. Besides, the contamination during the manipulation of the honeycomb must be controlled with good manufacturing practices (Snowdon and Cliver, 1996). Among the microorganisms isolated from the honey and pollen of the stingless bee *S. jujuyensis*, **(Fig. 1A)** bacteria belonging to the *Bacillus* genus were the more abundant (40%) and were identified using their 16 S rRNA genes (**Fig. 1B**).

The *Bacillus* strains showed different enzymatic activities, e.g., protease, amylase, lipase, cellulase and xylanase (**Fig. 2 A-D**). Protease activity was found in 82.0% of the isolates, followed by amylase (63.0%), xylanase (54.0%), cellulase (36.0%) and lipase (27.0%), (**Table 1**). The presence of the *Bacillus* genus was previously reported by other authors particularly in the stingless bee's products such as honey. (Ngalimat et al., 2019; Pajor et al., 2018; Zulkhairi et al., 2019). *Bacillus* strains are spore-forming bacteria, stable in acidic pH with the ability to colonize different environments, such as honey.

Although these bacterial species are associated with bees, their biological functions are not clear. In stingless bees colonies, bacteria such as *Bacillus* strains could be involved in the degradation the bees' products through enzyme production (Gilliam et al., 1990). These microorganisms could have an important role in the fermentation and conversion of pollen constituents by secreting hydrolytic enzymes (Gilliam et al., 1985; 1989; 1990). High protease, amylase, and xylanase activities were evidenced in the isolates (**Table 1**). These enzymes could be involved in the breakdown of complex biomolecules, such as carbohydrates and proteins which have an essential role in the composition of the bees' products (Gilliam et al., 1990; Lee et al., 2015; Vásquez and Olofsson, 2009). These hydrolase properties are a useful biotechnological tool to improve the efficiency of industrial processes. Enzymes produced by microorganisms with tolerance for a wide range of temperatures, pH, and salinity might be used industrially (Combey, 2017; Syed et al., 2018).

After the evaluation of enzyme production, three *Bacillus* strains were selected for more studies (**Fig. 2**). The isolates 4A, 86B, and 230-p were grown in the presence of bile salts (**Fig. 2E**). The production of the extracellular polymer matrix in the solid medium was also evaluated. Only the isolates 86B and 4A showed the ability to grow in the presence of bile salts and to produce an extracellular polymer matrix (**Fig. 2E and F**). The strain 4A was selected to evaluate the EPS production **(Fig. 2F**). The microorganism grew in the presence of sucrose as a carbon source and the EPS was partially purified using ethanol precipitation.

The sugar profile of the EPS was measured using HPLC for EPS and hydrolyzed EPS **(Fig. 3A** and **B).** Fructose was the more abundant sugar (110±10 mg/g EPS) followed by glucose (31±2 mg/g EPS). After hydrolysis, the fructose concentration increased 4 times its value (450±20 mg/g EPS), showing that it was the main fructose polymer (**Table 2**). The functional groups in the EPS were identified using FTIR spectroscopy. **Fig. 3C** shows the EPS spectrum, which is characterized by a dominant band at 3430 cm^{-1}, corresponding to the hydroxyl stretching vibration of the polysaccharide. The band at 2935 cm^{-1} could be due to the C-H stretching vibration corresponding to the methyl and methylene groups. The region between 1000 and 200 cm^{-1} is considered the fingerprint region for carbohydrates. This region is dominated by the ring vibration overlapping the stretching vibration of the C-OH side group and by the glycosidic band vibration (C-O-C). Considering the high fructose concentration observed using HPLC, the FTIR spectrum was compared with a commercial levan, a polymer-rich in fructose, and produced by the *Bacillus* genus (**Fig. 3C**). Both spectra were similar, and few differences were observed. Although polysaccharide production is known in *Bacillus*, its composition can vary significantly between different strains. The levan structure is not uniform and is determined by the microorganism and the culture conditions (Chaves et al., 2020; Romero et al., 2018). The *Bacillus* strains from honey of different bees are a source of different types of levan (Hamdy et al., 2017; Ragab et al., 2020).

Levan has interesting applications as a polymer of fructose for coating different compounds such as food or drugs (Tomulescu et al., 2016).

The emulsifier properties of levan could be used to stabilize two phases distributed in a gel. Levan can be used to form hydrogels by emulsification for the encapsulation of both oil and water, two immiscible phases in one system (Rong et al., 2020). Hydrogels using emulsification have attracted increasing attention due to their intrinsic properties that can be used for different biotechnological applications (Rong et al., 2020). The emulsifying activity of levan from *Bacillus* sp. 4A was evaluated with kerosene, fish oil, and chia oil **(Fig. 3D).**

The EPS was more effective in forming stable emulsions for 24 h with chia oil followed by fish oil and a low stability of the emulsion was observed with kerosene (**Table 3**).

Thus, the stability for emulsions with oils could be related to the effect of the hydrophilic components of the EPS that increase the viscosity, which can reduce the movement of the oil droplets and therefore their flocculation (Dickinson, 2009; von Staszewski et al., 2014). Levan could be used for the delivery of nutritional compound such as omega-3 PUFA Recent studies suggested that human gut microbiota, host immune cells, and omega-3 PUFA, work together to ensure the intestinal wall integrity (Parolini, 2019).

The antimicrobial activity of levan has also been reported (Esawy et al., 2011; Hamdy et al., 2018; Ragab et al., 2020). Therefore, the antimicrobial activity of levan from *Bacillus* sp. 4A was evaluated.

The *in vitro* antibacterial activity of EPS against *E. coli* (Gram-negative bacterium), *S. aureus* (Gram-positive bacterium), and *L. monocytogenes* (Gram-positive bacterium), was investigated. The size of the clear zone around the inoculum was measured. Levan was shown to be effective as an antimicrobial agent for all three at all concentrations (1.0, 0.5 and, 0.25 mg/mL). These results were similar to those observed by Koşarsoy Ağçeli and Cihangir (2020). The antibacterial activity was similar in both Gram positive and Gram-negative pathogens (**Table 4**). A few studies showed that microbial EPS had antimicrobial activity (Pajor et al., 2018; Yaşar Yildiz et al., 2019). EPS-producing microorganisms are promising strains that could be exploited for the production of added-valuable compounds (Aullybux et al., 2019; Salama et al., 2019; Wang et al., 2019). Several antibacterial mechanisms could be used to explain EPS activity, such as cell wall and cytoplasmic membrane disruption, alteration of cell division and DNA (He et al., 2010; Wu et al., 2010).

The prebiotic activity of levan from *Bacillus* sp. 4A was studied on strains of *L. casei* 1232. The effect of omega-3 PUFA and its emulsion with levan were also evaluated. The strain *L. casei*, showed better growth in the presence of the biopolymer whether hydrolyzed or not, observing a slight improvement, ~35% more growth with the hydrolyzed EPS (**Fig. 4).**

The effect of the levan and omega-3 PUFA blend on microbial growth was also evaluated. Hydrogels using emulsification slightly improved the growth of *L. casei* between 12 and 24 h of culture, compared to the growth of the microorganism in the presence of omega-3 PUFA but without the biopolymer (**Fig. 4).** Both omega-3 PUFA or levan affected the human and animal gut microbiota composition and the control of related diseases (Costantini et al., 2017). These natural components showed good compatibility and an interesting effect on the probiotic microorganism. A possible connection between hydrogels using emulsification with the microbiota suggested the need for further study.

To evaluate the effect of the EPS on the metabolism of the *Bacillus* sp. 4A this microorganism was growth using a simulated GIT. The results showed that the survival of *Bacillus* sp. 4A in gastric juice was independent of the pH. The microorganism was viable after 120 min with all three conditions. In the most extreme condition (pH = 2.0), a reduction of 5 log units was observed after 120 min, while in the less extreme condition (pH = 3.0) no significant changes in the number of cells were observed (**Fig. 5A**).

Fig. 5B shows that the simulated enteric juice was able to reverse the effect of the simulated gastric juice. The cells from the lower pH were able to recover, increasing 2 logarithmic units after 120 min and retained those viable cells for 24 h (**Fig. 5B**). In the case of pH = 2.5, an increase of 4 logarithmic units was observed at 240 min but decreased 2 units at 24 h. While in the least extreme condition, no significant changes were observed in the viable count.

The ability of this strain to survive the extreme conditions in the simulated GIT could be related to the ability of *Bacillus* sp. 4A to form an extracellular polymeric matrix that favors its adherence to the intestinal epithelium and the possibility to trigger biochemical effects such as enzymatic activities (Barbosa et al., 2005; Hamdy et al., 2017; Jeżewska et al., 2018) Besides, this polymeric matrix enables the anaerobic sporulation and the ability to retain antimicrobial activity (Elshaghabee et al., 2017).

On the other hand, *Bacillus* had a positive influence on the growth and composition of the commensal and beneficial species in the intestine with the production of enzymes, vitamins, and extracellular peptides (Elshaghabee et al., 2017; Lee et al., 2019).

Conclusions

A *Bacillus* strain was isolated from the honey of *S. jujuyensis*, a native stingless bee from northern Argentina.

Bacillus sp. 4A was able to produce hydrolytic enzymes, an abundant extracellular polymer matrix (EPS), and grow in bile salts. The EPS was partially purified and identified as levan. The biopolymer showed antimicrobial activity, emulsification properties, and prebiotic activity.

In addition, the strain was resistant to gastric conditions which could be related to the protective effect of the extracellular polymer matrix.

The properties reported make EPS a potential prebiotic compound and *Bacillus* sp. 4A a potential probiotic strain.

Considering that the microorganism comes from the stingless bee honey, a natural product highly beneficial due to its features from its composition, the microorganism and its bio-products are considered as promising nutraceutical compounds that could be added to the honey of *S. jujuyensis*. This strain could provide an added value to the products of the native stingless bees colonies which are traded around the world.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Figure Legends

Figure 1. A- *S. jujuyensis* belong to the meliponids group, insects of the family Apidae (Hymenoptera: Apidae: Meliponini). **B-** phylogenetic tree of *Bacillus* species based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method.

Figure 2. Properties of the *Bacillus* spp. isolated from the native stingless bee products, *S. jujuyensis*. **A-** proteolytic enzyme, **B-** amylolytic enzyme, **C-** lipolytic enzyme, **D-** xylanolytic enzyme, **E-** growing of the *Bacillus* strains in bile salts**, F-** extracellular polymer matrix production in solid medium by *Bacillus* strains.

Figure 3. Sugars composition detected with HPLC with a refractive index detector: **A-** exopolysaccharide EPS (on the upper right margin shows a chromatogram of a mixture of commercial standards of fructose, glucose, sucrose, maltose, trehalose and melezitose) and **B-** hydrolyzed exopolysaccharide; **C-** FTIR spectrum of the exopolysaccharide from *Bacillus* sp. 4A and commercial levan as control; **D-** emulsification index (EI_{24}) of the EPS with hydrophobic compounds: kerosene, fish oil and chia oil.

Figure 4. Prebiotic activity on *Lactobacillus casei* 1232 with EPS (5%) (■); EPS hydrolized (5%) (▲); omega-3 PUFA (×); omega-3 PUFA plus EPS (●); *Lactobacillus casei* 1232 growing with a limited carbon source (♦).

Figure 5. Effect of extracellular polymeric matrix on the *in vitro* resistance to the gastrointestinal tract (GIT) of *Bacillus* sp. 4A. **A-** gastric juice; **B-** enteric juice.
pH 2.0 (♦); pH 2.5 (■); pH 3 (▲).

Table 1. Enzymatic activities of bacteria isolated from honey and pollen of *Scaptotrigona jujuyensis*

		Hydrolytic enzyme activity*				
Number of Isolate	**Origin**	**Proteolytic**	**Lipolytic**	**Amylolytic**	**Xylanolytic**	**Cellulolytic**
41	honey	0.9	-	-	1.5	-
43	honey	0.5	-	-	0.4	0.8
46	honey	-	-	-	-	-
49	honey	-	-	-	-	-
51	honey	-	-	-	-	-
63	honey	-	-	-	3.2	3.4
73	honey	-	0.2	-	0.5	1.2
74	honey	-	0.1	-	2.4	1.6
77	honey	1.5	-	0.3	1.8	4.0
78	honey	1.0	0.7	0.1	2.8	-
84	honey	0.7	-	-	-	-
85	honey	1.6	-	0.4	1.8	3.9
86	honey	1.6	0.5	0.3	3.0	3.5
2A	honey	1.6	-	0.1	-	-
3A	honey	1.4	-	0.3	-	-
4A	honey	1.3	-	0.9	-	0.5
12A	honey	0.3	0.1	-	0.1	-
16A	honey	0.8	-	0.3	0.2	-
86B	pollen	0.5	-	0.7	-	-
11B	pollen	-	0.1	0.2	-	-
21 p	pollen	0.9	-	-	-	-
30-p	pollen	0.5	-	0.3	-	-
230-p	pollen	0.4	-	0.2	0.7	-
199 p	pollen	1.1	-	0.3	-	-

*****Hydrolytic activities were measured in mm and express as the halo/colony diameter (h/c) ratio.

Table 2. Main sugars in the exopolysaccharide (EPS) and in the hydrolyzed EPS determined with HPLC - refractive index.

Sugar	EPS (mg/g EPS)	Hydrolyzed EPS (mg/g EPS)
Fructose	110±10	450± 20
Glusose	31±2	35± 2
Sucrose	8 ±1	ND*

*ND: No determined

Table 3. Emulsification index (EI_{24}) of the EPS against hydrophobic compounds

Hydrophobic material	EI_{24} (%)*
Chia oil	30±2 b
Fish oil	20±1 b
Kerosene	5.5±1 a

Tukey test was made to evaluated the significant difference between the variables. Different letters indicated that the variables showed different behaviors ($p<0.05$).

Figure 1

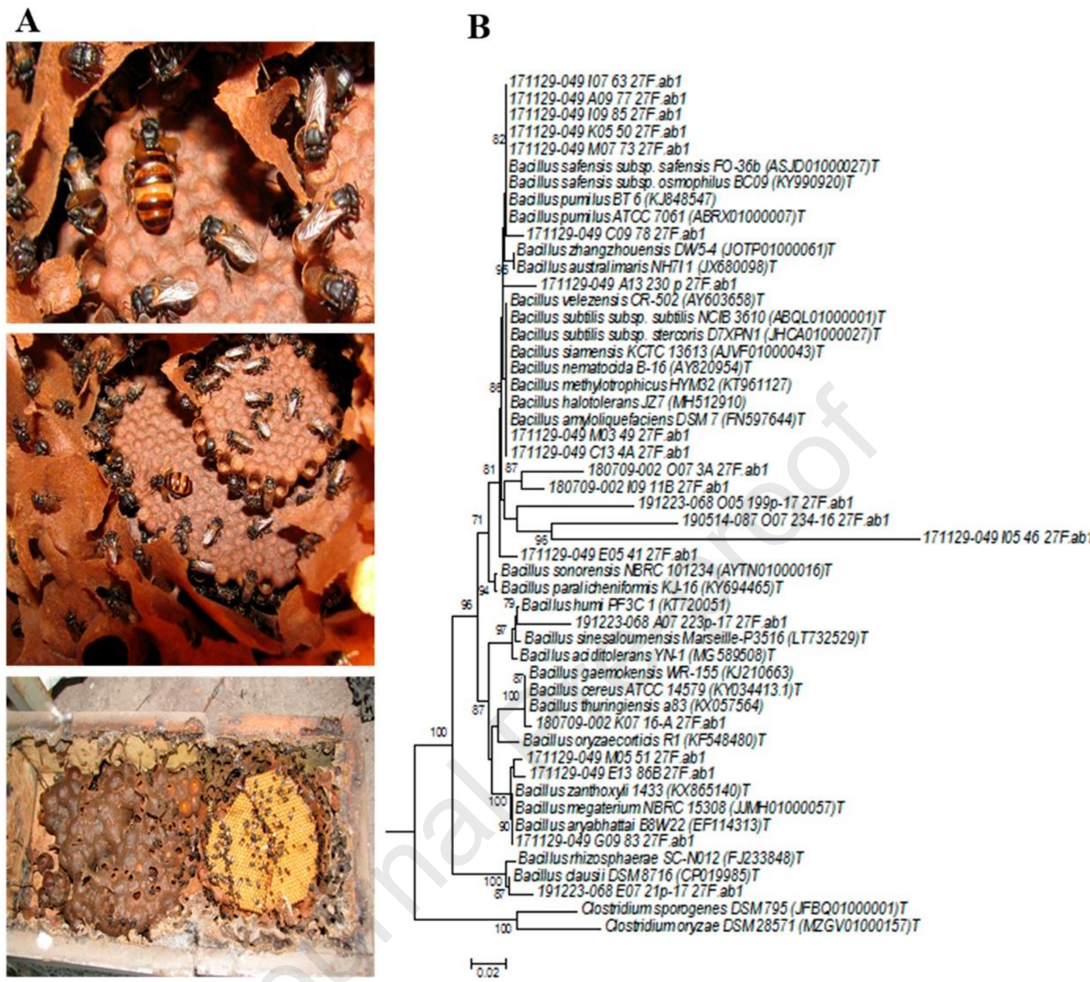
A
B
171129-049 I07 63 27F.ab1
171129-049 A09 77 27F.ab1
171129-049 I09 85 27F.ab1
171129-049 K05 50 27F.ab1
171129-049 M07 73 27F.ab1
Bacillus safensis subsp. safensis FO-36b (ASJD01000027)T
Bacillus safensis subsp. osmophilus BC09 (KY990920)T
Bacillus pumilus BT 6 (KJ848547)
Bacillus pumilus ATCC 7061 (ABRX01000007)T
171129-049 C09 78 27F.ab1
Bacillus zhangzhouensis DW5-4 (JOTP01000061)T
Bacillus australimaris NH7I 1 (JX680098)T
171129-049 A13 230 p 27F.ab1
Bacillus velezensis CR-502 (AY603658)T
Bacillus subtilis subsp. subtilis NCIB 3610 (ABQL01000001)T
Bacillus subtilis subsp. stercoris D7XPN1 (JHCA01000027)T
Bacillus siamensis KCTC 13613 (AJVF01000043)T
Bacillus nematocida B-16 (AY820954)T
Bacillus methylotrophicus HYM32 (KT961127)
Bacillus halotolerans JZ7 (MH512910)
Bacillus amyloliquefaciens DSM 7 (FN597644)T
171129-049 M03 49 27F.ab1
171129-049 C13 4A 27F.ab1
180709-002 O07 3A 27F.ab1
180709-002 I09 11B 27F.ab1
191223-068 O05 199p-17 27F.ab1
190514-087 O07 234-16 27F.ab1
171129-049 I05 46 27F.ab1
171129-049 E05 41 27F.ab1
Bacillus sonorensis NBRC 101234 (AYTN01000016)T
Bacillus paralicheniformis KJ-16 (KY694465)T
Bacillus humi PF3C 1 (KT720051)
191223-068 A07 223p-17 27F.ab1
Bacillus sinesaloumensis Marseille-P3516 (LT732529)T
Bacillus aciditolerans YN-1 (MG 589508)T
Bacillus gaemokensis WR-155 (KJ210663)
Bacillus cereus ATCC 14579 (KY034413.1)T
Bacillus thuringiensis a83 (KX057564)
180709-002 K07 16-A 27F.ab1
Bacillus oryzaecorticis R1 (KF548480)T
171129-049 M05 51 27F.ab1
171129-049 E13 86B 27F.ab1
Bacillus zanthoxyli 1433 (KX865140)T
Bacillus megaterium NBRC 15308 (JJMH01000057)T
Bacillus aryabhattai B8W22 (EF114313)T
171129-049 G09 83 27F.ab1
Bacillus rhizosphaerae SC-N012 (FJ233848)T
Bacillus clausii DSM 8716 (CP019985)T
191223-068 E07 21p-17 27F.ab1
Clostridium sporogenes DSM 795 (JFBQ01000001)T
Clostridium oryzae DSM 28571 (MZGV01000157)T
82
95
86
81
87
71
96
95
94
79
97
87
100
87
100
90
100
87
100
0.02

Figure 2

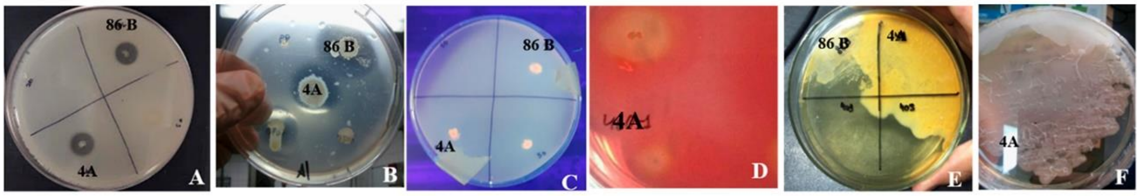

Figure 3

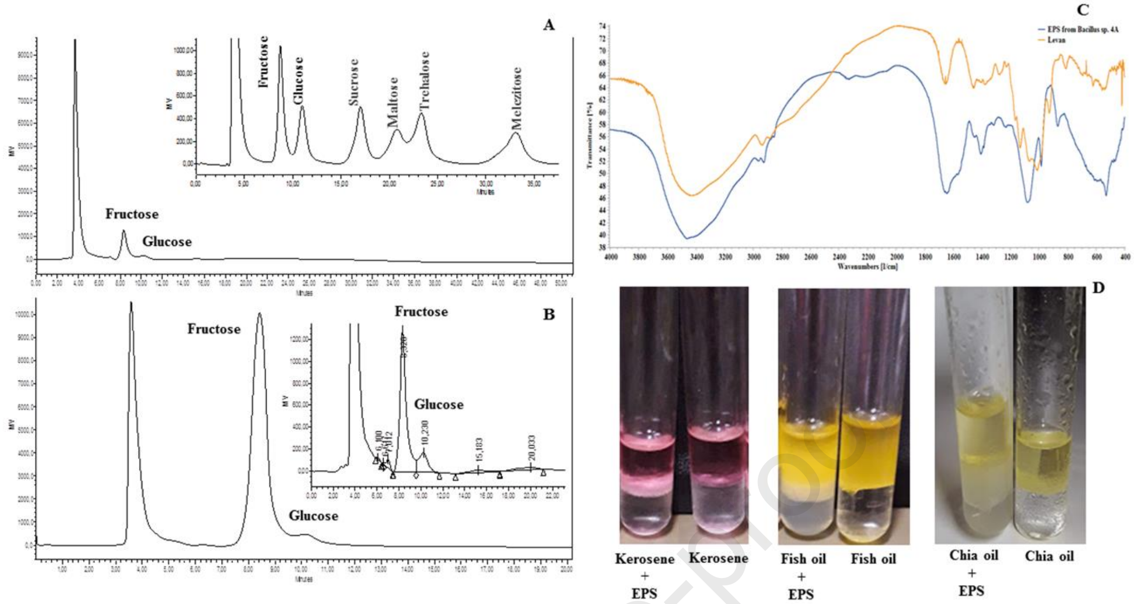
A
Fructose
Glucose
Sucrose
Maltose
Trehalose
Melezitose
Fructose
Glucose
B
Fructose
Glucose
Fructose
Glucose
C
EPS from Bacillus sp. 4A
Levan
Transmittance [%]
Wavenumbers [1/cm]
D
Kerosene + EPS
Kerosene
Fish oil + EPS
Fish oil
Chia oil + EPS
Chia oil

Figure 4.

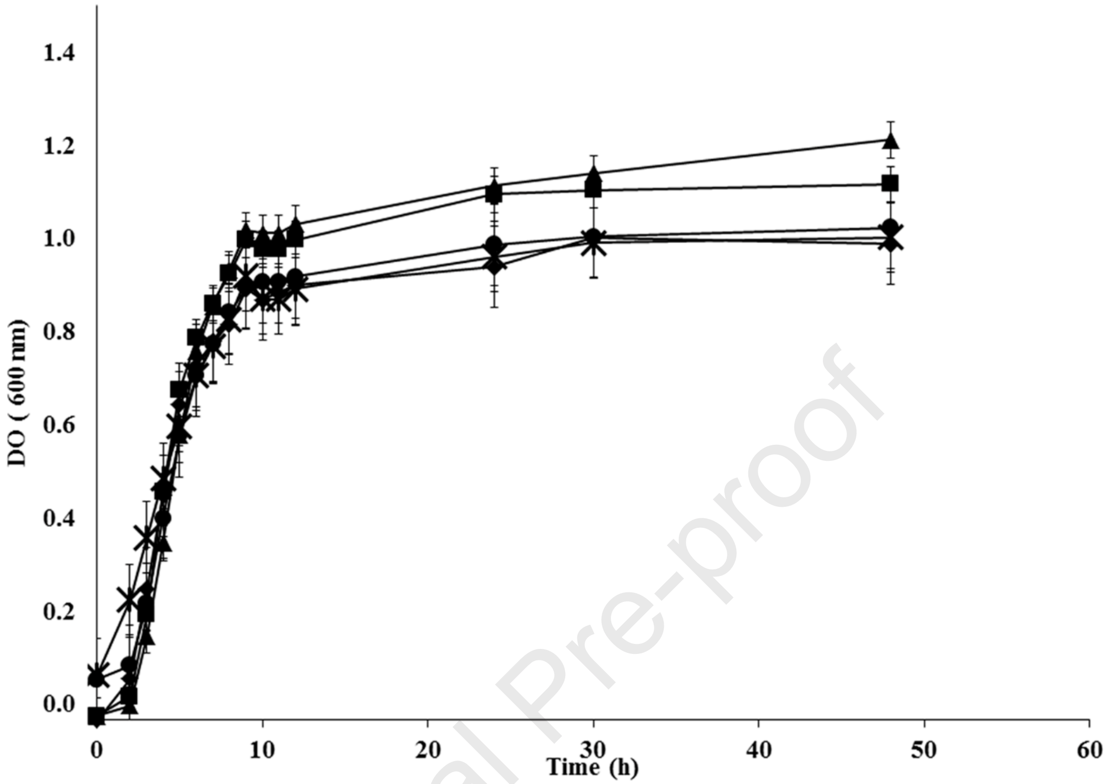

Figure 5

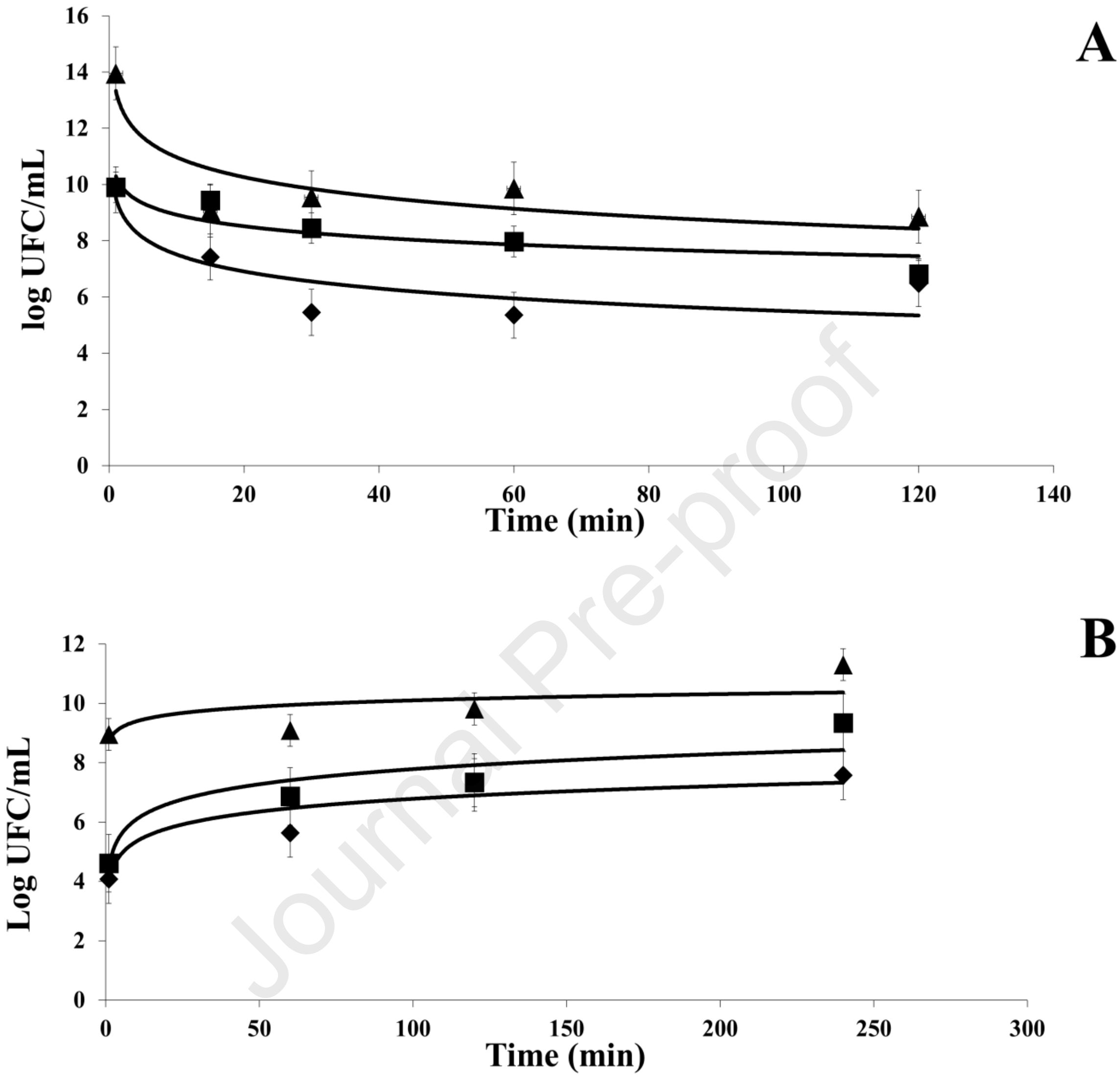
A
log UFC/mL
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (min)
B
Log UFC/mL
12
10
8
6
4
2
0
0
50
100
150
200
250
300
Time (min)

Table 4. Antibacterial activity of EPS- inhibition zones. Values are expressed as mean ±

EPS (mg/mL)	Diameters of inhibition zone (cm)		
	L.monocytogenes Scott A	*E.coli ATCC 25922*	*S. aureus ATCC 35217*
1	1.4± 0.28 a	2.05± 0.5 a	1.45± 0.3 a
0.5	1.3± 0.17 a	1.5± 0.4 a	1.3± 0.1 a
0.25	1.1± 0.14 a	1.2± 0.3 a	1.2± 0.1 a

standard deviation, n=3.

Values with no letters in common within each row are significantly different ($p<0.005$)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: