



## Characterization of Novel Biosurfactant Produced from Probiotic *Enterococcus faecium* NM113

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### Abstract

This work aims to produce, identify, and characterize the bioactive surfactant from *Enterococcus faecium* NM113, previously isolated as probiotic from the feces of breast-fed infants according to its immunomodulatory properties. The yield of the biosurfactant (BS) production was as 0.2 g L<sup>-1</sup>. Functional and structural characterizations of the produced biosurfactant were achieved by TLC followed by post chromatographic detection. Silica plate or replica plate was sprayed with anthrone produced gives a yellow spot indicated the presence of sugar moieties. Lipopeptide residues were detected while stained with Ninhydrin reagent. According to FTIR spectrum obtained, the extracted BS is composed of carbohydrates and lipid. The hydrophobic chain of the BS is composed of lipid and hydrophilic part is mainly composed of sugar. Furthermore, Identification of BS features was done by <sup>1</sup>H NMR and <sup>13</sup>C NMR and confirmed as glycolipid. The evaluation of the antimicrobial activity of BS produced from *E. faecium* NM113 was done against five pathogens. The biosurfactants have a great opportunity to be novel agents to compete the antimicrobial-resistance microorganisms.

**Key words:** Biosurfactants, *E. faecium* NM113, Antimicrobial, Antioxidant and Probiotics.

### Introduction

In nature, numerous sources and products are available for medicine and treatment preparations. The natural products comprise extracts or pure chemical compounds created by living organisms providing biological properties on further organisms [1].

Natural resources are found in bacteria, yeast, plants, marine sources, and animals. Probiotics and beneficial bacteria might have the ability to exert natural compounds providing anti-inflammatory and immunomodulatory properties.

In our previous work, we screened numbers of lactic acid bacteria isolated from breast-fed infant for their immunomodulatory properties; one of those is the probiotic bacterium *Enterococcus faecium* NM113 which also meet the safety issues and probiotic properties [2]. *E. faecium* NM113 expressed ability to activate human immune cells by induction the release of interleukin 12 (IL-12), which is responsible for generating T helper 1 cells in the innate immune system and downregulating the inflammation. Hence the strain *E. faecium* NM113

showed the activation for Toll-like receptor 2 (TLR-2), we expected lipopeptides would be responsible for its immunomodulatory properties [2].

The biosurfactants (BS) are known as the bioactive molecules that formed through microbes including bacteria, fungi and yeasts. The forming of the BS could be either associated to the cell membrane or out the cells [3,4]. Many of BS have been isolated from different bacteria and characterized for their structure and bioactivities [5-10]. The element structures of BS comprising of (carbohydrate, polysaccharides, lipids and proteins) providing many applications in the biomedical, pharmaceutical and food industries such as emulsification, antioxidant, antimicrobial, and anti-biofilm activities.

Here we present the isolation of the surfactant from the *E. faecium* NM113 and characterization its chemical structure. In addition, the isolated surfactant was evaluated in respect to antioxidant, antimicrobial, cytotoxic, and anti-inflammatory. The results confirm the surfactant isolated from *E. faecium* NM113 as a promising biosurfactant for

consideration in pharmaceutical, medicine, and food industries.

## Materials and methods

### Bacterial strains and growth conditions

*Enterococcus faecium* NM113 strain was isolated previously as probiotic in our laboratory from infant feces [2]. It was grown on deMan Rogosa and Sharpe (MRS) agar medium contains (20 g D-Glucose, 10 g Peptone, 10 g Beef extract, 5 g Yeast extract, 2 g Di-potassium phosphate, 2 g Tri-ammonium hydrogen citrate, 5 g Sodium acetate, 0.2 g Magnesium sulphate, Manganese sulphate and 1 g Tween-80 per liter) (Difco, Franklin Lakes, NJ, USA) and incubated at 37°C for 48 h.

### Extraction of biosurfactant

The isolation of the surfactant from *E. faecium* NM113 was performed as described previously [11], whereas 600 mL of MRS broth in 1 L Erlenmeyer flask were inoculated by 1 % of an overnight culture *E. faecium* NM113 followed by incubation for 72 h at 37 °C on a rotatory shaker at 120 rpm. Then the cells were harvested by centrifugation (10,000×g for 5 min at 10 °C), washed twice in demineralized water, and suspended in 100 mL of phosphate-buffered saline solution (PBS) pH 7.0. Cell suspensions were incubated at room temperature for 2 h with gentle stirring to release the biosurfactant then followed by centrifugation. The supernatant was dried in an oven at 70 °C and the biomolecules were extracted by acid precipitation as described by Van Hoogmoed *et al.* [12]. The acidified samples were incubated at 4 °C for 2 h, and the precipitates were collected by centrifugation (10,000×g for 15 min at 4 °C) and washed twice with acidic water (pH 2.0). The precipitates were dissolved in distilled water and adjusted to pH 7.0 using 1 M NaOH [13].

### Chemical and biological characterization of the isolated biosurfactants

The chemical structure was identified by (TLC, FT-IR, and NMR). The surfactant was also evaluated for its antimicrobial activity using the well agar diffusion method, antioxidant ability targeting the DPPH free radical and cytotoxicity ability using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. The characterization of biosurfactant was achieved at Central Laboratories Network, National Research Centre, Giza, Egypt (<https://www.nrc.sci.eg/central-labs/>).

### Thin layer chromatography (TLC)

The composition of the cell-bound partially purified biosurfactants was determined by TLC followed by post chromatographic detection. Briefly, 4 ml of PBS extract was extracted twice with ethyl acetate 1:1.25 (Rankem, India). Upper phase was

extracted two times with ethyl acetate and the ethyl acetate was allowed to evaporate at room temperature [14, 15]. One mL aliquot of BS extract was concentrated and separated on a precoated silica gel plate (Merck, India) [16]. Chloroform, methanol and water in the ratio of 70:10:0.5 was used as developing solvent system with different color developing reagents. Two such reagents like, ninhydrin reagent (0.5 g ninhydrin in 100 mL anhydrous acetone) was used for lipopeptide biosurfactant detection as red spots and anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) to detect glycolipid biosurfactant as yellow spots [17].

### Fourier-transform infrared spectroscopy (FT-IR)

The infrared spectra (with wave numbers spanning from 4000 to 400 cm<sup>-1</sup>) were recorded in a Shimadzu FT-IR-8400 spectrometer using 2 mg of the extracted BS combined with 200 mg KBr (Spectroscopic Grade). The data acquired represented an average of 50 scans across the whole range [18].

### Nuclear magnetic resonance (NMR) spectroscopy

The 1–3 mg BS was dissolved in 100% CDCl<sub>3</sub> and <sup>13</sup>CNMR analysis was carried out using a Bruker Av II-400 spectrometer. Both proton and carbon NMR chemical shifts were stated in ppm relative to the solvent shift as chemical standard. Peaks were compared and predicted with the data reported previously [19].

### Antimicrobial activity assay

The antibacterial activity of partly purified biosurfactants was investigated against Gram positive bacteria *Staphylococcus aureus* (ATCC 29213) and gram-negative bacteria (*Escherichia coli* (ATCC 25922), *Helicobacter pylori* and *Pseudomonas aeruginosa* (ATCC 27953) also tested against yeast *Candida albicans*. The identified pathogenic bacteria and yeast were obtained from the Microbiology Laboratory of eco-friendly compound, National Research Center, Giza, Egypt. The pathogenic strains were cultivated as follows: *S. aureus* on BHI agar; *E. coli*, *H. pylori* on blood agar, *Ps. aeruginosa* on LB agar and *C. albicans* on nutrient agar. The antibacterial activity was evaluated by the agar well diffusion method [19]. 200 microliters of the test bacteria (10<sup>7</sup> CFU/mL) were spread on the selected medium to test their antibacterial activity. After 2 hours at 37 °C incubation, a well (7 mm) was picked and filled with 1 µg biosurfactant (50 µL of 20 µg / mL concentration). As a negative control, sterile distilled water was put into the well. To observe the zone of inhibition, the plates were incubated at 37 °C for 24 hours. The experiment was carried out in triplicate.

### Cytotoxicity activity (MTT assay)

MTT assay is colorimetric assay based on enzymatic activity of cellular oxidoreductase enzymes in metabolically active cells to reduce the yellow water-soluble substrate tetrazolium dye MTT into an insoluble purple formazan. Formazan is an insoluble blue crystalline product that can be dissolved with an appropriate detergent and measured spectrophotometrically. The amount of formed formazan is directly proportional to the number of viable cells [20].

Briefly, MDA-MB-231 cells (8000 cells/well) were seeded onto 96-well plates in a total volume of 200  $\mu$ l and left overnight to form a semi-confluent monolayer. Cell monolayers were treated in quadruplicate with BS samples (100  $\mu$ g/mL) for an exposure time of 48 h. Doxorubicin hydrochloride was used as reference cytotoxic drugs. At the end of exposure, 30  $\mu$ l/well of MTT solution in DPBS (5 mg/ml) was added to all wells and left to incubate for 90 min. The formation of formazan crystals was visually confirmed using phase contract inverted microscope (Olympus CK2, Japan). DMSO (100  $\mu$ l/well) was added to dissolve the formazan crystals with shaking for 10 min after which the absorbance was read at 492 nm against blank (no cells) on a Tristar LB2 microplate reader (Berthold, Germany). The percentage of viability was calculated using the following formula: % viability =  $\frac{At-Ab}{Ac-Ab} \times 100$

Whereas, **At**: Absorbance of treated cells, **Ab**: Absorbance of blank (medium only without cells), **Ac**: Absorbance of control. IC<sub>50</sub> values were calculated using the dose response curve fit to non-linear regression correlation using Graph Pad Prism® V6.0 software (GraphPad Inc., San Diego, USA).

### 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity

The scavenging activity for DPPH free radical was measured according to Zhao *et al.* (2006) [20] with some modifications. Sample solutions of BS (100  $\mu$ L) at different concentrations (2.5, 5.0, 10.0, 20.0, and 40.0 mg/ml) were mixed with 900  $\mu$ L of 0.1mM DPPH solution in methanol. The mixture was shaken vigorously and allowed to reach a steady state for 30 min in dark at temperature 37°C. Decolourization of DPPH was determined by measuring the absorbance at 517 nm, and the DPPH radical scavenging was calculated according to the following equation: % Scavenging activity =  $(A_1 - A_2/A_1) \times 100$

Where **A<sub>1</sub>**: was the absorbance of the DPPH solution without sample and **A<sub>2</sub>**: was the absorbance of DPPH with the sample. Ascorbic acid was taken as the standard. All the tests were performed in triplicate.

## Results and discussion

### Production of biosurfactant

The surfactant was produced from probiotic strain *E. faecium* NM113 and the yield production

was 0.2 g L<sup>-1</sup> by using shaking 150 – 300 rpm. The observations by other researchers that indicated the yields of the BS from lactic acid bacteria was in low quantities and this could be improved by modification of the growth media, conditions and temperature [13, 21, 22]. In this work the aim was isolation and characterization of the produced BS from *E. faecium* NM113, we would optimize the yield production of the BS in future work.

### Chemical composition of the produced biosurfactant

To accurate determination of the chemical composition of the produced BS, the use of combination of different methods as TLC, FTIR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and GC-Mass were used.

#### Thin-layer chromatography (TLC)

BS was initially characterized by TLC followed by post chromatographic detection. Silica plate or replica plate was sprayed with anthrone giving a yellow spot which indicated the presence of sugar moieties Figure 1 (A). Lipopeptide residues were detected while stained with Ninhydrin reagent Figure 1 (B). The results of TLC characterization confirmed the presence of the glycolipid BS. Generally, glycolipids are carbohydrates in amalgamation with long-chain aliphatic or hydroxy aliphatic acids [14, 15].

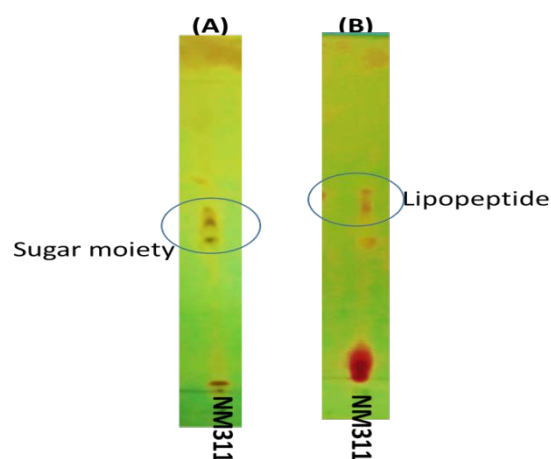


Figure 1. Thin-layer chromatography (TLC)

#### Fourier Transform InfraRed (FTIR) analysis.

Analysis of FTIR spectrum of BS produced from *E. faecium* NM113 revealed the composition as lipid and polysaccharide fractions. The molecular composition of the BS as indicated in Figure (2) and Table (1) revealed the presence of OH stretching vibration around 3380 cm<sup>-1</sup> of the sugar moiety, adsorption bands were located at 2900 cm<sup>-1</sup>–2950 cm<sup>-1</sup> (C–H stretching bands of CH<sub>3</sub> and CH<sub>2</sub> groups), 1725 cm<sup>-1</sup> and 1638 cm<sup>-1</sup> (C = O stretching vibrations of the carbonyl groups). A single band

near  $1457\text{cm}^{-1}$  was observed due to (CH) bending. Another peak at  $1384\text{cm}^{-1}$  was noticed and caused by -COC- anti-symmetric stretching, which also, corresponded to groups characteristic of carbonyl groups in unsaturated aliphatic carboxylic acids and C-H bending of the  $\text{CH}_2$  and  $\text{CH}_3$  groups,  $1045\text{cm}^{-1}$  corresponds to -COC- stretching in carbohydrate cyclic structures and indicated the presence of bonds formed between carbon atoms and hydroxyl groups

in the sugar. These results suggests the presence of glycolipid type of the surfactant [11].

According to FTIR spectrum obtained, the produced BS is composed of carbohydrates and lipid. The hydrophobic chain of the surfactant is composed of lipid and hydrophilic part is mainly composed of sugar. Comparison of the spectra obtained revealed it is closely similar to Xylolipid (glycolipid) reported earlier from different LAB strains [23- 25].

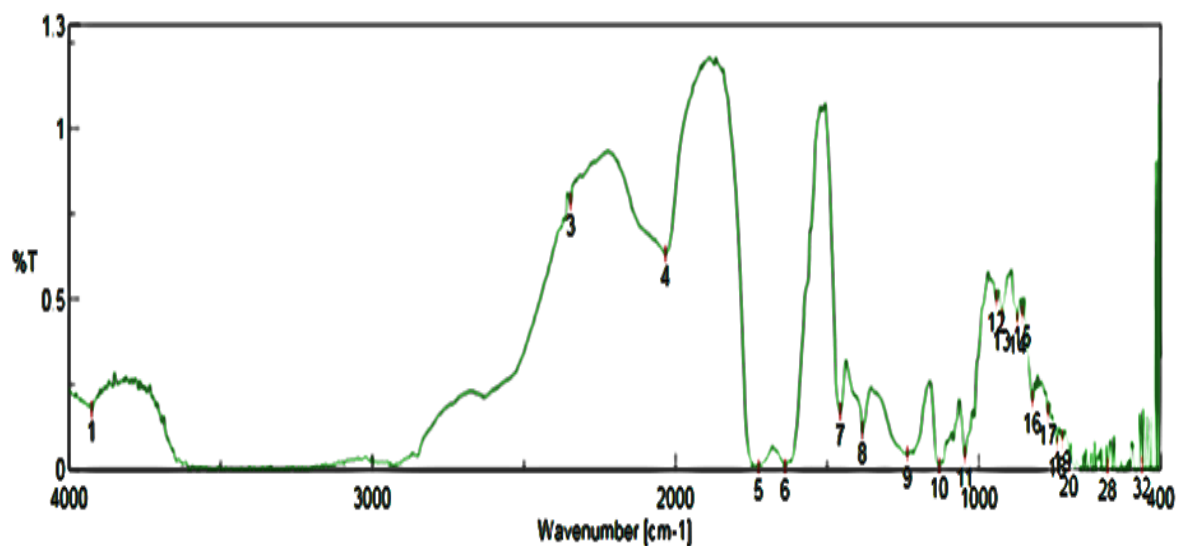


Figure (2): The spectral analysis of produced BS from *E. faecium* NM113 by FTIR

Table 1. The function groups detected by FTIR of BS produced by *E. faecium* NM113

Absorbance range ( $\text{cm}^{-1}$ )	Functional groups detected
3000–3600	OH stretching, typical polysaccharides
2900–2950	C-H (stretching) groups $\text{CH}_2$ and $\text{CH}_3$
1725, 1638	C = O (stretching of carbonyl group)
1457	CH stretching
1384	-COC- anti-symmetric stretching
1000–1300	C-O sugar stretching
675-1000	C-H bending

#### Characterization by NMR

The surfactant was further analysis by  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR and the results are shown in Figure 3 and Figure 4 in addition to Tables (2 and 3).  $^1\text{H}$  NMR results showed the occurrence of  $-\text{CH}_3-$  ( $\text{CH}_2$ ) – at ( $\delta$  0.91 – 0.93 ppm),  $-(\text{CH}_2)-$  at

( $\delta$  1.27, 1.28 ppm),  $-(\text{CH}_2-\text{OH})-$  at ( $\delta$  3.55 ppm). Also, the  $^1\text{H}$  NMR spectrum showed the anomeric proton of the sugar moiety as a doublet at  $\delta$  4.96 ppm. The other protons of the sugar ring resonated at  $\delta$  2.00–4.22 ppm.

Table 2. Chemical shift assignment of BS produced by *E. faecium* NM113 ( $^1\text{H}$  NMR)

Assignments	Chemical shift (ppm)
$\text{CH}_3-(\text{CH}_2)-$	0.91-0.93
$-(\text{CH}_2)-$	1.27,1.28
$-\text{CH}_2\text{OH}$	3.55
1-H (sugar)	4.96

The results from <sup>13</sup>C NMR indicated the presence of fatty acid derivatives as indicated from the carbonyl groups signals located at δ□167.67 – 176.92 ppm. The signals between δ□60.74 and 73.01

ppm may be attributed to the oxygenated carbons of the sugar moieties. The results propose that BS produced by *E. faecium* NM113 is glycolipid.

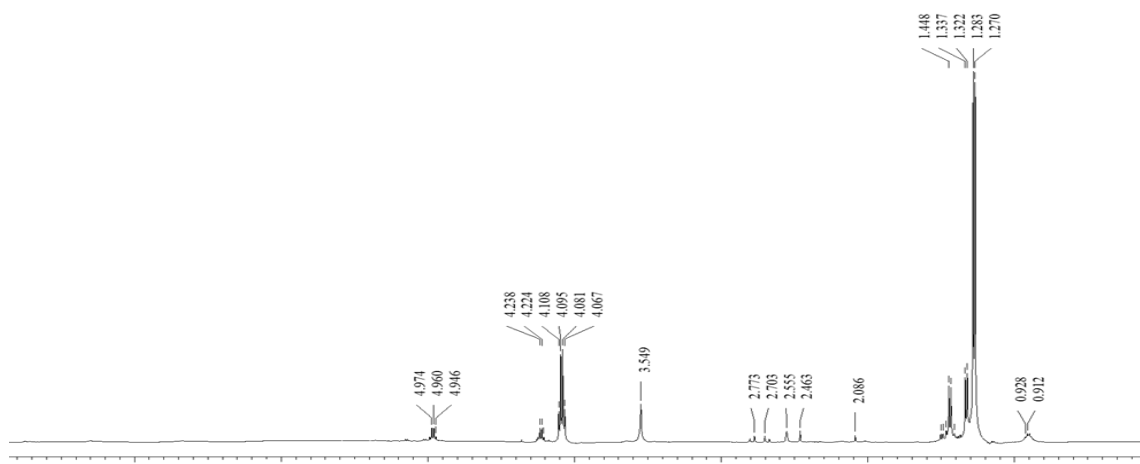


Figure 3 <sup>1</sup>H NMR Spectra of BS produced by *E. faecium* NM113

Table 3. Chemical shift assignment of BS produced by *E. faecium* NM113 (<sup>13</sup>C NMR)

Assignments	<sup>13</sup> C NMR (ppm)
C-1	128.52
C-2	60.74
C-3	60.74
C-4	69.61
C-5	55.51
OCH	55.51
COO	167.67-176.92

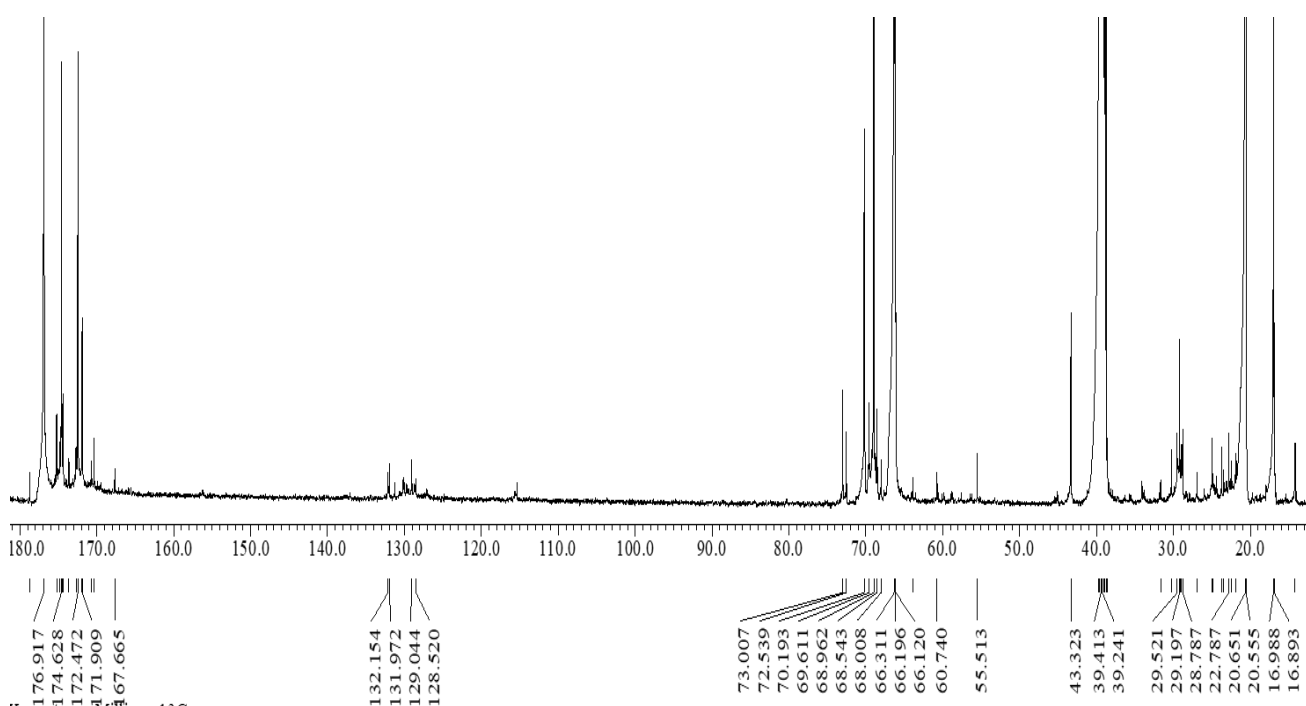


Figure 4 <sup>13</sup>C NMR Spectra of *E. faecium* NM113

### DPPH radical Scavenging activity

The scavenging activity for BS produced from *E. faecium* NM113 was determined by DPPH method. Five concentrations i.e. (2.5, 5, 10, 20, 40 mg/mL) were evaluated and results are indicated in Table (4) where the activity increased by increasing the concentration the surfactant showed  $88 \pm 1.02\%$  radical scavenging ability at a concentration of 40 mg/mL. The antioxidant activity of BS produced by

lactic acid bacteria were confirmed by other researchers. Fariq and Yasmin (2020) [26] reported 85 % scavenging activity of eight BS produced from halophilic bacteria. Also, Nageshwa and coworkers (2022) [27] showed that BS they got from their probiotic *Lactococcus lactis* LNH70 has anti-oxidative activity represented by scavenging activity.

**Table 4.** Scavenging activity of Bs produced by *E. faecium* NM113. Data are presented as mean $\pm$ SE.

Concentration (mg/ml)	Scavenging activity (%)
40	88 $\pm$ 1.02
20	85 $\pm$ 0.86
10	57 $\pm$ 0.6
5	36 $\pm$ 1.2
2.5	17 $\pm$ 0.98
IC50(mg/ml)	11.72

### Cytotoxicity assessment

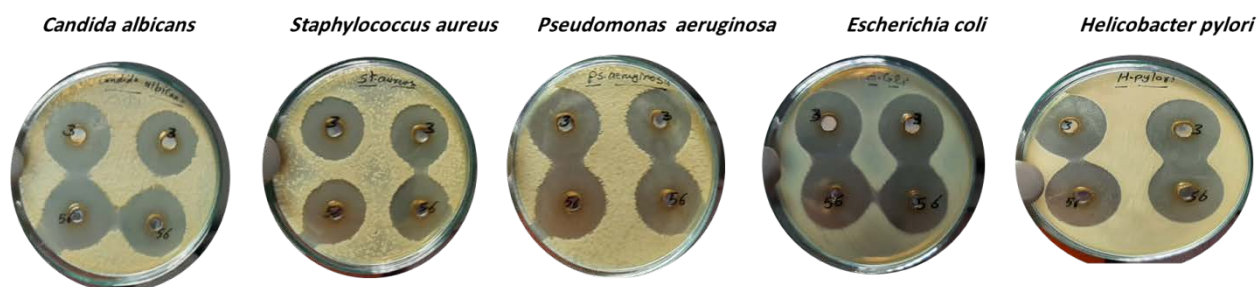
Cytotoxicity assessment was done for the produced BS to address its ability to affect the cell viability. Initial screening of the produced BS with concentration 100  $\mu$ g/mL was done by MTT assay against the proliferation of MDA-MB-231 cancer cell line revealed 100 % viability. Sharma and colleagues [28] isolated surfactant from *E. faecium* that showed low cytotoxicity. The low cytotoxicity is necessary for application the surfactant in medical purposes or food processing. The low cytotoxicity is necessary for application the surfactant in medical purposes or food processing.

### Antimicrobial activity

The evaluation of the antimicrobial activity of the produced BS from *E. faecium* NM113 was done against the following pathogens (*C. albicans*, *St.*

*aureus*, *P. aeruginosa*, *E. coli*, *H. pylori*) using agar well diffusion test (Figure 5) and the antimicrobial zones listed in Table (5). The tested BS has a great opportunity to be novel agents to compete the antimicrobial-resistance microorganisms.

Morais and coworkers [29] stated the antimicrobial activity of the BS produced by *L. gasseri* P65 and *L. jensenii* P6A against *C. albicans*, *Klebsiella pneumonia* in addition to G+ and G- pathogens. Also, Sakr and coworkers [30] showed that BS produced by *Lactobacillus plantarum* 60 FHE expressed antimicrobial activity against the tested pathogens G+ and G- but no antimicrobial activity was recorded against *C. albicans*. Therefore, the antimicrobial activity of the BS is confirmed as specific action for each BS as the same for the bacterial strains.



**Figure 5** Antimicrobial zones of the tested BS produced from *E. faecium* NM113 (encoded 3) against the pathogenic indicator strains using an agar well diffusion assay.

**Table 5.** Antimicrobial zones of BS produced from *E. faecium* NM113 using an agar well diffusion assay. Diameters of zones of inhibition are presented in mm. (a, b, c) represent three independent experiments.

	Inhibition zone (mm)				
	<i>E. coli</i>	<i>St. aureus</i>	<i>C. albicans</i>	<i>Ps. aeruginosa</i>	<i>H. pylori</i>
NM113 (a)	30	26	28	30	30
NM113 (b)	29	27	30	31	31
NM113 (c)	30	26	29	31	28

## Conclusion

Here we present the biosurfactant produced by the probiotic bacterium *E. faecium* NM113. The chemical structure identification of the isolated biosurfactant confirmed it as glycolipid by using TLC, FTIR, and NMR. The surfactant showed outstanding antioxidant and antibacterial activities. The results suggest it as novel biosurfactant for promising future applications in medical and pharmaceutical sectors.

## Conflicts of interest

The authors declare there are no conflicts of interest.

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## المخلص

تهدف هذه الدراسة إلى إنتاج وتحديد وتوصيف المتجانس الحيوي النشط لـ *Enterococcus faecium* NM113 ، الذي عزل سابقًا كمحفز معوي من أمعاء الأطفال الرضع الذين يتغذون على حليب الأم بناءً على خصائصه المناعية المعدلة. بلغ إنتاج المتجانس الحيوي 0.2 (BS) جرام / لتر. تم تحقيق التوصيف الوظيفي والهيكلية للمتجانس الحيوي المنتج باستخدام الكروماتوغرافيا التطبيقية المستتبعية بالكشف الكروماتوغرافي اللاحق. تم رش صفيحة سيليكات أو صفيحة مطبوعة بواسطة مركب الأنترون، حيث أن البقعة الصفراء المظهرة تشير إلى وجود مجموعات سكرية. تم اكتشاف بقايا الليبوبيبتيد عند تلويئها بمستحضر النينهيدرين. ووفقاً للطيف الرنين المغناطيسي النووي (NMR) المستحصل عليه، يتكون المتجانس الحيوي المستخلص من الكربوهيدرات والدهون. تتكون سلسلة BS الهيدروفوبية من الدهون والجزء المحب للماء يتكون أساساً من السكر. علاوة على ذلك، تم تحديد خصائص BS بواسطة الطيف الرنيني النووي للبروتونات والكربون وتأكيده وجود الجلايكوليبيد. تم إجراء تقييم للنشاط المضاد للميكروبات للمتجانس الحيوي المنتج من *E. faecium* NM113 ضد خمسة مسببات أمراض. يتمتع المتجانسات الحيوية بفرصة كبيرة لتكون عوامل جديدة تنافس الميكروبات المقاومة للمضادات الحيوية.

الكلمات الرئيسية: المدعمات الحيوية، *E. faecium* NM113 ، مضاد للميكروبات، مضاد للأوكسدة ومحفزات معوية.