ANTIMICROBIAL, ANTIOXIDANT, AND OPTIMAL CONDITION FOR EXOPOLYSACCHARIDES SECRATED FROM NEW LOCAL STRAIN-ISOLATE *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3

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ABSTRACT

Metabolic products of exopolysaccharide (EPS) generated by LAB strains have several biomedical effects. Our study focused on the isolation of a new strain of *Bifidobacterium* spp. From the sources of children's feces, examining them to choose the best EPS-producing strain, studying all the optimal conditions to increase the efficiency of producing more EPS, knowing the stage of their production, and finally introducing them into biomedical applications to test their antimicrobial and antioxidant activities. EPS is secreted by the most efficient new local *strain Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 was extracted and purified, resulting in the highest production (2.08 ± 0.41 g/L) in 1% (w/v) starch, which is the best carbon source with the best inoculum size of 1% (v/v) in 18 h at 37 °C and a pH value of 6.5. Coinciding with the best production phase, the generation time for the new strain was 32.2 min, and maximum production occurred after 16 h (2,45 g/L) to 20 h (2.14 g/L) between the end of the logarithmic phase and the early stationary phase. EPS exhibited antimicrobial activity, where S. aureus (17.24 ± 1.2 mm) and E. coli (16.6 ± 0.94 mm) were immediately resistant to EPS. In contrast, other pathogens were resistant to EPS, whereas strong antioxidant activity was observed at a concentration of 1500 µg/ml (84.43 ± 0.95%). Through this study, we have proven that optimal conditions directly affect the quantity and production of EPS and that it has an inhibitory effect on some microbes in addition to being a natural antioxidant for DPPH free radicals.

Keywords: *Bifidobacterium longum* subsp. *infantis*, Optimal condition, New local strain, Generation time, Production time, Antioxidant activity, antimicrobial activity

INTRODUCTION

In recent years, researchers have focused extensively on the natural metabolic products produced by nonpathogenic bacteria (lactic acid bacteria: LAB) and their inclusion in many applications in the food industry, such as hyaluronic acid as a natural antioxidant produced from *Streptococcus thermophilus*, reuterin produced from *Limosilactobacillus reuteri* as a food preservative and a natural antibacterial against enteric pathogens, and bacteriocin and antibiotics produced from *Lactobacilli* spp. as a food preservative instead of chemical preservatives (**Niamah et al., 2020; Mohammed et al., 2022; Niamah et al., 2023**). Furthermore, exopolysaccharides (EPS) are produced by most LABs, such as *Lactobacillus* spp. and *Streptococcus* spp. *Lactococcus* spp., *Pediococcus* spp., *Leuconostoc spp.*, *Bifidobacterium* spp., and *Weissella* are known to produce EPSs frequently (**Adebayo-Tayo et al., 2018**). LAB considers one part of the human microbiota in the mucous membranes of the urinary and gastrointestinal tract and has a positive stimulating effect in restoring the intestinal flora of the human intestine, stimulating immunity, and improving resistance to pathogenic microbes. Therefore, the advantages of EPS derived from LAB strains are its natural, safe, and non-toxic properties, and the physicochemical properties display diversity to be closely related to their chemical and structural composition, molecular weight (MW), electrical charge, and linkage patterns, either homoexopolysaccharides (HoEPS), whose subunits consist of one repeated sugar, or heteroexopolysaccharides (HeEPS), whose subunits consist of more than one repeated sugar subunit; furthermore, EPS composition includes a significant proportion of carbohydrate in addition to noncarbohydrate ingredients such as protein phospholipids and nucleic acids. Furthermore, it lends it a lot of attractiveness in the food industry as an additive (**Al-Roomi & Al-Sahlany, 2022**), biomedical activities including anticancer, immunomodulatory effects, antioxidants, antibiofilm, antimicrobials, antidiabetics, prebiotic, and pharmaceutical applications. In addition, it is useful in wastewater treatment as a coagulant, thickener, and emulsifying agent (**Riaz et al.,2020; Sajna et al., 2021; Yang et al., 2021**).

Free radicals are extremely harmful to many living organisms, and the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), n-propyl gallate (PG), and butylated hydroxytoluene (BHT) can pose potential risks to the human

system because they have strong antioxidant activity that can cause liver damage and carcinogenesis. Therefore, to protect humans from free radicals, bio-source compounds that are safe and natural, such as EPS, can be used against free radicals produced from plants or beneficial microbes such as fungi or bacteria. EPS enhances defense against cellular oxidation either by creating enzymes such as superoxide dismutase (SOD) or by producing non-enzymatic antioxidants such as glutathione (GSH). EPS secreted from strains of lactic acid bacteria replaces synthetic antioxidants and can be safe and protect human health in addition to treating disease (**Liu and Pan, 2010; Zhu et al., 2012**). EPS has also been used as an antimicrobial, along with some other compounds, to control microbes and inhibit the growth of pathogenic and food-borne microbes through the production of compounds such as hydrogen peroxide, D-isomers of amino acids, bacteriocin, CO_2, diacetyl, acids, and reuterin (**Abdalla et al., 2021**). Given the beneficial roles that EPS provides, our focus is on obtaining the best strain of *Bifidobacterium* spp. that produces EPS, isolating the bacteria from its sources of infant stools, genetically diagnosing it, and studying the production capacity by studying the optimal conditions for producing high-efficiency EPS, knowing the time of polymer production, and introducing EPS to possess many distinctive features in many applications, the most important of which is as an antimicrobial and natural antioxidant.

MATERIAL AND METHODS

Sample collection

A total of 52 samples were collected from breastfed human infant stools that were not sick (aged from 10 days to 3 months) as a source of bacterial isolates. Samples were placed in sterile tubes containing 5 ml of thioglycolate broth (Oxoid, England) and transferred to the laboratory for anaerobic incubation at 37 °C for 24 h. Samples were then streaked onto NNL-MRS agar with 0.5 L-cysteine HCl (100 mg/L Neomycin sulfate, 15 mg/L Nalidixic acid, and 3 g/L Lithium chloride) and incubated anaerobically at 37 °C for 24 h. Subsequently, all colonies were sub-cultured on selective *Bifidobacterium* agar medium (Hi-media, India) and incubated anaerobically at 37 °C for 24 h. After incubation, individual, pure colonies were selected and transferred into sterile MRS broth mediums with 0.05%

L-cysteine for other biochemical tests after being microscopically examined using Gram staining, depending on the shape, color, size, and arrangement of the bacterial cell, as well as using a malachite green dye solution for spore-forming examination.

Biochemical test

Oxidase Test

Spreading a fresh single colony using a sterile wooden stick to a filter paper that had been saturated with 1% N, N, N, N-tetramethyl-p-phenylene diamine hydrochloride. Follow up on the positive result by changing the color to purple within 5–10s **(Shields and Cathcart, 2013)**.

Catalase Test

A fresh colony of 18–24 h in age was transferred using a wooden stick on a clean glass slide and one drop of 3% H_2O_2, then noted the presence of oxygen bubbles indicated a positive result **(Harley and Prescott, 2002)**.

Primary screening for exopolysaccharide (eps) production

Congo Red Agar (CRA) Method for Biofilm Formation

Based on **Freeman *et al.* (1989**) with slight modifications to their methods, MRS agar base supplemented with 50g glucose and 0.8g Congo red in 1 L of distilled water, PH 8, after autoclaving, cooled to 45 °C and supplemented with filter-sterilized 0.5 g/L of L-cysteine-HCL, then streaked pure, fresh colony on CRA media, and incubated anaerobically overnight at 37°C.

Carbohydrate fermentation test

Carbohydrate fermentation broth medium is composed, per liter, of yeast extract (5 g), peptone (10 g), Tween 80 (1 ml), sodium acetate (5 g), ammonium citrate (2 g), magnesium sulfate (0.2 g), manganese sulfate (0.05 g), tri-ammonium citrate (2 g), and dipotassium hydrogen phosphate (2 g). A chlorophenol red reagent at a concentration of 0.05% was added to the basic medium. After sterilization, 1% of 0.45 Millipore filter-sterilized sugars (Lactose, Glucose, Cellobiose, Fructose, Galactose, Maltose, Melibiose, Sorbitol, Raffinose, Mannose, Sucrose, Trehalose, Mannitol, xylose, Salicin, Inulin, arabinose) were added independently to each tube **(Parte *et al.*, 2012)**.

Molecular identification

Using the genomic DNA mini kit (Geneaid, Taiwan) according to the manufacturer's instructions for extraction DNA, as the template for the amplification of universal 16SrDNA primers from all positive isolated *Bifidobacterium* spp. for strain identification, using B 27 F 5′-AGAGTTTG ATCCTGGCTCAG-3′ and U 1492R 5′-GGTTACCT TGTTACGACTT-3′, the cycling condition was set: 92°C for 2min to initial denaturation, then, 30 cycle at 94°C for 30 sec, 51.8°C for 45 sec, 72°C for 1.5 min lastly 72 °C for 5 min to final extension. The results of *16S rDNA* sequencing for all *Bifidobacterium* species were compared with the sequences in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov) **(Mashak, 2016)**.

Secondary screening for eps production

Extraction, Isolation, and quantification EPS using Ethanol Precipitation Method

The extraction and isolation methods for quantitative EPS production were performed on all strong-forming biofilm strains of *Bifidobacterium* spp. Based on the methods of **Bajpai *et al.* (2016)**, fresh *Bifidobacterium* spp. colony in 1 L of sterilized MRS broth with glucose (10 g/L), broth supplemented with 0.5 g/L L-cysteine, and incubated anaerobically at 37 °C for 24 h. Then, centrifuged broth culture at 8,000×*g* for 20 min at 4°C, the supernatant was collected and added with a final concentration of 14% trichloroacetic acid (TCA) and homogenized at 90 rpm for 30-40 min, after which centrifugation at 8,000×*g* for 20 min at 4°C. The supernatant was collected and mixed by the addition of 2 volumes of cold absolute ethanol and incubated at 4°C for 24-48 h for precipitation, followed by centrifugation at 8000×*g* at 4°C for 20 min. Quantitative determination of partial-purification EPS production was performed and expressed as mg/L after drying. For purification, EPS was dissolved in deionized water and dialyzed against ultrapure water (using 12-14 kDa) for 24–48 h (3 days) at 4°C, then freeze-dried to obtain lyophilized EPS and stored at 4°C.

Optimal condition determination for eps production

Different variables for determining the optimal conditions to obtain a higher production of the extracted EPS level for *Bifidobacterium* spp. The special conditions of production are subject to change or modification to obtain the best EPS production, with triplicate for each variable and all seven cultured in the modified MRS broth production medium with 0.05% L-cysteine-HCl and inoculum 1% (v/v) of size cell density ~3x10^8 CFU/ml from fresh *Bifidobacterium* spp. the suspension was grown anaerobically using the following: carbon source 1% (W/V) of (glucose, sucrose, lactose, fructose, and soluble starch) Different concentrations of the best carbon source (1, 1.5, 2, 2.5, and 3%), different incubation times (18 h, 24 h, 36 h, 48 h, and 60 h), incubated at different temperatures (30 ºC, 37 ºC, 45 ºC, 52 ºC and 60ºC), pH values averaged at (4.5 5.5, 6.5, 7.5, and 8.5), and optimal inoculum size (0.5,1,4, 7, and 10 % (v/v) cell density ~3x10^8 CFU/ml).

Generation time for *Bifidobacterium* spp. and determination of eps production

The relationship between EPS production activity and the bacterial growth curve can be determined based on generation time and compound production. Using the method of **Duboux *et al.* (2023)** with slight modifications, 1 ml of inoculated fresh *Bifidobacterium* spp. in 1L of modified MRS broth production medium with 0.05% L-cysteine-HCl and the best carbon source with its optimal concentration, the culture was incubated anaerobically at the optimal temperature and incubation time considering the zero time for the start of generation time and production of EPS. The sample was collected periodically every 2h for enumerated viable *Bifidobacterium* spp. by the pour-plating method, and the extraction of EPS for quantitative determination was then expressed as g/L after weighting (the methods were described above) until 26 h. Then, we calculated the generation times to determine the relationship between the logarithmic phase of the growth cell and time in hours using the following equation **(Stanier *et al.*, 1985)**: $n = log\,(b/B) \times 3.3$

Where: **n**= Number of generations, B=Logarithm of the number of bacteria at zero-time (t_0) **b**= Logarithm of the number of bacteria at time (t)**;** so, Generation time (G) calculated as follows: $G = t / n = t / 3.3 \times log\,b/B$, **t** = Total development time

In vitro: biomedical effect for eps

Antimicrobial activity

The antimicrobial activity of crude EPS was assessed using the agar-well diffusion method **(Hossain, 2023)**. A 0.1 ml aliquot of the bacterial and yeast suspension inoculum size of ~1.5x10^8 CFU/ml was spread on the Mueller Hinton agar surface for pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus, Pseudomonas aeruginosa*) and on Sabouraud dextrose agar for yeasts (*Saccharomyces cerevisiae*, *Candida albicans*). Wells of 6 mm diameter were aseptically scratched on an agar plate using a 6 mm sterile stainless steel cork borer of size 6 mm. Subsequently, 60 µL of the EPS solution (100 µg/ml) was added to every well, and the plates were incubated at 37 °C for 24h for aerobic bacteria and 4 days for yeast. Negative control was normal saline added in the center well, and the diameter of any inhibition-resulting regions around the wells was measured in millimeters **(Tarannum, *et al.*, 2023)**, and the diameter was compared with interpretive standards (resistance (R) ≤16, intermediate (I) 17-20, and sensitive (S) ≥21) based on **Cavalieri (2005).** The method was performed in triplicate.

Antioxidant activity

Based on **Yin *et al.* (2010)** with some modification, the DPPH free radical scavenging activity of the different concentrations of EPS (250, 500, 750, 1000, 1250, and 1500 µg /ml) was measured by freshly prepared DPPH (dissolving 0.1 mM in 95% methanol) a volume of 1.0 ml of DPPH solution was mixed with 1.0 ml of different concentrations of EPS and then incubated in the dark for 30 min. Then, the samples were measured at 517 nm. The standard was ascorbic acid (10 mg /ml), and the method was repeated in triplicate. The following equation was used to determine the capacity to scavenge the DPPH radical **(Paul *et al.*, 2023)**::
$Scavenging\ activity\ (\%) = [1-(A\ sample - A\ blank)/$
$A\ control]\ x\ 100\ [A\ control = EPS +$
$distilled\ deionized\ water, A\ blank = distilled\ deionized\ water +$
$DPPH\ solution].$

Statistical analysis

All data were analyzed with ANOVA (one-way analysis) to compare the results with statistical significance between the data at $P<0.05$ using GenStat version 12, and mean± SD was expressed, all experiments were accomplished in triplicate.
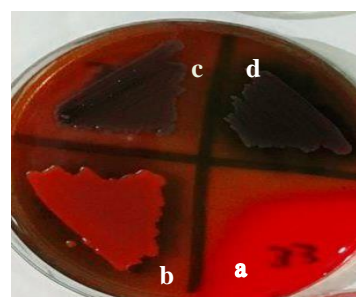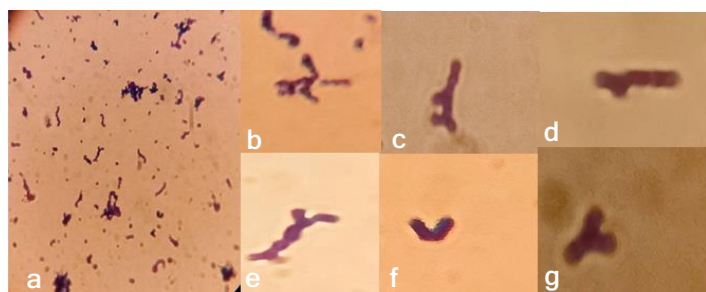
RESULT AND DISCUSSION

Phenotypic identification

47 isolates (90.38%) from 52 samples on NNL-MRS agar were gram-positive and non-spore-forming bacilli, picked and streaked onto selective Bifidobacterium agar medium. The results show 34 (72.34%) of 47 isolates of *Bifidobacterium* spp.

grew on their selective medium. Identification was achieved morphologically according to Bergey's Manual of Systematic Bacteriology (**Parte** *et al.***, 2012**) as smooth, convex with entire edges, cream to white, glistening, and soft consistency. The biological factors produced from the mother's milk were the only reason for the preponderance of Bifidobacterium spp. in the fecal microbiota of healthy breast-fed infants (**Modler** *et al.***, 1990**). In this case, MRS agar supplemented with L-cysteine HCl was used as an O_2 scavenger (**Prasanna** *et al.***, 2014; Singh** *et al.***, 2021**). It provides an anaerobic condition and support for the growth of *Bifidobacterium* spp., as well as the presence of antibiotics that are employed in the recovery of *Bifidobacterium* spp., such as nalidixic acid, which is considered a narrow-spectrum agent for enteric bacteria; neomycin sulfate, which has a broad spectrum against gram-negative bacteria; and lithium chloride, which is used for the synthesis of organic substances. All these agents inhibit a high percentage of microorganisms during the isolation process and growth control of *Bifidobacterium* spp. and some *Lactobacillus* spp. from infant stools. Therefore, NNL-MRS is not a selective or differential media for Bifidobacteria but enhances their growth well on it, which is evidence of the existence of other types of *Lactobacillus* spp. This agrees with the previous study by **Vinderola and Reinheimer (1999)**, where they noticed *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were able to grow at low counts on NNLP-MRS media. However, in this study, we proved that *Bifidobacterium* agar is a selective medium for its isolation from yogurt and infant stools. The pre-identification of *Bifidobacterium* spp. according to aspects of morphological and biochemical tests, phenotypic diagnosis was as described above, it is small colonies, and pleomorphism was observed, this is mostly associated with culture medium composition as in the researcher's previous results for **Kojima** *et al.* **(1968)**. Also, no activity was observed for oxidase and catalase, which were indicated to be strictly and fastidiously anaerobic bacteria. Under microscopic analysis, a positive rod for Gram staining was grouped as irregular, non-spore-forming, and bifid V- or Y-shaped, as shown in Figure 1A.

Primary screening: *in vitro*-biofilm formation on cra

There was a direct relationship between EPS production and biofilm formation. The first step to the formation of slim layers and adherence to surfaces depended on EPS production. selected CRA method to estimate EPS production and measure the variation of *Bifidobacterium* strains to form biofilms as well as the effect of EPS production on biofilm formation. Most *Bifidobacterium* strains formed biofilms. *In vitro* observations revealed that 6 (17.68%) isolates of 34 were identified as strong biofilms forming during 18 h after incubation. They also noticed the formation of ropy and sticky colonies on their texture-CRA medium, which were considered to produce large quantities of EPS that played an essential role in the enhancement of biofilm. A positive result as black colonies with dry crystalline (slime producers), intermediate results as dark-pink colonies (weak slime producers), and negative results as pink-white or no growth (not slime producers). As shown in Figure 1B. Because of the instability in the rob formation characteristic results, CRA is considered the presumptive method only for primary screening. This method was used in previous research by **Rühmann** *et al.* **(2015); Siddharth** *et al.* **(2021); Tarannum** *et al.* **(2023)**, and the results agreed with theirs for the variation of biofilm formation for other lactic acid bacteria.

A-

B-

Figure 1 A- Morphological shapes of *Bifidobacterium* spp. under a light microscope (oil lens =100x), **a:** Gram-positive, **b**: Irregular cell arrangement for *Bifidobacterium* spp., **c,d,e,f,g:** cell shape,V,Y, or Bifide shape. **B-** Shows the variation of biofilm formation using the CRA method. **a:** not slime producers, **b:** moderate slime producers, **c:** slime producers but no strong, **d:** strong slime producers; with ruby and sticky colonies.

Carbohydrate fermentation and molecular identification

Isolates that passed the CRA method successfully selected the best strain for biofilm formation, which was reflected in the production of quantities of EPS subjected to carbohydrate fermentation and confirmed diagnosis by genetically determining their strains. as Table 1. Based on carbohydrate fermentation patterns and genetic identification, the six isolates are *B. longum* subsp. *infants*, *B. adolescentis, B. breve,B. longum* subsp. *longum,* and *B. bifidum*. Record these species as new local isolates in Iraq-Basrah as a new strain, as shown in Table 1.

Table 1 The carbohydrate fermentation test and genetic identification and accession number in GenBank for new local isolates of *Bifidobacterium* strains

Sample ID	Glucose	Arabinos	cellobiose	fructose	Galactose	Inulin	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Sample ID	*Bifidobacterium* strain name	Accession number in Gene Bank
B1	+	-	+*	+	+	+*	+	+	-	-*	+	+	-	-	+	-	+*	B1	*B. longum* subsp. *infantis* strain Iraq-Basrah 6	OQ737781.1
B2	+	+	-	+	+	-	+	+	-	-*	+	+	-	-	+	-	+*	B2	*B. longum* subsp. *longum* strain Iraq-Basrah 1	OQ737778.1
B3	+	-	-	+	+	+*	+	+	+*	+	+	+	+	+*	+	-*	-	B3	*B. breve* strain Iraq-Basrah 2	OQ737779.1
B4	+	-	-	+	+	-	+	-	-	+*	-	-	-	-	+*	-	-	B4	*B. bifidum* strain Iraq-Basrah 5	OQ743510.1
B5	+	-	+	+	+	+*	+	+	-	+*	+	+	-	-	+	-	+	B5	*B. longum* subsp. *infantis* strain Iraq-Basrah 3	OQ738864.1
B6	+	+	+	+	+	+*	+	+	+*	+*	+	+	+	+*	+	+*	+	B6	*B. adolescentis* strain Iraq-Basrah 4	OQ737780.1

Listed sugar symbols +* or -*, meaning results are erratic.

Most *Bifidobacterium* strains in Table 1 have variables (+* or -*) for the fermentation of most sugars and were given mainly erratic results, where a ratio of 90% of strains were positive for carbohydrate, indicating that the ability of *Bifidobacterium* spp. for degradation of most carbohydrate was even complex, and the other 10% had a negative result. This variation is dependent on the gene content of the genome of bifidobacterial strains that encode for the transfer and fermentation of a variety of sugars and reflect their adaptation to the broth medium environment; this variation agrees with **Parte** *et al.* **(2012)**. Therefore, the strains were genetically confirmed using universal *16S rDNA*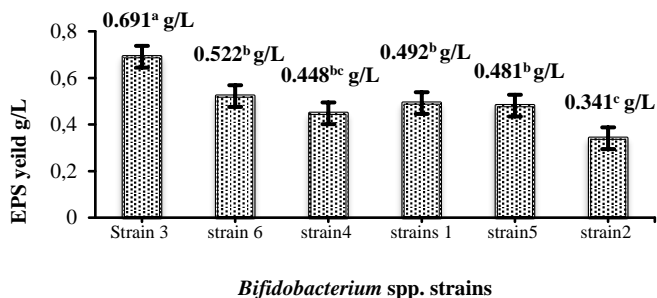 amplification, followed by sequencing and alignment with the reference strain in the GenBank because it is more accurate in diagnosis without loss or avoiding any error in the identification of any bacterial strain.

Secondary screening: eps extraction, isolation, and eps quantification

EPS isolation and extraction were screened on six new local *Bifidobacterium* strains for their ability to produce maximum yields of EPS using the ethanol precipitation method in a modified MRS broth with glucose as the production medium. The dry weight and yield of EPS after extraction and isolation were calculated for all strains and compared. Strain Iraq-Basrah 3 showed a high yield

(0.691±0.07g/L) from EPS with a significant difference of $p<0.05$, followed by (0.522±0.11 g/L) for strain Iraq-Basrah6, strains Iraq-Basrah1 (0.492±0.06g/L), strain Iraq-Basrah5 (0.481±0.05g/L), strain Iraq-Basrah4 (0.448±0.075 g/L), and strain Iraq-Basrah2 (0.341±0.08g/L). There is no significant difference between strains Iraq– Basrah 6, 4, 1, and 5 and between strains Iraq– Basrah 4 and 2, whereas there is a significant difference between strains Iraq– Basrah 6, 1, and 5 and strain Iraq– Basrah 2, as shown in Figure 2. The first step is to choose a suitable strain based on the end yield of the EPS produced. This difference is due to several reasons. The bacterial genetic background that affects the end yield of EPS production and composition is also influenced by the components of the production medium and the ability of strains to produce EPS. Successful high-production EPS yields depend primarily on the choice of a suitable culture medium and its components for production, as well as the methods used for detecting and quantifying EPS. The previous study by researchers **Alhudhud *et al.* (2014)** proved that MRS mediums are complex, nutrient-rich media with nitrogen sources (peptones and yeast extract) and some supplementation and are therefore used to regularly culture LAB either on agar or in broth for producing EPS.

0.691ᵃ g/L 0.522ᵇ g/L 0.448ᵇᶜ g/L 0.492ᵇ g/L 0.481ᵇ g/L 0.341ᶜ g/L

Bifidobacterium spp. strains

Figure 2 Screening between strains for production (EPS), LSD$_s$: 0.1367, symbol [abc] meaning significant deference p<0.05. elucidate the mean ± SD for the data of a triplicate experiment.

Optimal condition for eps production

Determination of maximum yield and production EPS using different variables for the best isolate that gave a high yield in primary screening is *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3, as shown in Table 2. *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 was grown under different conditions to obtain the best conditions for EPS production. Table 2 shows that the strain was producing a high yield of EPS in the modified MRS medium with starch as the preferable carbon source (1.512 g) at a concentration of 1% (w/v) and an inoculum size of 1% (v/v) in 37 ºC at 18-24 h and a pH value between 5.5 and 6.5 with a high significant difference p< 0.05 where is non-significant between incubation time 18h and 24h (2.08g and 1.51g) and pH values of 5.5 and 6.5 (1.92g and 2.08g), respectively, but there is an increase in EPS concentration at 18 h with a pH of 6.5. The ability of LAB strains to produce EPS depends on several factors and the species and strains of the same species. Therefore, the change in the EPS composition subunit and its concentration are due to the use of different carbon sources. Producing *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 to high yield EPS in the medium containing starch is the first key factor in the achievement of producing EPS. Second, it depends on the

ability of the strain's metabolic capacity, degradation, hydrolysis, and gene coding to transport the system for carbohydrate utilized, Therefore, *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 utilized starch as a specific prebiotic substrate to produce EPS. This is due to the hydrolysis of starch, which has been split into glucose, leading to an increase in glucose subunits in the medium to enhance its growth and increase fermentation and production. This finding is in agreement with previous research (**Zarinah *et al.*, 2018; Soumya *et al.*,2019**). The maximum yield of EPS for *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 at 37 °C may reflect the temperature of the primary isolation source of infants as well as the enzyme responsible for polymerization and production of EPS is more influenced by high temperatures. This result agrees with that of the researcher. **Bennama *et al.* (2011)** reported higher production of EPS in *Streptococcus thermophilus* at 37 ºC. Furthermore, the production of EPS at 18–24 h indicates that EPS is synthesized and produced throughout the late exponential phase (logarithmic) or in the early stationary phase (prolonged incubation). The nutrients are finished in the late stationary phase, and the bacteria use the EPS as a prebiotic by producing glycopyrrolates, which may degrade polysaccharides and decrease the resulting (**Degeest *et al.*, 2001**). Inoculum size about production time (1% v/v) appropriate with the amount of volume of nutrient medium prepared for production to make nutrients available to the bacteria during EPS synthesis by providing the largest sufficient amount of nutrients. In addition, the activity of bacteria increases at pH 5.5–6.5, this is an authentic pH for MRS medium, which gives it the ability to transport amino acids and peptides in the synthesis process in the cytoplasm, specifically the amino acid glutamate, it is possible to obtain the largest amount of the last amino acid, and then the largest amount of polysaccharide can be obtained (**Van Den *et al*, 1995; Van Niel *et al.*, 1999; Ryan et al., 2015**). These results demonstrate that the production of polysaccharides is influenced by the medium composition, pH, temperature, inoculum size, carbon, and concentration, among other factors.
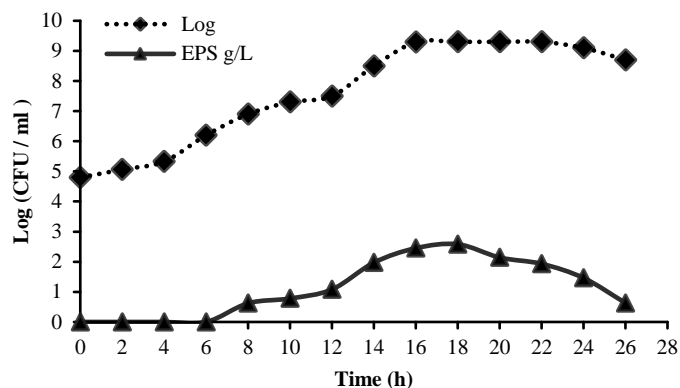
Generation time and time determination of EPS production

The EPS yields of *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 and the growth features of the bacteria were investigated and are shown in Figure 3. The growth curve of bacterial cells was followed in the production medium composed of 1% starch as a carbon source at 37°C for 18 h with a pH of 6.5 to determine the growth phases and the start time of EPS production during this period. The growth phases were followed by counting the number of viable cells per ml (CFU/ml). The results showed the generation time for strain was 32.2 min, and showed after 8h started producing EPS in the late logarithmic phase (0.62g/L) and maximum producing after 16 h (2.45 g/L) to 20 h (2.14 g/ L) between end of logarithmic phase to early of stationary phase then started to reduce due to excrete glycohydrolases, which lead to the degradation of polysaccharide. This results in agreement with the researcher group **Audy *et al.* (2010)**. Another researcher, **Rütering (2020),** proved that the polymer was initiated to produce during the entry of *the Paenibacillaceae* spp. strain at the stationary phase after 17 h of fermentation and reduced after 20 h of fermentation. Formation of EPS is either associated with cell growth or the late logarithmic or stationary phase, and maximum EPS production is mostly influenced by several factors, especially sugar as a substrate unit and the *EPS* gene cluster on the *pGTF* gene, which is considered an indicator of transcription EPS, as well as the transcription genes encoding for enzymes of Leloir involved in the biosynthesis of EPS, which starts at the logarithmic phase to the early stationary phase (**Svensson et al., 2005; Audy *et al.*, 2010**).

Table 2 Survey the optimal condition for the production high yield of EPS by *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah3.

Carbone source 1%	End yield of EPS g/L (mean)	LSD$_S$	±SD	Optimal best carbon source concentrations	End yield of EPS g/L (mean)	LSD$_S$	±SD
Sucrose	0.331ᵇ g		0.09	1 %	1.512ᵃ g		0.72
fructose	0.449ᵇ g		0.08	1.5 %	0.828ᵇ g		0.07
lactose	0.352ᵇ g	0.604	0.08	2 %	0.335ᵇ ᵍ	0.594	0.07
Starch	1.512ᵃ g		0.72	2.5 %	0.00 g		0.00
glucose	0.672ᵇ g		0.08	3 %	0.00 g		0.00
Optimal inoculum size	**End yield of EPS g/L (mean)**	**LSD$_S$**	**±SD**	**Different incubation times in an hour at 37 ºC**	**End yield of EPS g/L (mean)**	**LSD$_S$**	**±SD**
0.5 %	0.850ᵇ g		0.02	18 h	2.08ᵃ g		0.41
1 %	1.512ᵃ g		0.72	24 h	1.51ᵃ g		0.72
4 %	0.732ᵇ g	0.592	0.03	36 h	0.63ᵇ g	0.678	0.08
7 %	0.364ᵇ g		0.06	48 h	0.04ᵇ g		0.01
10 %	0.309ᵇ g		0.01	60 h	0.00 g		0.00
Incubated in different temperatures at 18 h	**End yield of EPS g/L (mean)**	**LSD$_S$**	**±SD**	**Different pH values (37 ºC-18h)**	**End yield of EPS g/L (mean)**	**LSD$_S$**	**±SD**
30 ºC	0.831ᵇ g		0.72	4.5	0.89ᵇ g		0.06
37 ºC	2.08ᵃ g		0.06	5.5	1.92ᵃ g		0.85
45 ºC	0.331ᶜ g	0.34	0.08	6.5	2.08ᵃ g	0.765	0.41
52 ºC	0.00 g		0.00	7.5	0.80ᵇ g		0.03
60 ºC	0.00 g		0.00	8.5	0.00 g		0.00

Symbol [a,b,c] were arranged gradually according to significant difference between means end yield of EPS p<0.05
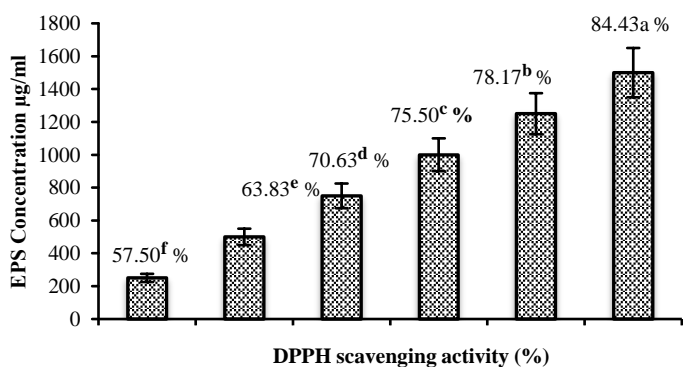
Figure 3 Logarithmic growth kinetics of *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 and determination of time production of EPS

Biomedical effect of EPS

Antimicrobial activity

The antimicrobial activities of EPS produced from *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3 against selected pathogen microbes are shown in Figure 4. There was a significant difference between them ($p<0.05$), where *S. aureus* and *E. coli* were intermediately resistant to EPS (17.24±1.2 mm and 16.6±0.94 mm, respectively), whereas the other pathogens *P. aeruginosa* (13.2±0.84mm), *C. albicans* (11.02±2.64 mm), *B. cereus* (8.8±5.3 mm), and *S. cerevisiae* (6.66±2.7mm) were observed to be resistant to EPS. Other research in the same line observed in vitro that EPS has a remarkable antimicrobial effect against pathogens due to its several mechanisms acting as bactericidal or bacteriostatic substances. **Wu *et al*. (2010)** and **He *et al*. (2010)** noticed from their research that Bifidobacterium longum weakens cell division for pathogens such as *Vibrio parahaemolyticus*, *Staphylococcus aureus, S. Typhimurium*, and *Bacillus cereus*, as well as rupturing the cell wall and cytoplasmic membrane and disintegrating DNA. **Rani *et al*. (2018)** proved that heteroexopolysaccharides (HeEPS) exhibit activity against the pathogen *Listeria monocytogenes* MTCC 657, and **Ayyash *et al*. (2020b)** noticed EPS from *Lactiplantibacillus plantarum* C70 led to a decline in viability for pathogens *E. coli* and *S. aureus*. Therefore, **Li *et al*. (2014)** showed that EPS during their studies did not affect *C. albicans*. EPS presents different levels of activity for pathogens: high, moderate, or no effect. This may be due to the subunit composition of EPS for lactic acid bacteria being different from strain to strain or the concentration used in *in vitro* tests against pathogens. Therefore, all studies excluded in the protocol discussion exactly the antagonistic mechanisms for EPS against pathogens.
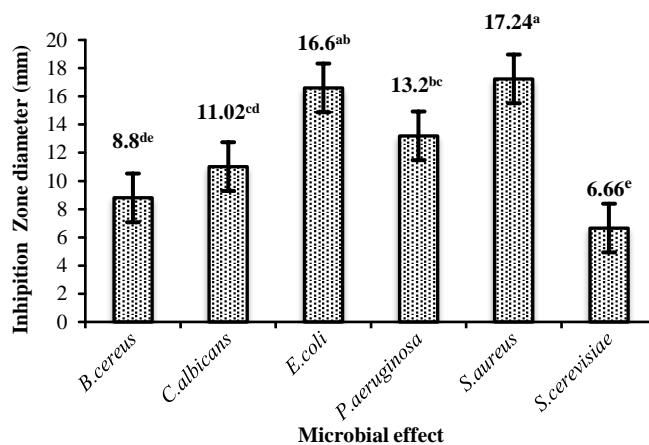

Figure 4 EPS effects on selected pathogens; the symbol [abcde] indicates a significant difference for the mean ± SD between them (p > 0.05),
LSD$_s$: 3.568

Antioxidant activity

DPPH is considered a relatively constant free radical and can be used to deter the antioxidant capacity of scavenging free radicals. Therefore, EPS can be used to demonstrate its ability to act as a free radical at different concentrations. The effectiveness of antioxidants increases with increasing EPS concentration, as observed by the high significant ($p<0.05$) difference between them, the strong antioxidant exhibits with the concentration of 1500 µg/ml (84.43±0.95%), then capacity as an antioxidant varies till low concentration 250 µg/ml (57.5±0.5%) when comparison with standard ascorbic acid (88.43±1.2%) shown in figure 5. These results proved that EPS possesses a natural capacity as an antioxidant to combat free radical-induced oxidative stress. The disadvantages of synthetic antioxidants can also be reduced by natural compound antioxidants, as well as the fact that EPS is produced from *Bifidobacterium longum* subsp. *infantis* strain Iraq-

Basrah3 exhibits a higher scavenger for DPPH free radicals than other research groups **Benattouche *et al*. (2018)** conclude that their EPS derived from LAB isolates from different sources of yogurt display at concentrations of 1000 µg/ml as antioxidant effected range from 16% to 56%, and **Khalil *et al*. (2022)** demonstrated through their research that the EPS derived from LAB and Bifidobacterium at concentrations of 20 mg/ml exhibit antioxidant scavenging DPPH free radical ranging from 33.3% to 77.0%, So researcher group **Saif *et al*. (2020)** showed EPS scavenger ability and inhibited DPPH free radical at concentrations estimated at 0.0 to 10 mg/mL ranging from 28.30 to 61.67, and in agreement with researchers **Tarannum *et al*. (2023)**, finding EPS producing from LAB isolated from bovine milk display antioxidant effect range nearly 82–89%.
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Figure 5 EPS scavenging effects; the symbol [abcdef] indicates a significant difference for the mean ± SD between them ($p > 0.05$), LSDs:1.18

CONCLUSION

A lot of research has been conducted in recent years on all metabolic products produced by lactic acid bacteria (LAB), indicating that they have many benefits and applications in food and biomedicine as an antimicrobial against pathogens, a natural antioxidant for many compounds that produce free radicals, an anticancer, an antitumor, an antimicrobial that forms biofilm, and against intestinal ulcers formed by chemical antibiotics as well. Many studies are concerned with natural extracts that have a biomedical effect or their application to foods and have natural benefits without significant side effects. Research has shown that EPS produced from lactic acid bacteria has entered many areas of food and biomedical applications, and the results were variable because the compound is specific to a strain of bacteria that differs from other strains of the same bacterial species, resulting in different subunit-form polymers depending on the carbon source used as a substrate in the production of polysaccharides and environmental conditions that have a direct effect on the quantity of production. We have proven through this study that, firstly, the carbon source and its concentration, in addition to the pH value, temperature, incubation period, and inoculum size, have a direct effect on the quantity and production of EPS, and secondly, the compound has an inhibitory effect on some microbes in addition to being an antioxidant for free radicals.

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