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Immunomodulation capability and cytotoxicity activities of novel exopolysaccharides produced by a new local strain, *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3

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ABSTRACT

Exopolysaccharides (EPS), metabolic products secreted by lactic acid bacteria (LAB) strains, have several biomedical effects. Our study focused on the extraction and purification of EPS from a new local strain, *Bifidobacterium longum* subsp. *infantis* strain Iraq-Basrah 3, which was characterized by analyzing its monomer subunits, assessing its impact on cytotoxic testing, and investigating its immunostimulatory activity in vivo. The results show that the biopolymer for EPS is composed of five sugar subunits: glucose, galactose, ribose, xylose, and glucosamine. It exhibits non-cytotoxic and RBC membrane stabilization. Furthermore, EPS demonstrated immunomodulatory capability, stimulating the anti-inflammatory cytokine IL-10 and suppressing the pro-inflammatory IL-6 in treated groups (administered orally and via intraperitoneal injection). When induced with LPS, there was no significant change in hematological parameters compared to normal groups. In contrast, there was no observed effect in the group treated with yogurt fermented with the starter *B. longum* subsp. *infantis* strain Iraq-Basrah 3 producing EPS, which showed increased IL-6 levels and hematological parameters (WBC, neutrophils, and PCV) while decreasing monocyte, lymphocyte, and PT levels. We conclude that EPS is a novel compound that can be used directly as an immunotherapy agent to treat inflammation and is immunomodulatory; furthermore, it is safe for health and nontoxic.

1. INTRODUCTION

The immune system is the first line of defense. It is composed of a complex of cells and molecules that interact to protect against any infection or disease. Inflammation resulting from infection causes a disorder of immune system balance. Therefore, one method for preventing disorders in immune cells is providing immunomodulatory therapy, which restores the immune system's balance and changes its response by preventing and normalizing abnormal immune cell reactions [1,2]. 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. For example, xanthan gum is produced by *Xanthomonas campestris* and used as a thickener, stabilizer, emulsifier, and gelling agent [3]. Hyaluronic acid, a natural antioxidant, is produced from *Streptococcus thermophilus*. Reuterin, produced from *Limosilactobacillus reuteri*, serves as a food preservative and a natural antibacterial against enteric pathogens. Additionally, bacteriocin and antibiotics are also produced from *Lactobacillus* spp. as food preservatives; it is used instead of chemical preservatives, making them highly attractive in the food industry as an additive [4,5]. Furthermore, exopolysaccharides (EPS) are produced frequently by most LABs, such as *Lactobacillus* spp., *Streptococcus* spp. *Lactococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Bifidobacterium* spp., and *Weissella* [6]. LAB refers to one component of the microbiota found in the mucous membranes of the urinary and gastrointestinal tracts in most organisms. It has a beneficial effect on restoring the intestinal flora, enhancing immunity, and increasing resistance to pathogenic microbes [7].

The term exopolysaccharide (EPS) was coined by the scientist Sutherland in 1978 and has since become a common designation for all types of EPS found outside the microbial cell wall [8]. The study of bacterial EPS began in the mid-nineteenth century with the discovery of dextran, an EPS produced by the bacteria *Leuconostoc mesenteroides* in wine. Over time, other sugars were discovered, including cellulose, xanthan, inulin, altenam, levan, reuteran, kefiran, alginate, and others [9]. EPS is a metabolic substance that can either be capsule-like and tightly attached to the cell wall or loosely attached and released into the environment. It serves as a

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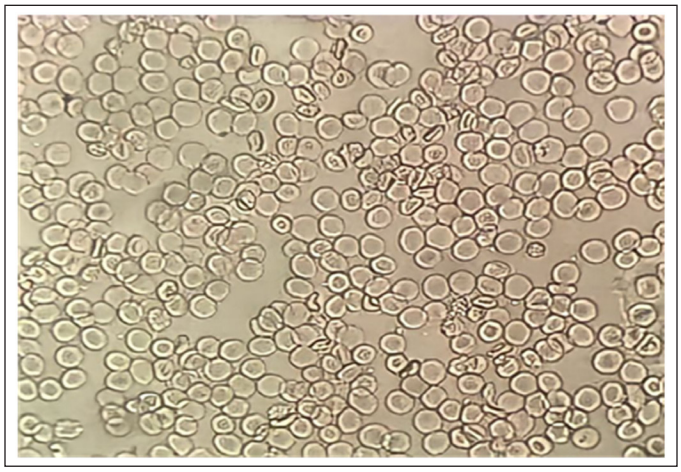

Figure 1: Red blood cells without hemolysis (40×).

key mediator of communication between the host immune system and probiotics, acting as a postbiotic and providing health benefits. Most species of LAB have the ability to produce EPS, including *Bifidobacterium* spp. [10]. The advantages of EPS derived from LAB strains include its natural origin, safety, and nontoxicity for cells. Other researchers have demonstrated its efficacy in vitro against the proliferation of various cancer cell lines, showing significant cytotoxic properties by inhibiting the formation and growth of tumors in human hepatoma and lung cancer cell lines [11]. Additionally, it exhibits strong cytotoxic effects against HeLa cells and cervical carcinoma cell lines, while being nontoxic to normal healthy cells (HEK-293) [12].

The physiochemical properties display diversity closely related to their chemical and structural composition, molecular weight (MW), electrical charge, and linkage patterns. They can be categorized as either homo-exopolysaccharides (HoEPS), whose subunits consist of one repeated sugar, or hetero-exopolysaccharides (HeEPS), more than one repeated sugar subunit. Furthermore, EPS composition includes a significant proportion of carbohydrates along with noncarbohydrate ingredients such as proteins, phospholipids, and nucleic acids [13]. The characteristics and monosaccharide composition analysis for EPS include size exclusion chromatography (SEC), ion-exchange chromatography, nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR), scanning electron microscopy (SEM), high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), and transmission electron microscopy (TEM). These are some of the more advanced techniques that can be used to investigate the structure and monomer subunit of EPS [14].

Furthermore, the significance of biomedical activities for EPS is evident in a distinguished number of ways. It serves as an anticancer agent from natural sources, ensuring safety with minimal harmful effects on the immune system. This is crucial for immunopharmacology as an alternative to synthetic anticancer therapies (chemotherapy). EPS also possesses potent immunostimulatory properties that can trigger both humoral and cellular immunological responses against antigens. It achieves this by promoting the proliferation of T/B lymphocytes, enhancing the phagocytic activity of macrophages, and increasing the tumoricidal activity of natural killer (NK) cells. Additionally, the antioxidant activity of EPS surpasses that of synthetic antioxidants, which can potentially lead to cancer and cytotoxicity. EPS also acts as an antibiofilm agent, altering the bacterial coat and preventing the attachment of pathogenic bacteria to surfaces. Moreover, EPS exhibits antibacterial properties against gram-positive and gram-negative dietary pathogens, anti-tumor activities, cholesterol-lowering

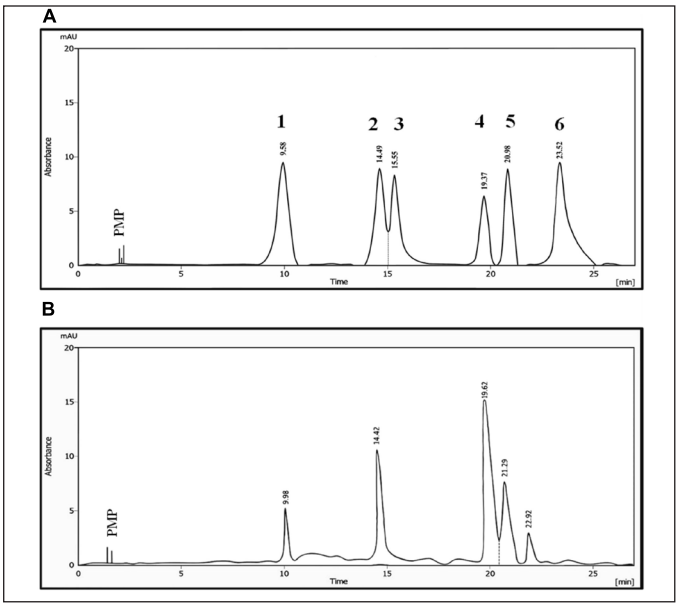

Figure 2: HPLC analysis: **A**: mixed standard monosaccharide derivatives, **B**: subunits of EPS derivative hydrolysate, PMP: 1-phenyl-3-methyl-5-pyrazolone.

effects, antidiabetic properties, prebiotic characteristics, and various pharmaceutical applications [13,15,16].

The aim of the study is to isolate and identify *Bifidobacterium* spp. In addition, the study aims to extract polysaccharides, characterize their sugar moiety subunits, and then determine their characteristics and potential as stimulants, modulators, and regulators of interactions between immune system cells to combat inflammation caused by pathogenic infections, as well as their effects as anti-cytotoxic agents.

2. MATERIALS AND METHODS

2.1. Sample Collection and *Bifidobacterium* Strain Isolation

Samples were collected from breastfed human infant stools in Basrah, Iraq, who were not ill (aged from 10 days to 3 months) to obtain bacterial isolates. Samples were placed in sterile tubes containing 5 mL of thioglycolate broth and transferred to the laboratory to incubate anaerobically at 37°C for 24 h. Subsequently, they were streaked onto selective *Bifidobacterium* agar medium (India, Hi-Media) and incubated anaerobically at 37°C for 24 h. Following incubation, individual pure colonies were selected and transferred into sterile MRS broth mediums with 0.05% L-cysteine for strain diagnosis. This was done after microscopic examination using gram staining to assess the shape, color, size, and arrangement of the bacterial cells. Additionally, a malachite green dye solution was used for spore-forming examination.

2.2. Molecular Identification of *Bifidobacterium* spp. Isolate

Using the genomic DNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's instructions for DNA extraction, as the template for the amplification of universal *16S rDNA* primers for positive isolated for *Bifidobacterium* spp. and strain identification, using B 27 F 5'-AGAGTTTGATCCTGGCTCAG-3' and U 1492R 5'-GGTTACCTTGTTACGACTT-3'. The cycling condition was set

Table 1: HPLC result, number of monosaccharide names in HPLC analysis peaks, and retention time for standard in Figure 2A compared with the retention time of EPS subunits in Figure 2B.

	No. of Monosaccharide Names					
	1-Mannose	2-Glucose amine	3-Rhamnose	4-Glucose	5-Galactose	6-Xylose
Standard retention time (min)	9.58	14.49	15.55	19.37	20.98	23.52
EPS subunit retention time (min)	9.98	14.42	---------	19.62	21.29	22.29

at 92°C for 2 min to initiate denaturation, followed by 30 cycles at 94°C for 30 s, 51.8°C for 45 s, 72°C for 1.5 min, and finally at 72°C for 5 min to final extension. The results for *16S rDNA* sequencing for all *Bifidobacterium* spp. were compared with the sequences in the GenBank database using BLAST: http://www.ncbi.nlm.nih.gov [17].

2.3. Exopolysaccharides Isolation, Extraction, Quantification, and Purification

The extraction and isolation methods were performed according to Bajpai *et al.* [18] with a slight modification: the inoculum I% v/v with a size of ~1.5 × 10^8 CFU/mL from a fresh strain was inoculated in 1 L of sterilized MRS broth with starch (10 g/L), then incubated anaerobically at 37°C for 18 h. with a pH of 6.5 Then, the broth culture was centrifuged 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 then homogenized at 90 rpm for 30–40 min, followed by centrifugation at 8,000 ×*g* for 20 min at 4°C. The supernatant was collected and mixed with the addition of 2 volumes of cold absolute ethanol and incubated at 4°C for 24–72 h for precipitation, followed by centrifugation at 8000 ×*g* at 4°C for 20 min. The quantitative determination of partial purification EPS production was weighted to be expressed as mg/L after drying. For purification, EPS was dissolved in deionized water and dialyzed against ultrapure water using a cut-off of 12–14 kDa for 24–48 h at 4°C, and then freeze-dried to obtain lyophilized EPS and stored at 4°C.

2.4. Protein Determination for EPS

To estimate the presence and quantity of protein content in the EPS extracted from *B. longum* subsp. *infantis* strain Iraq-Basrah 3, the microorganism was analyzed according to the Bradford methods [19]. The total protein content was determined using bovine serum albumin (BSA) to create a standard calibration curve.

2.5. Determination of Purified EPS Cytotoxicity and RBC Membrane Stabilization

The cytotoxicity activity of EPS was determined according to the method of Nair *et al*. [20], with a slight modification where each concentration of EPS (250, 500, 750, 1000, 1250, and 1500 µg/mL) was dissolved in distilled deionized water (1 mL). Then, 38 mL of physiological Ringer's solution was mixed with 2 mL of red blood cell (RBC) drawn from a human in a tube containing an anticoagulant, 0.2 mL of the previously prepared mixture was distributed to each sterilized tube, and 0.8 mL of each EPS concentration was added to complete preparation. The final volume of each tube was 1 mL, and then the tubes were incubated at a temperature of 37°C for 8 h. The examination of the hemolysis of RBC was observed under a light microscope each hour. Deionized water + human blood was used as a positive control laboratory.

2.6. Characterization of EPS

2.6.1. Acid hydrolysis and monosaccharide analysis of purified EPS

Lyophilized EPS 10 mg was dissolved in 8 mL of 2 M trifluoroacetic acid (TFA) in a sealed tube and heated at 110°C for 6 h. After that, 200 µL of hydrolyzed EPS was added and mixed with 240 µL of 0.3 M NaOH, then 240 µL of a methanolic solution of 0.5 M from 1-phenyl-3-methyl-5-pyrazolone (PMP) was added and mixed for 10 s thoroughly using the vortex mixture and incubated in the oven at 70°C for 2 h. Accordingly, 240 µL of 0.3 M HCl was added to neutralize the mixture to stop the reaction after incubation, and then it was cooled to 25°C. The impurities were removed by subjecting the mixture three times to chloroform extraction; the chloroform layer was later discarded and the aqueous layer was collected and thoroughly filtered using syringe filter paper with a pore size of 0.22 µm for analysis by HPLC using 1 mg of the monosaccharide reference standard, including xylose, glucose, glucosamine, galactose, rhamnose, and mannose, dissolved in 1 mL of water. A total of 200 µL from the mixture of monosaccharides were derivatized using the above steps to obtain hydrolyzed EPS [21].

2.6.2. HPLC analysis

The derivative EPS and monosaccharides reference standards were analyzed using an HPLC device (Dionix, Germany). The C-18 reverse phase column and UV/visual detector wavelengths were set at 250 nm. The mobile phase consisted of HCl (0.01%) and acetonitrile in distilled water (10%) with a flow rate of 1.0 mL/min, a pressure less than 200 psi, and a temperature of 30°C. Peaks for the analyzed EPS have been compared with reference standard peaks [22].

2.7. Experimental Animals

2.7.1. Ethical approval

The animals used in this research were approved and handled by the College of Veterinary Medicine, University of Basrah, in accordance with research ethics unit report number 7/37 in 2022 [23].

2.7.2. Condition of the rearing of experimental animals

The experiment lasted 4 weeks and involved male white rats of the *Rattus norvegicus* species that were of age ~5 months, with an average initial body weight of 149 ± 5.7 g. They were randomly distributed into five different groups. Before the experiment began, the subjects were housed in controlled laboratory conditions for 2 weeks to acclimate. The environmental laboratory conditions were controlled, with an air temperature maintained at 22–25°C, a 12-h light–dark cycle, and a relative humidity of approximately 55%. The rats were fed a commercial diet and had access to water ad libitum.

Table 2: Effect of EPS on serum IL-10 and IL-6 levels of LPS-induced groups.

Cytokines	Grp.1	Grp.2	Grp.3	Grp.4	Grp.5
IL-10 pg/mL↑	$213.9^{b} \pm 88$	$227.4^{b} \pm 76$	$366.6^{a} \pm 36$	$322.1^{a} \pm 31$	$231.7^{b} \pm 51$
IL-6 ng/L↓	$3.18^{b} \pm 1.2$	$20.8^{a} \pm 1.5$	$2.75^{b} \pm 1.2$	$2.60^{b} \pm 1.1$	$20.34^{a} \pm 1.3$

↑: Increase; ↓: Decrease. Data are displayed as mean ± SD. symbols ^{ab} small letters indicate significantl difference ($p \leq 0.05$) between groups.

Table 3: Hematological parameters for treated and untreated groups of rats.

Hematology Parameter	Grp.1	Grp.2	Grp.3	Grp.4	Grp.5
WBC, $10^3/\mu L$	$2.3^{b} \pm 0.3$	$6.4^{a} \pm 0.4$	$2.3^{b} \pm 0.4$	$2.2^{b} \pm 0.3$	$5.8^{a} \pm 0.2$
Neutrophils, $10^3/\mu L$	$0.7^{b} \pm 0.2$	$3.8^{a} \pm 0.3$	$0.6^{b} \pm 0.2$	$0.7^{b} \pm 0.2$	$3^{a} \pm 0.1$
Lymphocytes, $10^3/\mu L$	$3.1^{a} \pm 0.4$	$1.5^{b} \pm 0.2$	$2.7^{a} \pm 0.3$	$2.6^{a} \pm 0.1$	$1.8^{b} \pm 0.1$
Monocytes, $10^3/\mu L$	$0.08^{a} \pm 0.01$	$0.013^{b} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.02^{b} \pm 0.01$
RBC, $10^6/\mu L$	8.3 ± 0.5	8 ± 0.3	8.1 ± 0.1	8 ± 0.3	7.8 ± 0.3
HB, g/dl	14 ± 0.4	13.9 ± 0.5	14 ± 0.6	14 ± 0.2	14.2 ± 0.5
PT, $10^3/\mu L$	$573^{a} \pm 24.6$	$346^{b} \pm 31.9$	$507^{a} \pm 18.9$	$506^{a} \pm 46.3$	$325^{b} \pm 44.3$
PCV, %	$39^{b} \pm 0.4$	$48^{a} \pm 0.5$	$38^{b} \pm 0.3$	$39^{b} \pm 0.2$	$42.6^{a} \pm 0.5$

Data are shown as mean ± SD, symbols ^{ab} were significant differences ($p \leq 0.05$) for parameters between treatment groups.

2.7.3. Experimental design of the animal

Rats were randomly grouped into five different groups (six rats in each group). The experiment was proceeded for 14 days based on the study by Kukihi and Afiati [24], but the other experimental conditions are subject to change or modification according to the test specified in the current study—Grp1: normal group; Grp2: control groups, orally administered with PBS; Grp3: intraperitoneal (IP) injection with 1500 µg/mL of an aqueous solution of purified extracted EPS; Grp4: orally administered with 1500 µg/mL of an aqueous solution of purified extracted EPS; and Grp5: orally administered with 1 mL of yogurt suspensions prepared based on [25,26]. Yogurt suspensions containing *B. longum* subsp. *infantis* strain Iraq-Basrah 3 secreted EPS as a starter; the viability of the strain starter in yogurt was 8.83 ± 0.4 log CFU/mL.

On the 14th day, 4 h after the end of the experiments, all test groups of animals except the normal group (Grp1) received extracted LPS (2 mg/mL) for 24 h (LPS was extracted from *Escherichia coli* according to methods employed by Lee and Inzana [27]) orally administered by gavage tube. On the 15th day, the health condition of the rats was observed, then all test groups of animals were anesthetized, and blood samples were taken by intracardiac puncture and promptly transferred into EDTA tube (ethylenediaminetetraacetic acid-coated vials) for the determination of hematological tests and gel tubes for the determination of cytokine (pro-inflammatory IL-6 and anti-inflammatory IL-10) level tests.

2.8. Determination of IL-6 and IL-10 Cytokine Test

The levels of IL-6 (pro-inflammatory) and IL-10 (anti-inflammatory) in serum were determined by commercial rat interleukin 10 (IL-10) and interleukin 6 (IL-6), using Bioassay Technology Laboratory ELISA kits, China, kit contents, and reagents prepared according to their companies.

2.9. Hematological Analysis Parameter

A blood sample was placed in an EDTA tube for the determination of the hematological assay, including white blood cell (WBC), RBC, total plate count (TPC), packed cell volume (PCV), lymphocyte, monocyte, neutrophil, and hemoglobin (Hb) analysis on each sample and compared with the control group.

2.10. 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.

3. RESULTS AND DISCUSSION

3.1. Morphological and Genetic Identification of *Bifidobacterium* Strain

Identification of *Bifidobacterium* spp. was achieved morphologically on selective *Bifidobacterium* agar medium according to Parte et al. [28], where the species formed smooth, convex with entire edges, cream to white, glistening, and of soft consistency colonies; furthermore, microscopically, it exhibited gram-positive and non-spore-forming bacilli. The bifidogenic factors produced from the mother's milk were the only reason for the preponderance of *Bifidobacterium* spp. in the fecal microbiota of healthy breastfed infants [29]. In addition, the strains were confirmed genetically by using universal *16S rDNA* amplification, and then sequenced and aligned with the reference strain in the GenBank. It turns out that the isolate is *B. longum* subsp. *infantis*. This species was recorded as the new local isolate in Iraq-Basrah 3 as a new strain, and the accession number assigned in the GenBank is OQ738864.1. The genetic diagnosis is more accurate in diagnosis without loss or avoiding any error in the identification of any bacterial strain.

3.2. Total EPS Quantification and Content of Protein

The total dry weight and yield of crude EPS for *B. longum* subsp. *infantis* strain Iraq-Basrah 3 were 2.08 ± 0.41 g/L in the modified MRS medium, with starch being the preferred carbon source using the ethanol precipitation method. Then, the total protein content in crude EPS was displayed (0.6 ± 0.1 µg/mL). High-production EPS yields depend primarily on the choice of a suitable culture medium and its components for production. This finding is in agreement with other research [30], as well as the optimal conditions, and the fact that using starch as a carbon source has an effective role for high-quality products. Furthermore, the methods used for purified EPS have trichloroacetic acid, which precipitates proteins during extraction [31,32].

3.3. In vitro Activity of EPS as Cytotoxic and RBC Membrane Stabilization

The RBC hemolysis test is a method of analyzing RBC eligibility and the toxicity of materials. Validation of the hemocompatibility of the EPS non-hemolytic activity, membrane stabilization, and non-cytotoxicity was assessed under a light microscope. The result in Figure 1 shows the stabilization ability of membrane RBC and demonstrates all concentrations of EPS isolated from *B. longum* subsp. *infantis* strain Iraq-Basrah 3. Protection for the RBC membrane against hypotonic-triggered hemolysis, which indicates the EPS is not toxic to RBCs, has the potential to stabilize membrane RBC, and is safe at low concentrations. This result is similar to the previous results identified: 1000 µg/mL of EPS extracted from endophytic fungus Fusarium solani SD5 prevent hyposaline-induced hemolysis of RBC [33], and 100 and 500 µg/mL of EPS produced from halophilic bacterium Virgibacillus dokdonensis also protect RBC hemolysis [34] as well, during their experiment, they showed that EPS does not harm the vitality of healthy Caco-2 cells [35].

Membranes for human RBC are similar to lysosomal membranes and help maintain cell integrity against osmotic stress and heat-induced lysis. Hypotonic solutions can cause excessive fluid accumulation within the cells, leading to membrane rupture and the osmotic loss of cell membranes. Therefore, making the membrane firm can prevent the leakage of fluid and serum proteins into the tissue during the process of enhancing cell permeability and preventing inflammation [36]. Furthermore, the binding of EPS to membrane proteins is considered to be a possible mechanism for the stabilizing characteristic; this could change the RBC membrane's surface volume ratio, changing the surface charge and calcium flux in the membranes, and inhibiting the aggregating agents [37]. Therefore, this shows that the EPS is active, exhibits potential as a novel biocompatibility agent, and can be used instead of bioactive synthetic molecules such as butylated hydroxytoluene and butylated hydroxyanisole, which have been proven to have toxicity [38].

3.4. Determination of Monosaccharide Subunits for Hydrolyzed EPS

Diagnosis of monomer subunits for EPS produced has been achieved by liquid chromatography (HPLC) on EPS hydrolysate derivatives, and comparison with mixed standard monosaccharide derivatives is displayed in Figures 2A and 2B. There are five peaks in the HPLC results for EPS compared with the retention time of the derivative monosaccharide standard, as shown in Table 1.

The HPLC result in Figure 2B display that EPS has a five-peak, and the retention time due to the five monosaccharide subunits, compared with the standard retention time for carbohydrates in Figure 2A. This result indicates that this biopolymer is a hetero-polysaccharide secreted by a new local strain, *B. longum* subsp. *infantis* strain Iraq-Basrah 3. It depends on the carbon source used in the product medium; previous workers reported the biosynthesis of hetero-polysaccharides from sugar glucose and the glucose portion acted as a carbon source [39]. This result follows the same line of research by previous researchers on wild and mutant *Lactobacillus delbrueckii*; their EPSs were hetero-polysaccharides, a subunit composite of eight different types of sugars: ribose, xylose, arabinose, rhamnose, fructose, glucose, mannose, and galactose (6).

3.5. Serum IL-10 and IL-6 Cytokines Level Determination

Purified EPS showed potential to reduce the anti-inflammatory cytokine IL-10 and suppress the pro-inflammatory cytokine IL-6 in the serum of rats. In the groups induced with LPS and treated with EPS, there was a significant increase in IL-10 (Grp.3: 366.6 ± 36 pg/mL and Grp.4: 322.1 ± 31 pg/mL) compared to the control group (227.4 ± 88 pg/mL) and a significant decrease in IL-6 (Grp.3: 2.75 ± 1.2 ng/l and Grp.4: 2.60 ± 1.1 ng/l) compared to the control group (20.8 ± 1.5 ng/l). Furthermore, there was no significant difference between the control group and the group treated with yogurt-containing strains that produce EPS, as shown in Table 2.

This novel biopolymer, EPS, is a natural compound that stimulates the immune response against inflammation when used as an immunostimulatory compound. Table 2 exhibits groups induced with LPS and treated with purified EPS Grp.3 and Grp.4 (IP injection and oral administration, respectively), stimulating anti-inflammatory cytokines (IL-10) and suppressing pro-inflammatory cytokines (IL-6). Nevertheless, the mechanisms behind how the EPS stimulates anti-inflammatory activity remain poorly understood.

The innate immune response to microbial infections is known to be critically influenced by toll-like receptor 2 and 4 (TLR2, TLR4), and dysfunction of this receptor is associated with a wide range of diseases. TLR4 activation triggers the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways, which in turn promote the expression of genes related to inflammation, including cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) [40]. LPS is one of the components of the outer membrane of gram-negative bacteria, which has a molecular pattern associated with pathogenic bacteria and is recognized by TLR, which binds to it when bacteria are degraded in the host, causing inflammation and activation of the innate immune system in response to the inflammatory reactions (pro-inflammatory) caused by LPS, causing fever, diarrhea, and dysfunction of the heart. TLR may therefore be a therapeutic target for the treatment of immune diseases [41,42].

The ability of LPS to stimulate innate immunity by binding to TLR causes a rapid response and secretion of pro-inflammatory cytokines (IL-1 ß, IL-6, and TNF-α) to healthy rats; even in low dosages, within 24 h through two TLR4 signaling pathways: the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway and the MyD88-dependent pathway. When these pathways interact, they result in a complex inflammatory response [39,43]. Gavzy et al. [44] demonstrated the synthesis of high-molecular-weight EPS by *Bifidobacterium* spp., which is mediated by the induction of TLR-2 and the ability to induce $Foxp3^{+}T_{reg}$ cells. This induction increases the production of anti-inflammatory cytokines (IL-10) and suppresses pro-inflammatory cytokines (IL-6). Kwon et al. [45] demonstrated that EPS inhibits the inflammatory response by interacting with TLR4 using TAK-242 (Resatorvid is a TLR4 inhibitor) and blocking the interaction between LPS and TLR4, as well as reducing the expression

of genes responsible for secreting pro-inflammatory cytokines such as nitric oxide synthase (iNOS), basic mediator of inflammation; IL-1 ß, IL-6, and TNF-α induced by LPS. This finding agrees with other researchers' reports that polysaccharides secreted by *Bacteroides fragilis* can *in vivo* suppress the pro-inflammatory IL-17 production by intestinal immune cells induced by *Helicobacter hepaticus* [46].

In Grp.5, inflammatory symptoms were observed, but there were no statistically significant differences ($p \geq 0.05$) compared with control groups (Grp.2): increased IL-6 (20.34 ± 1.3) and decreased IL-10 (231.7 ± 51). Grp.5 were treated orally with yogurt fermentation by starter *B. longum* subsp. *infantis* strain Iraq-Basrah 3, which produced EPS and was induced with LPS. This increased inflammation due to LPS stimulating pro-inflammatory cytokines (IL-6), while IL-10 cytokines were not affected by yogurt fermentation, indicating no immune stimulation for anti-inflammatory purposes. Several explanations are possible, including (1) the skim milk media and optimal conditions for fermentation are unfavorable for high production of EPS; (2) the amount of EPS produced in yogurt is used again as a prebiotic for starters *Bifidobacterium* in fermented yogurt; (3) degradation by carbohydrate digestion enzymes produced by the small intestine [47] may not absorb a sufficient amount of EPS into the systematic circulation to stimulate the immune system. Additionally, the acidic environment in the stomach may have a more significant effect on the viable cell count for *Bifidobacterium* spp. or any microbial species, which may reflect on colonization and adhesion, and the ability to produce EPS during shorter periods, thereby modifying the intestinal flora and stimulating the host's immunity.

3.6. Hematological Parameters for Rats Treated with EPS and Untreated Groups

The hematological parameters of the rats induced with LPS and treated with EPS and the untreated control rat groups were compared. The level of WBCs, neutrophils, and PCV (5.8 ± 0.2, 3 ± 0.1, 42.6 ± 0.5, 14.2 ± 0.5, respectively) were significantly increased ($p \leq 0.05$), while the levels of lymphocytes, monocytes, and PT parameters (1.8 ± 0.1, 0.02 ± 0.006, 325 ± 44.3, respectively) were significantly decreased ($p \leq 0.05$) in Grp.5 compared with normal group (Grp.2). In contrast, Grp.3 and Grp.4 showed no effect from inflammation inducement and there were no significant differences in parameter Hb and RBC levels for all groups, as exhibited in Table 3. All hematological parameters for the inducement groups and those treated with EPS (Grp.3 and Grp.4) are in the normal range compared with the normal group (Grp.1), whereas Grp.5 was in the abnormal range, indicating stimulation of inflammation compared with untreated group (Grp.2). This result agrees with previous studies (6). There are no previous studies on the mechanism of the effect of polysaccharides on maintaining physiological blood parameters against inflammation. Blood and bone marrow are composed of a complex mixture of cells that respond to different inflammations, including bacterial endotoxin (lipopolysaccharide) that alters (increase or decrease) most bone marrow cell types by interacting LPS with the cell membrane and affecting the function and growth of cells (18). EPS binding with TLR and blocking the interaction of LPS with TLR [48] leads to engulfment of LPS by macrophages, which improves bone marrow and blood cells.

4. CONCLUSION

A lot of research has been conducted in recent years on all metabolic products produced by LAB, indicating that they have many benefits and applications in food and biomedicine. Numerous studies focus on natural extracts that have a biomedical effect. Our research has proven that the novel EPS produced from a new local strain, *B. longum* subsp. *infantis* strain Iraq-Basrah 3, is an anti-cytotoxic and anti-inflammatory compound that stimulates IL-10 cytokines and suppresses pro-inflammatory IL-6 cytokines in groups of white rats treated with EPS and induced with LPS. Additionally, inflammatory symptoms were observed in groups treated with yogurt fermentation. So, EPS can be used as an immune therapy to treat inflammation in small doses over a short period of time. It is safe for health as an immune-stimulating supplement.

5. ABBREVIATIONS

EPS: Exopolysaccharides; HoEPS: Homo-exopolysaccharides; HeEPS: Hetero-exopolysaccharides; IL: Interleukin; LAB: Lactic acid bacteria; MW: Molecular weight; MRS: de Man, Rogosa, and Sharpe; TCA: Trichloroacetic acid; BSA: Bovine serum albumin; TFA: Trifluoroacetic acid; HPLC: High-performance liquid chromatography; Grp: Group; IP: Intraperitoneal; PMP: 1-phenyl-3-methyl-5-pyrazolone.

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7. AUTHORS' CONTRIBUTIONS

KH, AN, and KM conceived and designed the experiments. KH carried out laboratory experiments, analyzed the data, analyzed the genome sequence, and contributed to the manuscript writing and preparation. AN extensively revised the manuscript; and finally, all authors read and approved the final manuscript.

8. FINANCIAL SUPPORT AND SPONSORSHIP

There is no financial support and sponsorship.

9. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The animals used in research were approved and handled by the College of Veterinary Medicine, University of Basrah, in accordance with research ethics unit report number 7/37 in 2022.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

13. PUBLISHER'S NOTE

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