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The Effects of Biotics Derived from *Lactiplantibacillus* plantarum EIR/IF-1 on the Modulation of Periodontal Inflammation

Lactiplantibacillus plantarum EIR/IF-1 Kaynaklı Biyotiklerin Periodontal İnflamasyonun Modülasyonu Üzerindeki Etkileri

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ABSTRACT

Objective: This study aims to investigate the immunomodulatory effects of postbiotics and paraprobiotics derived from Lactiplantibacillus plantarum EIR/IF-1.

Materials and Methods: Human periodontal ligament fibroblast (hPDLF) cells were co-incubated with the release and bound forms of exopolysaccharides (10-1,000 µg/mL), cell lysate (0.1-1,000 µg/mL), cell surface proteins (0.1-100 µg/mL), and inactivated cells (10^6-10^{10} CFU/mL) derived from Lactiplantibacillus plantarum EIR/IF-1 for 24 hours. Cell viability was assessed by MTT assay. Lipopolysaccharide (LPS) from Porphyromonas gingivalis, a key pathogen involved in periodontal disease, was used to induce inflammation in hPDLF cells. Non-toxic doses of the biotics were subsequently tested for their impact on cytokine production in LPS induced hPDLF cells. Cytokine levels (IL-10, IL-8, and IFN-γ) were quantified using the ELISA protocol.

Results: After 24 hours incubation of hPDLF cells with 1 µg/mL LPS and selected concentrations of the biotics derived from the EIR/IF-1 strain that did not exhibit toxic effects, it was observed that LPS from P. gingivalis induced IL-8 production. However, the biotics extracted from the EIR/IF-1 strain significantly reduced IL-8 production (p<0.0001). Furthermore, these biotics increased the production of the anti-inflammatory cytokine IL-10 (p<0.0001).

Conclusion: The obtained data suggest that the biotics have the potential to counteract the pro-inflammatory effects induced by LPS. The findings highlight the potential of postbiotics and paraprobiotics, containing biologically active and effective components, as a promising natural and reliable approach for reducing and preventing periodontal inflammation. In conclusion, our results suggest that microbiota-derived biotics could serve as effective adjuncts in the management of periodontal diseases.

Keywords: *Inflammation, Periodontal Diseases, Cytokine, Probiotic*

ÖZET

Amaç: Bu çalışma, Lactiplantibacillus plantarum EIR/IF-1 suşundan elde edilen postbiyotiklerin ve paraprobiyotiklerin immünmodülatör etkilerini araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: İnsan periodontal ligament fibroblast (iPDLF) hücreleri, Lactiplantibacillus plantarum EIR/IF-1 kaynaklı salınan ve bağlı formlarda bulunan eksopolizakkaritler (10–1.000 µg/mL), hücre lizatı (0,1–1.000 µg/mL), hücre yüzeyi proteinleri (0,1–100 µg/mL) ve inaktif hücreler (10^6–10^{10} KOB/mL) ile 24 saat süreyle birlikte inkübe edilmiştir. Hücre canlılığı MTT testi ile değerlendirilmiştir. Periodontal hastalıkla ilişkili önemli bir patojen olan Porphyromonas gingivalis'ten elde edilen lipopolisakkarit (LPS), iPDLF hücrelerinde inflamasyonu indüklemek için kullanılmıştır. Biyotiklerin, LPS ile uyarılmış iPDLF hücrelerinde sitokin üretimine olan etkisi, toksik olmayan dozlarla birlikte inkübasyon neticesinde test edilmiştir. Sitokin seviyeleri (IL-10, IL-8 ve IFN-γ) ELİZA protokolü ile belirlenmiştir.

Bulgular: Hücreler 1 µg/mL LPS ve toksik etkisi olmayan seçilen biyotik dozları ile 24 saat inkübe edildikten sonra, P. gingivalis kaynaklı LPS'nin IL-8 üretimini indüklediği gözlemlenmiştir. Ancak, EIR/IF-1 suşundan elde edilen biyotikler, IL-8 üretimini önemli ölçüde azaltmıştır (p<0.0001). Ayrıca, bu biyotikler anti-inflamatuvar bir sitokin olan IL-10 üretimini artırmıştır (p<0.0001).

Sonuç: Elde edilen veriler, biyotiklerin LPS'nin oluşturduğu pro-inflamatuvar etkileri azaltabilecek potansiyele sahip olduğunu göstermektedir. Bu bulgular, biyolojik olarak aktif ve etkin bileşenler içeren postbiyotikler ve paraprobiyotiklerin periodontal inflamasyonu azaltmak ve önlemek için umut verici, doğal ve güvenilir bir yaklaşım olarak kullanılabileceğini vurgulamaktadır. Sonuç olarak, mikrobiyota kaynaklı biyotiklerin periodontal hastalıkların yönetiminde etkili yardımcı bir tedavi olarak kullanılabileceği önerilmektedir.

Anahtar Kelimeler: *İnflamasyon, Periodontal Hastalıklar, Sitokin, Probiyotik*

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Introduction

Periodontal diseases, which refer to inflammation and infection of the tissues that surround and support teeth, are reported as the second most prevalent chronic disease worldwide, after cardiovascular diseases. Additionally, periodontal diseases are recorded as the sixth most common disease among 291 diseases reported within the scope of Global Burden of Disease studies and are considered an important current public health problem when their prevalence, treatment costs, and negative effects on the patients are taken into consideration.[1] In 2022, World Health Organization (WHO) highlighted the fact that more than 1 billion people worldwide suffer from periodontal diseases.[2] In general, it is estimated that 15% of the human population has experienced periodontitis at some point in their lives, and more than 47% of adults aged 30 and over have been diagnosed with periodontal disease at least once. Periodontal diseases, which increase with age, affect more than 70% of adults aged 65 and older. Among individuals between the ages of 35 and 44, tooth loss occurs at a rate of 5% to 15% following aggressive periodontitis.[3] In addition to the damage they cause to oral tissues and tooth loss, periodontal diseases have also been linked to various systemic conditions such as cardiovascular diseases, diabetes, and Alzheimer's disease.[4] Therefore, considering both their direct effects on dental or oral tissues and their indirect role in predisposing individuals to other diseases, as well as their high global prevalence and socio-economic impact on societies, periodontal diseases are currently referred to as a "silent" global pandemic.[5]

Periodontal diseases are generally characterized as chronic, infectious, and inflammatory pathological conditions initiated by a dysbiotic subgingival biofilm (plaque). Although the etiopathology of the disease is influenced by various factors, it can be broadly described by the interactions between microorganisms responsible for dental biofilms and host's immune system response.[6] In light of these processes, periodontal diseases are classified into categories: gingivitis and periodontitis. The accumulation of bacteria from microbial dental plaque in the subgingival tissues, followed by inflammation of the surrounding tissues, is referred to as gingivitis (gum inflammation). When the dental biofilm caused by microorganisms is not eradicated, the infection in the gum progresses to other tissues of the periodontium, ultimately reaching the alveolar bone and leading to periodontitis, which is defined as an aggressive pathological condition. The progression of the disease results in advanced attachment loss and alveolar bone resorption, culminating in progressive periodontal bone loss. Failure to implement an effective treatment approach for healing or biofilm eradication leads to early tooth loss.[7] In conclusion, both tooth loss resulting from periodontal inflammation and its connection with systemic diseases underline the need for development of new and effective treatment strategies for periodontal diseases.[8] Considering that the existing damage is caused by inflammation resulting from excessive activation of the host's immune cells, targeted treatment strategies are expected to not only focus on biofilm removal but also aim to reduce inflammation.

Biotics, defined as an umbrella term encompassing probiotics, prebiotics, postbiotics, and paraprobiotics, have gained considerable attention in recent years within both health and food industries. The terms postbiotics and paraprobiotics are emerging as current concepts that are not included in the traditional definitions of probiotics (microorganisms that confer health benefits to the host when consumed in adequate amounts) and prebiotics (non-digestible dietary components).[9] Postbiotics, a novel area within the biotics family, are defined as "bacterial or metabolic end products produced by microorganisms that possess biological activity in the host", and may include any substance or combination of substances that provide synergistic benefits to the host, either directly or indirectly.[10,11] These metabolic end products may consist various components such as short-chain fatty acids, microbial cell fractions, functional proteins, exopolysaccharides (EPS), cell lysates, teichoic acids, peptidoglycan-derived muropeptides, and pili-type structures. It has been noted that postbiotics produced by

beneficial microorganisms can exert their positive effects attributed to the producer strain through the similar mechanisms.[12] They have been reported to possess antibacterial, anti-biofilm, anti-inflammatory, hypocholesterolemic, antioxidant, and immunomodulatory effects on the host organism.[13] Additionally, they contribute to microbiota homeostasis by positively influencing host metabolism and signaling interactions.[14] The fact that postbiotics exert their potential effects on the host by modulating biological signaling pathways underscores their significance as key mediators of the beneficial effects of microbiota members. Another group of biotics that has attracted attention in recent years, "paraprobiotics," is defined by the Food and Agriculture Organization/World Health Organization as "inactive (non-living, ghost) microbial cells that provide benefits to consumers when applied in sufficient amounts".[10,15]

This study aims to investigate the potential effects of postbiotics and paraprobiotics derived from Lactiplantibacillus plantarum EIR/IF-1 on inflammation associated with periodontal disease under *in vitro* conditions. This bacterium was selected due to its previously observed biological activities (antimicrobial, anti-biofilm, and anti-quorum sensing) against significant periodontal pathogens, as reported in our earlier studies.[16-18]

Materials and Methods

Lactiplantibacillus plantarum EIR/IF-1 (NCBI GenBank Accession Number: MW057714.1), isolated from the infant fecal microbiota and identified through 16S rRNA sequencing, was kindly provided by the bacterial culture collection of the Pharmabiotic Technologies Research Laboratory (Faculty of Science, Ankara University) and used as the source of biotics. Bacterial glycerol (50%) stock solution was transfered to De Man-Rogosa-Sharpe (MRS) broth medium and incubated at 37ºC for 24 hours (Nüve, Turkey). After two passages for activation, the purity and phenotypic validation were performed through streak plating and Gram staining and the strain was used for further assays.[19]

To obtain inactive cells, EIR/IF-1 strain was inoculated into MRS broth at a 1% concentration and incubated at 37ºC for 24 hours. After incubation, the culture was centrifuged at 15,000x*g* for 20 minutes at 4ºC (Nüve, Turkey) and the pellet was suspended in sterile physiological saline solution. Serial dilutions were performed to determine the number of cells. 10 µL from each dilution were drop-plated on MRS agar plates and incubated at 37ºC for 24 hours. Following incubation, the colonies were counted and calculated as colony-forming units (CFU)/mL. The cells were then inactivated by heat treatment in a water bath (Nüve, Turkey) at 60ºC for 3 hours. Inactivation efficacy was confirmed by culturing the cells in MRS broth and agar for 48 hours at 37ºC.[20]

Following the culture procedures mentioned in the previous step, the pellet obtained from the EIR/IF-1 strain was dissolved in 1 mL of a solution containing 5 mM ethylenediaminetetraacetic acid (EDTA, Merck, USA) and 5 mM magnesium chloride ($MgCl_2$, Merck, USA) in 50 mM Tris (pH 7.5) buffer (Merck, USA). Sonication was performed three times at 40W (QSonica, USA). The cell lysate was obtained after centrifugation at 14,000×*g* for 10 minutes at 4ºC. To confirm cell lysis, cell lysate was inoculated into MRS broth and onto MRS agar, and incubated for 48 at 37ºC. After confirming the absence of microbial growth, the cell lysate was subsequently lyophilized (Buchi, Switzerland).[21]

Exopolysaccharides (EPS) from the EIR/IF-1 were obtained in two different stages. To extract the release form of exopolysaccharides (EPS-r), the EIR/IF-1 strain was inoculated into MRS broth and incubated at 37ºC for 24 hours. After incubation, the culture was centrifuged at 15,000×*g* for 20 minutes at 4ºC, and supernatant (upper phase) was subsequently heated in a water bath at 100ºC for 15 minutes. After centrifugation at 15,000×*g* for 15 minutes, 20% trichloroacetic acid (TCA, Sigma, USA) was added to the supernatant. The mixture was incubated with shaking at 4ºC for 2 hours, and then centrifuged at 25,000x*g* for 20 minutes at 4ºC. The supernatant was mixed with two volumes of 95% ethanol (Merck, USA) and incubated overnight. After a final centrifugation

at 6,000×*g* for 30 minutes at 4ºC, the obtained EPS-r was lyophilized.[22] For the extraction of cell surface-bound exopolysaccharides (EPS-b), the cells obtained from the previous step were washed with 10 mL of sterile physiological saline and resuspended in 5 mL of 1 M sodium chloride (NaCl, Merck, USA). The suspension was sonicated at 40W for 3 minutes. After centrifugation at 6,000×*g* for 30 minutes at 4ºC, the supernatant was mixed with two volumes of 95% ethanol (Merck, USA) and incubated overnight. After a final centrifugation at 6,000×*g* for 30 minutes at 4ºC, the obtained EPS-b was lyophilized.[22] The total glucose content of EPS-b and EPS-r was determined according to the method of Dubois et al.[23] 1 mL of 5% phenol solution (Merck, USA) and 5 mL of 96% sulfuric acid (Merck, USA) were mixed with the EPS-b, EPS-r and standard solutions containing different concentrations of glucose, separately. The mixtures were incubated at room temperature for 10 minutes, followed by incubation at 37ºC for 15 minutes. The color change was measured by spectrophotometrically (BioTek Epoch, USA) at a wavelength of 490_{nm}.

For the extraction of cell surface proteins, the EIR/IF-1 strain was inoculated into 1% MRS broth and cultured at 37ºC for 24 hours. Following incubation, the culture was centrifuged at 15,000×*g* for 20 minutes at 4ºC. The obtained pellet was washed twice with 25 mL of cold phosphate-buffered saline (PBS; 0.12% K_2HPO_4, 0.022% Na_2HPO_4, 0.85% NaCl, pH: 7.4). Next, 10 mL of 5 M lithium chloride (LiCl, Merck, USA) solution was added, and the mixture was stirred at 4ºC for 15 minutes. After centrifugation at 9,000×*g* for 10 minutes at 4ºC, the supernatant was transferred to a molecular weight cut-off (MWCO) membrane with a 12,000 Da pore size (Sigma, USA), and dialysis was performed against cold distilled water (dH_2O) for 24 hours. During the dialysis process, water was exchanged every 2 hours. The dialyzed solution was centrifuged at 20,000×*g* for 30 minutes at 4ºC. The resulting pellet was resuspended in 10 mL of 1 M LiCl solution and stirred at 4ºC for 15 minutes. After centrifugation at 20,000×*g* for 10 minutes at 4ºC, the supernatant was transferred to the dialysis membrane and dialyzed again for 24 hours. The final dialyzed solution was centrifuged at 20,000×*g* for 30 minutes at 4ºC and the obtained cell surface proteins were lyophilized.[24] The total protein content of the cell surface proteins was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Smith method; TaKaRa, Japan) according to the manufacturer's instructions. Briefly, standard solutions of bovine serum albumin (BSA) at concentrations of 125-2,000 µg/mL were prepared from a 2 mg/mL BSA stock solution. Standard solutions and cell surface proteins were separately mixed with 200 µL of BCA reagent and incubated at 37ºC for 30 minutes. Following incubation, a purple-colored product was formed as a result of the reaction between the copper ions of the BCA reagent and the peptide bonds of the proteins. The color change was measured spectrophotometrically at a wavelength of 562_{nm}.

The immunomodulatory effects of the biotics were evaluated using human periodontal ligament fibroblast (hPDLF) cells, which were kindly provided by the cell culture collection of the Pharmabiotic Technologies Research Laboratory (Faculty of Science, Ankara University). These cells were isolated and characterized from healthy human periodontal tissues during our previous studies.[25] The cells were cultured in alpha-MEM medium (Sartorius, Germany) supplemented with 10% fetal bovine serum (BI, Germany), 1% penicillin-streptomycin (Gibco, USA), 1% L-glutamine (Sartorius, Germany), and 1% non-essential amino acids (Gibco, USA) at 37°C with 5% CO^2 and 80-90% relative humidity in T75 flasks. The medium was refreshed every 3 days. During culturing, cells were visualized under an inverted phase contrast microscope (Olympus, Japan), as shown in Figure 1. When 85-90% confluence was observed, the culture medium was removed, and the cells were washed with PBS buffer, followed by trypsinization. After centrifugation at 200×*g* for 5 minutes, the cells were counted using an automatic cell counter (Bio-Rad, USA) following trypan blue staining, and were subsequently seeded in 96-well tissue culture plate at a density of 10,000 cells per well to assess the non- cytotoxic doses of biotics. After a 24-hour incubation period, the culture

medium was completely removed, and media containing EPS-r (10-1,000 µg/mL), EPS-b (10-1,000 µg/mL), cell lysate (0.1-1,000 µg/mL), cell surface proteins (0.1-100 µg/mL), and inactivated cells (10^6-10^{10} CFU/mL) were added to the wells in triplicate. Wells without biotics used as the control. After an additional 24-hour incubation period, the culture medium was removed, and cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, 10 µL of MTT solution (5 mg/mL, Serva, USA) was added to each well, and after a 4-hour incubation at 37ºC, the MTT solution was removed. Subsequently, dimethyl sulfoxide (DMSO, Sigma, USA) was added to each well, and the optical density was measured at a wavelength of 550_{nm} using a microplate reader (BioTek Epoch, USA).[26]

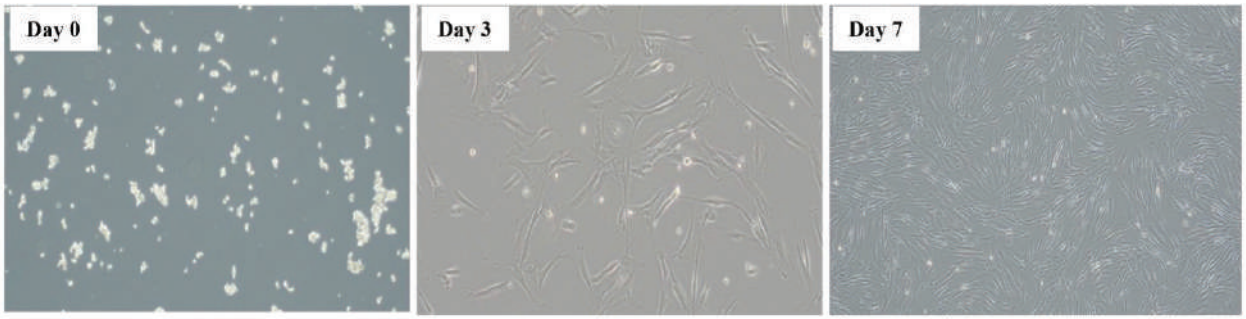

Figure 1. Microscopic images of human periodontal ligament fibroblast cells

To determine the immunomodulatory effect of the biotics, an inflammation was induced in hPDLF cells using lipopolysaccharide (LPS) from Porphyromonas gingivalis (Sigma, USA). First, the dose of LPS that did not exhibit cytotoxic effect on cells was determined through MTT analysis, as previously described. Next, the highest non-cytotoxic dose of LPS was co-incubated with biotics derived from the EIR/IF-1 strain. After a 24-hour incubation period, the levels of interleukin (IL)-10, IL-8, and interferon (IFN)-γ in the cell supernatants were determined using ELISA (Enzyme-Linked Immunosorbent Assay) kits (MabTech, Switzerland), following protocols recommended by the manufacturer.

All analyses in this study were performed in triplicate and analyzed using GraphPad Prism v.8.0 (GraphPad Software, San Diego, CA, USA). Differences between groups were evaluated with Tukey's test one-way analysis of variance (ANOVA) and $p<0.05$ was considered significant.

Results

Among the biotics extracted from EIR/IF-1 strain, heat-inactivated cells were applied in the cell culture studies with their concentration as CFU/mL. Therefore, the number of bacterial cells prior to inactivation was determined through microbiological assays. The results indicated that the viable cell count was 2×10^{11} CFU/mL, which was then used for dose calculations. After heat inactivation, no growth was observed in either broth or agar media which confirming the successful inactivation of the cells. Similar results were also observed for the lysed cells, indicating that cell lysis was also successfully achieved. The total glucose content of EPS-b and EPS-r was determined based on the glucose standard curve (Figure 2a). Our results showed that the glucose content of EPS-b was 140.71±2.4 mg/L, while that of EPS-r was 741.63±18.2 mg/L. Similarly, the total protein amount of cell surface proteins extracted from the EIR/IF-1 strain was evaluated using the BSA standard curve (Figure 2b). According to the results, the total protein content of the cell surface proteins was determined to be 1.1±0.4 mg/mL.

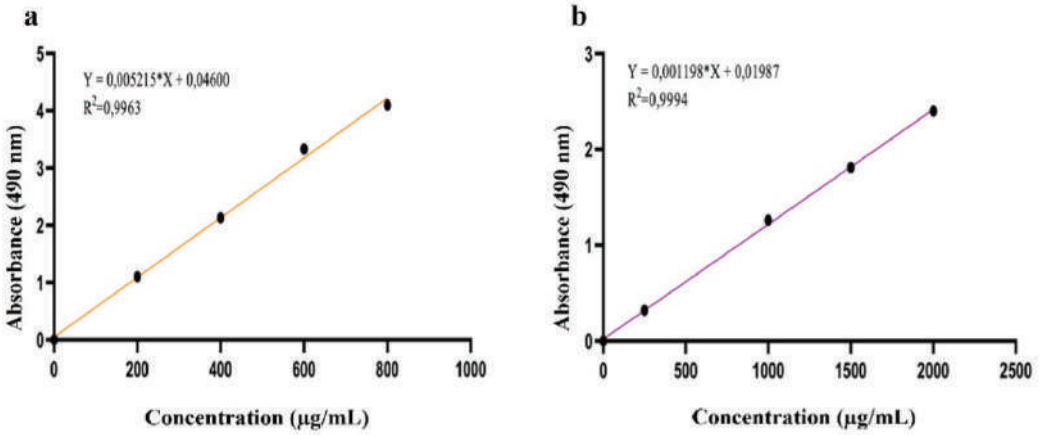

Figure 2. The standard curves used in the study for total sugar (a) and total protein content (b)

hPDLF cells were co-incubated with EPS-r (10-1,000 µg/mL), EPS-b (10-1,000 µg/mL), cell lysate (0.1-1,000 µg/mL), cell surface proteins (0.1-100 µg/mL), and inactivated cells (10^6-10^{10} CFU/mL) for 24 hours. Cell viability was assessed based on the absorbance values obtained from the test groups and control groups using the MTT assay. Upon evaluating the MTT data, it was observed that the 1,000 µg/mL doses of EPS-b, EPS-r and cell lysate, the 100 µg/mL dose of cell surface proteins and the 10^{10} CFU/mL dose of inactivated cells exhibited toxic effects on the hPDLF cells (Figure 3). Consequently, the highest doses that did not exhibit toxic effects were selected in the subsequent experiments.

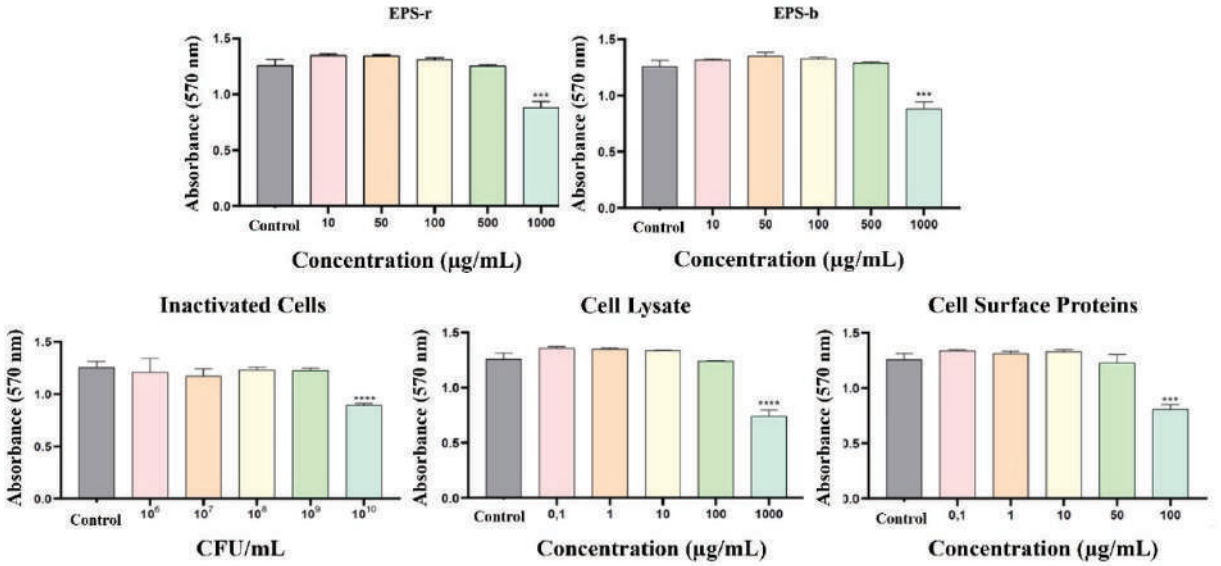

Figure 3. The effect of biotics derived from the *Lactiplantibacillus plantarum* EIR/IF-1 strain on the viability of hPDLF cells (***: $p<0.001$; ****: $p<0.0001$)

In order to determine the immunomodulatory role of biotics extracted from the EIR/IF-1 strain on hPDLF cells, an inflammation model was established in hPDLF cells. For this purpose, the effect of different concentrations of LPS from P. gingivalis on hPDLF cell viability was evaluated. The results indicated that LPS concentrations of 5 µg/mL ($p<0.05$) and 10 µg/mL ($p<0.001$) significantly reduced hPDLF cell viability. Based on these findings, a concentration of 1 µg/mL, which did not exhibit cytotoxic effects, was selected for cell stimulation assays (Figure 4).

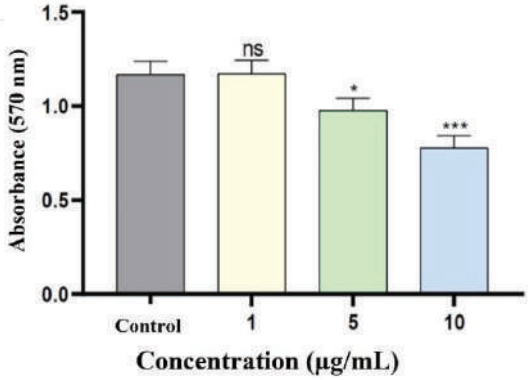

Figure 4. The effect of LPS derived from *Porphyromonas gingivalis* on the viability of hPDLF cells (ns: not significant; *: $p<0.05$; ***: $p<0.001$)

After co-incubating hPDLF cells with 1 µg/mL LPS and selected concentrations of the biotics derived from the EIR/IF-1 strain for 24 hours, the levels of IL-10, IL-8 and IFN-γ in the cell supernatants were determined using ELISA. The absorbance values obtained from the spectrophotometric measurements were evaluated using calibration curves of standards containing known amounts of recombinant cytokines. Regarding IL-8 production, a pro-inflammatory cytokine, the results indicated that LPS from P. gingivalis induced IL-8 production, while the biotics extracted from the EIR/IF-1 strain significantly reduced its production ($p<0.0001$). In contrast, when the results were evaluated in terms of the production of IL-10, an anti-inflammatory cytokine, it was found that biotics extracted from the EIR/IF-1 strain induced IL-10 production, with variations depending on the type of biotics (Figure 5). However, no signals were detected for IFN-γ production.

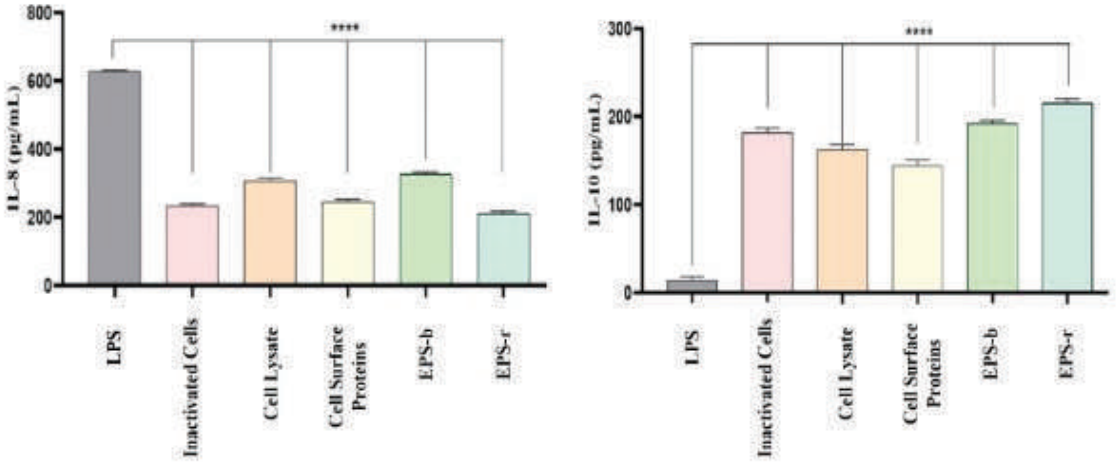

Figure 5. The effect of biotics derived from the *Lactiplantibacillus plantarum* EIR/IF-1 strain on IL-8 and IL-10 production in hPDLF cells induced by *P. gingivalis* LPS (****: $p<0.0001$)

Discussion

Microorganisms involved in the formation of dental plaque, which play a crucial role in the development of periodontal diseases, are considered important not only for their contribution to plaque formation through biofilm production but also for their ability to activate inflammatory responses.[27] Inflammation that occurs as a result of the host immune response ultimately leads to periodontal tissue destruction. During the progress of the disease, *Porphyromonas gingivalis* modulates the host's immune-inflammatory responses and contributes to periodontal tissue destruction by disrupting the homeostasis of the normal cell cycle. The LPS structure of *P. gingivalis* is chemically composed of multiple lipid A forms, including 3-hydroxy-15-methylhexadecanoic acid and

3-hexadecanoyloxy-15-methylhexadecanoic acid, acylated with a glucosamine beta-(1-6) disaccharide 1-monophosphate. This structure is recognized by the TLR-4 receptor on host cells, thereby triggering inflammation.[28] While inflammation initially serves a protective function for the host, if left unchecked, it can lead to tissue destruction.[29] The objective of this study was to examine the effects of postbiotics and paraprobiotics derived from *L. plantarum* EIR/IF-1 on the production of inflammatory cytokines in hPDLF cells stimulated by *P. gingivalis* LPS. We aimed to explore the potential of microbiota-derived biotics as adjunctive therapeutic agents for periodontal disease, a relatively underexplored area within contemporary periodontal research. This study is distinctive in its approach, as it investigates the capacity of postbiotics and paraprobiotics to modulate inflammatory responses in periodontal tissues, thereby offering novel insights into how microbiota-based interventions may provide therapeutic benefits for the management of periodontal disease. The results of this study contribute significantly to the expanding field of microbiota-based therapies, with potential implications for the development of innovative treatment strategies focused on mitigating periodontal inflammation.

Recent studies have indicated that bacteria possessing probiotic properties can effectively prevent inflammation associated with periodontal diseases through diverse immunological pathways including enhanced IgA production in the mucosa, increased macrophage activity, and augmented phagocytosis, all of which contribute to strengthening the immune response and preventing bacterial translocation in the mucosa.[30-32] Moreover, the ability of probiotics to inhibit the growth of major dental pathogens, such as *P. gingivalis*, reduce alveolar bone loss by modulating different signaling pathways, and exhibit immunomodulatory effects supports the hypothesis that probiotics may integrated into different therapeutic strategies for periodontal diseases.[33] However, challenges remain in the application of probiotics, as they require sustained colonization in the oral cavity, which can result in variability in their immunomodulatory effects. Additionally, probiotics can alter the host's microbial community, potentially leading to dysbiosis under certain conditions. The rapid colonization of exogenous probiotics within the established oral microbiota also presents a significant challenge. Furthermore, the use of live microorganisms in therapeutic applications carries risks, such as sepsis, and may present technical challenges related to cell viability, even if these risks are relatively low.[34] Therefore, the limitations associated with probiotics remain important challenges that need to be addressed. In this context, to overcome the disadvantages, an alternative approach has emerged that involves the use of probiotic-derived postbiotics or their inactive forms, such as paraprobiotics. These probiotic derived-mediators play key roles in the beneficial effects of live microorganisms and may offer potential for preventing or alleviating periodontal inflammation.[35] Current literature, however, indicates that the effects of postbiotics and paraprobiotics on inflammatory cytokine production in periodontal tissues remain inadequately explored highlighting a significant gap in the field. By investigating the impact of *L. plantarum*-derived postbiotics and paraprobiotics on inflammatory cytokine responses in hPDLFs exposed to *P. gingivalis* LPS, this study fills a critical gap in the literature. Our findings provide valuable insights into the potential application of microbiota-derived biotics for modulating inflammation in periodontal disease, paving the way for novel adjunctive therapies in clinical periodontal practice.

The mechanisms of action of postbiotics encompass modulation of the host cell-resident microbiota, regulation of systemic or local immune and metabolic responses, enhancement of epithelial barrier function, and signaling with the nervous system.[36] Immunmodulatory effects of postbiotics can be mediated by various mechanisms such as stimulating Th1 immune cells to increase cytokine production, while simultaneously reducing cytokine levels through Th2 cell stimulation.[37] Additionally, postbiotics interact with macrophages and inhibit the production of specific pro-inflammatory cytokines. A diverse range of metabolites within postbiotics act as mediators of these effects by

enhancing the production of anti-inflammatory cytokines and suppressing pro-inflammatory cytokines.[38,39] For instance, postbiotics derived from *Lactobacillus rhamnosus* GG have demonstrated anti-inflammatory properties by modulating the production cytokines such as IL-4, IL-5, and IL-10.[40] Similarly, other studies have shown that postbiotics influence the modulation of the anti-inflammatory cytokine IL-8 while affecting the production of IL-1β, IL-6, TNF-α, and IL-10 cytokines in macrophages.[41] Postbiotics derived from Bacillus coagulans have also been exhibite anti-inflammatory effects,[42] whereas those extracted from Bifidobacterium breve were shown to promote enteric cell maturation and viability, increase IL-10 production, and reduce TNF-α levels.[43] Considering that IL-1β, along with IL-6 and TNF-α, are critical mediators in periodontal tissue degeneration,[44-46] the ability of postbiotics to inhibit inflammation and promote angiogenesis in the epithelial tissues via the activation of α2β1 integrin collagen receptors holds significant therapeutic potential[47].

Bacterial EPS, one of the key component of postbiotics, are macromolecules known for their ability to facilitate interactions between bacteria and their environment, mediate adhesion properties, provide protection against pathogens, and exhibit anti-inflammatory effects.[48,49] Depending on the bacterial species, EPS can be classified as either homopolysaccharides or heteropolysaccharides.[50] Homopolysaccharides are composed of a single type of monosaccharide derivative and are synthesized within cells through glycosyltransferase activity.[51] In contrast, heteropolysaccharides consist of diverse monosaccharides, ranging from disaccharides to heptasaccharides.[52] Our findings in this study reveal that EPS-r derived from L. plantarum EIR/IF-1, which includes fractions with varying molecular weights (51 and 841 kDa) and contains glucose, galactose, and fructose monosaccharides,[17] exhibited more potent immunomodulatory effects compared to EPS-b.[53] Similarly, recent studies have also highlighted the capacity of EPS-r from various Lactobacillus spp. strains to modulate both systemic and mucosal immune responses. Purified EPS-r produced by *L.* rhamnosus RW-9595M has been reported to exert immunosuppressive effects on macrophages by inducing high levels of IL-10 (an anti-inflammatory cytokine) and low levels of TNF-α, IL-6, and IL-12 production.[54] Additionally, the acidic fraction of EPS-r produced by L. plantarum 14 was found to suppress the production of pro-inflammatory cytokines (IL-6, IL-8, and MCP-1) in porcine intestinal epithelial cells in response to enterotoxigenic E. coli.[55] *In vivo* studies corroborate these findings, demonstrating that administration of EPS-r produced by L. paraplantarum BGCG11 to mice led to reduced levels of IL-1β, TNF-α, and inducible nitric oxide synthase, alongside an increased production of IL-10.[49]

Paraprobiotics, a term encompassing inactive cells, cell lysates, and cell surface proteins, contain effector molecules that interact with host cells.[13] Inactive bacterial cells, rendered non-viable through methods such as heat treatment, chemical processing, sonication, or ultraviolet radiation, can exhibit immunomodulatory effects similar to those of probiotics or postbiotics. Among these inactivation techniques, heat treatment is widely regarded as the most common method.[15] In this study, microbial cells were fully inactivated using heat treatment. Our findings revealed that the inactive cells of the L. plantarum EIR/IF-1 strain significantly enhanced IL-10 synthesis.[53] Similar studies have demonstrated that inactivated cells of Lactobacillus spp. strains suppress the production of pro-inflammatory cytokines such as IL-6 and TNF-α while effectively promoting the production of anti-inflammatory cytokines like IL-10.[56] In another study, inactivation of *Lactobacillus* spp. strains via three different heat-treatment methods resulted in stimulation of dendritic and macrophage immune cells, leading to increased IL-12 production in mice.[57]

Bacterial lysates have been shown to exert modulatory effects on the host's immune mechanisms through TLR-mediated interactions with dendritic cells. Stimulation of dendritic cells with bacterial lysates leads to the release of chemokines and triggers the migration of polymorphonuclear neutrophils. Additionally, bacterial lysates play a key role in reducing

the levels of cytokines such as IL-4 and IL-13, which are released by Th2 cells, while increasing the levels of cytokines such as IFN-γ released by Th1 cells.[58] Our study demonstrated that the cell lysate from the L. plantarum EIR/IF-1 strain decreased IL-8 production while increasing IL-10 synthesis. Similar studies investigating the effects of bacterial cell lysates have reported that bacterial lysates stimulate the production of TNF-α, IL-1β, and IL-6 in the host. Furthermore, bacterial cell lysates interact with pattern recognition receptors (PRRs) in the host, playing a critical role in antibody production through their interaction with B cells.[59] Peptidoglycan structures from L. casei, L. johnsonii JCM 2012, and L. plantarum ATCC 14917 have been shown to suppress IL-12 production through the TLR-2 pathway.[60] Peptidoglycan purified from the L. salivarius Ls33 has been reported to exhibit anti-inflammatory properties by inducing IL-10 production.[61] Numerous studies have also demonstrated that teichoic acid derived from various Lactobacillus spp. strains reduced IL-8 expression and exhibited immunomodulatory properties by inducing anti-inflammatory effects on human intestinal epithelial cells.[62,63] Collectively, these findings highlight the importance of bacterial cell lysates in supporting host immunity.

Cell surface proteins are protein-based structures located on the outer membrane of cell, which regulate cellular material exchange, mediate various signaling pathways, and facilitate cell-cell interactions. Among the cell surface proteins, structures such as the S-layer protein, mucin-binding proteins, fibronectin-binding proteins, and collagen-binding proteins play essential roles in the mechanisms and actions of bacteria.[10] These cell surface proteins which enable contact with the host organism, thereby activating specific signal transduction pathways. Consequently, the activation of these pathways results in the secretion of chemokines and cytokines, which mediate the immunomodulatory effects of the cell surface proteins.[13,40] In line with the findings of our study, a similar investigation involving Lactobacillus spp. strains reported that cell surface proteins increased the production of the anti-inflammatory cytokine IL-10 in the host organism, while suppressing the production of the pro-inflammatory cytokine TNF-α. Furthermore, it was noted that cell surface proteins also reduced the expression of the pro-inflammatory cytokine IL-8.[64]

In this study, microbiota-derived biotics have demonstrated potential as adjunctive treatments for periodontal disease due to their ability to modulate inflammatory responses. The integration of probiotic-derived anti-inflammatory agents into various products and their eventual translation into clinical practice present a promising prospect. Although biotic-integrated products have not yet been widely implemented in clinical settings, numerous examples exist of probiotics as their natural producers, being successfully incorporated into clinical formulations. For instance, probiotic strains have been integrated into oral care products, including lozenges and mouthwashes, demonstrating their potential to reduce periodontal inflammation and support oral health.[65,66] Additionally, probiotic-based toothpaste has been shown to target local periodontal tissues during routine oral care, potentially reducing inflammation as reported by Amizic et al.[67] The short-term use of *Bifidobacterium animalis* subsp. *lactis* DN-173010 has a positive effect on plaque accumulation and gingival inflammatory parameters, even without oral hygiene measures.[68] However, challenges related to the viability and stability of probiotics in commercial formulations remain a significant concern.[34] In this regard, integrating biotics into oral healthcare products may offer a more stable and effective alternative for their translation into clinical practice. Despite our promising findings, the clinical relevance of our study is limited by its reliance on a restricted cytokine analysis and *in vitro* experimental conditions. While *in vitro* models are essential for preliminary investigations, they do not fully replicate the complexity of periodontal disease *in vivo*. Additionally, the limited cytokine panel analyzed constrains our understanding of the broader immune response involved in periodontal inflammation. Therefore, although our findings suggest the potential therapeutic effects of biotics, these effects must be validated through

preclinical studies before translation into clinical practice. To address these limitations, future research should focus on evaluating biotics in *in vivo* models that more accurately mimic the clinical environment. Conducting animal studies with a broader range of biomarkers will provide more comprehensive insights into the therapeutic potential of biotics in periodontal disease.

Conclusion

Understanding the importance of the oral microbiota in oral health has paved the way for the use of microbiota-derived biotics in the management of periodontal diseases. Although developments in this area are still emerging, current knowledge suggests that postbiotics and paraprobiotics may serve as potential candidates for controlling periodontal tissue inflammation. This study investigated the effects of EPS-b, EPS-r, cell lysates, cell surface proteins, and inactive cells derived from L. plantarum EIR/IF-1 on the inflammatory cytokine response induced by P. gingivalis infection in hPDLF cells. The results revealed that the active compounds extracted from the EIR/IF-1 strain, when applied at non-toxic doses, effectively reduced inflammation induced by P. gingivalis LPS. These findings suggest that biotics containing biologically active and effective components can be developed as a natural and reliable approach to mitigate and prevent periodontal inflammation. The integration of postbiotics, with their unique metabolite profiles, and paraprobiotics, with their excellent biological activities, into dental products holds promising potential for the treatment of periodontal diseases. In conclusion, this study highlights the potential of microbiota-derived biotics to make significant contributions to the management of periodontal diseases.

Ethical Approval

Ethical approval was not required for this study, as it does not involve human or animal subjects.

Conflict of interest

The authors have no conflict of interest to declare.

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Authorship Contributions

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