2219

INTERNATIONAL POST DOCTORAL RESEARCH FELLOWSHIP PROGRAMME

FINAL REPORT

TITLE OF THE RESEARCH: Evaluation of the Effects of *Lactobacillus plantarum* F.10 Strain Postbiotic Mediators on Periodontal Disease-Causing Pathogens with Antimicrobial, Antibiofilm and Antivirulent Approaches

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GENERAL INFORMATION

RESEARCH TITLE	Evaluation of the Effects of *Lactobacillus plantarum* F.10 Strain Postbiotic Mediators on Periodontal Disease-Causing Pathogens with Antimicrobial, Antibiofilm and Antivirulence Approaches
RESEARCHER NAME	Başar KARACA
RESEARCH FIELD	Natural Sciences/Microbiology
RESEARCH DURATION	12 months

1. INTRODUCTION

Research to develop products that can be effective in periodontal (gum) disease, which affects patients' quality of life, is gaining momentum with the development of technologies and scientific studies. However, considering literature reviews, global market analysis and expert opinions, it seems that the need for developing products with multifunctional properties and natural ingredients to combat periodontal disease continues to grow.

Periodontal diseases, whose damage is not limited to the dental tissues, also damage various systems of the body. Considering the significant impact and socioeconomic damage that periodontal diseases bring to the population, they are considered a "silent" but global epidemic. Periodontal diseases, caused mainly by pathogenic bacteria in the gingiva and teeth, are characterized by the loss of tooth supporting tissues over time. Gingivitis and periodontitis are the most common periodontal diseases in humans. Due to the accumulation of microbial biofilms that form under the gingiva, inflammation develops in the surrounding tissues, which is called gingivitis (inflammation of the gums). If the microbial biofilm formed cannot be removed from the surrounding area, the infection that develops in the gingiva can affect other tissues of the periodontium and spread to the alveolar bone (jawbone), leading to the formation of periodontitis, which is called aggressive pathology. If successful treatment cannot be carried out, early tooth loss is inevitable. Therefore, effective treatment of periodontitis is considered very important (Hajishengallis et al., 2011).

Postbiotics refer to products or by-products of metabolism secreted by living cells or soluble factors such as enzymes, peptides, teichoic acids, muropeptides, polysaccharides, cell surface proteins, and organic acids. Postbiotics, which are claimed to be as effective as living cells in terms of signalling pathways and barrier function, have clear chemical structures, reliable dosing parameters, are resistant to hydrolysis, have a long shelf life, and are antimicrobial, anti-inflammatory, immunomodulatory, antihypertensive, and have attracted much attention in recent years because they contain various signalling molecules (mediators) that may have anticarcinogenic and antibiofilm effects. Thus, postbiotics can contribute to improving host health in multiple ways by enhancing certain physiological functions (Aguilar-

Toala et al., 2018). There is an increasing trend in the literature to discover new uses for postbiotics. However, studies on the efficacy of postbiotics on periodontal microorganisms are still very limited. Moreover, current studies refer to single bacterial species when constructing a model of periodontal disease and evaluating the efficacy of postbiotics.

In this context, the fact that the postbiotics, which was the test material of the project, will also be tested on polymicrobial biofilms will allow a more integrated and realistic evaluation of periodontal diseases. In addition, similar studies evaluate only crude and complex forms of postbiotics. It was also planned to test various components of the postbiotics as part of this proposal. The proposed project involves an approach to prevent periodontal pathogens and their polymicrobial biofilms associated with periodontal disease through the use of postbiotics derived from lactic acid bacteria, which are the subject of current research. Periodontal disease is one of the most common diseases worldwide and an important public health problem. Periodontal diseases are considered as one of the new and interesting fields of application for the use of probiotics. However, there is a limited number of studies in the literature on the use of postbiotics for this purpose. In the proposed project, the effects of postbiotic mediators of the strain *Lactobacillus plantarum* F.10 (currently identified as *Lactiplantibacillus plantarum* EIR/IF-1 after taxonomic revisions) on planktonic and biofilm growth of periodontal pathogens, co-aggregation capacities, and protein and polysaccharide production in biofilm structures were determined using microbiological and biochemical methods. Later, the effects of postbiotics on cell membranes of important periodontal pathogens and some virulence properties of *Porphyromonas gingivalis* were determined by enzymatic and spectrophotometric analyzes. In addition, the possible cytotoxic activity of postbiotics of the related strain and postbiotic-derived protein and exopolysaccharide (EPS) fractions on the viability of periodontal ligament cell lines, their effects on the production of human beta-defensin 1, 2, and 3, and their effects on interleukin 1-β expression were investigated.

2. STUDIES IN REPORT TERMS

Term for 1st study

Postbiotics of the *Lactiplantibacillus plantarum* EIR/IF-1 strain show antimicrobial activity against oral microorganisms with pH adaptation capability

Theoretical background and hypothesis:

According to the International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics, postbiotics are defined as preparations of non-living microorganisms and/or their components that have a health benefit for the host (Salminen et al., 2021). In addition to inactivated non-living cells, postbiotics include metabolic by-products released from living cells as well as enzymes, peptides, teichoic acids, muropeptides, polysaccharides, and cell surface proteins released after cell lysis (Aguilar-Toalá et al., 2018). Beneficial effects of postbiotics on modulating the microbiota, strengthening epithelial barrier function, and modulating the immune response have been reported (Yan et al., 2013; Gao et al., 2019). Some secreted molecules of

postbiotics, such as organic acids and antimicrobial peptides, have direct antimicrobial effects against various pathogens (Stanojević-Nikolić et al., 2016; Meade et al.,2020). *Lactiplantibacillus plantarum*, formerly known as *Lactobacillus plantarum*, is a well-documented and extensively studied species of lactic acid bacteria. Its postbiotics consist of various useful metabolites, including organic acids, especially lactic acid. This species is also one of the preferred lactic acid bacteria species for the evaluation of the beneficial functions of postbiotics in recent years (Tsilingiri, & Rescigno, 2013; Aguilar-Toalá et al., 2018).

The symbiotic microbial community in the oral flora can deteriorate and transform into dysbiotic communities (Kreth et al., 2016). *Prevotella* is one of the most frequently identified genera in the oral microbiome (Liu et al., 2012). Comparative genomic analyzes show that there is a large gene repertoire of *Prevotella* strains in the human oral cavity that allows adaptation to different niches (Gupta et al., 2015; Ibrahim et al., 2017). *Prevotella* strains are non-spore forming, non-motile rods and most of them are saccharolytic and capable of utilizing complex carbohydrates (Shah et al., 2015). *Prevotella denticola*, *Prevotella loescheii*, and *Prevotella intermedia* accelerate biofilm formation by enabling interspecies adhesion and providing a suitable environment for late colonizers to build complex biofilms (Kolenbrander et al., 2010; Tett et al., 2021). *Fusobacterium nucleatum*, a commonly isolated bacterium of the oral cavity, promotes the coaggregation of early and late colonizers and generates an oxidoreduction potential low enough for the survival of e.g., *Porphyromonas gingivalis*, a potential periodontal pathogen (Diaz et al., 2000; Kolenbrander et al., 2002). *Streptococcus sanguinis*, another commensal member of the oral microbiota, is associated with healthy dental plaque formation (Belda-Ferre et al., 2012), but also facilitates the adhesion of some periodontal pathogens such as *P. gingivalis* and *F. nucleatum* (Bradshaw et al.,1998).

Inhibitory properties of postbiotics have been studied on periodontitis-associated pathogens. By-products of lactobacilli, recovered from cell-free supernatants of culture media reduce biofilm formation of *Aggregatibacter actinomycetemcomitans* (Ishikawa et al., 2021). Additionally, cellular response of epithelial cells against *P. gingivalis* can be normalized in the presence of metabolic by-products of *Lactobacillus rhamnosus* Lr-32 strain (Vale, & Mayer, 2021). However, it is important to bear in mind that periodontitis-associated pathogens are mostly sensitive to oxygen and pH, and require commensal oral bacteria to grow, to build biofilms, and to initiate destructive periodontal disease (Hajishengallis et al., 2011). Therefore, studies that focus only on the inhibitory effect of postbiotics on virulence of periodontal pathogens do not focus on how postbiotics may act against commensal group of bacteria, which have environmental adaptation capability. In our study, we hypothesized that oral bacteria with pH-adaptation capability resist to antimicrobial and antibiofilm effects of postbiotics. In this context, crude and neutralized forms of postbiotics obtained from the strain *L. plantarum* EIR/IF-1 were tested on individual strains of *P. denticola*, *F. nucleatum*, *S. sanguinis* and their multispecies consortia.

Material and methods:

Bacterial strains, culture media, and growth conditions

The previously isolated and identified *L. plantarum* EIR/IF-1 strain (NCBI GenBank Accession Number: MW057714.1) was available at the Pharmabiotic

Technologies Research Laboratory, Department of Biology, Faculty of Science, Ankara University. *P. denticola* ATCC 33185 (type strain), *P. denticola* AHN 32366 (clinical strain), *P. denticola* AHN 32484 (clinical strain), *F. nucleatum* ATCC 25586 (type strain), and *S. sanguinis* NCTC 10400 (type strain) were obtained from the culture collections of the Institute of Dentistry, University of Turku, and used as test microorganisms in antimicrobial screening assays.

Prior to postbiotics preparation, the *L. plantarum* EIR/IF-1 strain stored in 50% glycerol at 80 °C was first grown for 24 h at 37 °C under static conditions on De Man, Rogosa, and Sharpe agar (MRS agar, Merck, Germany). Prior to antimicrobial screening, *P. denticola* ATCC 33185, P. denticola AHN 32366, *P. denticola* AHN 32482, and *F. nucleatum* ATCC 25586 strains were grown on Brucella Blood agar medium supplemented with, 750 mg/ml cysteine, 5 mg/mL hemin and 10 mg/mL vitamin K1. *S. sanguinis* NCTC 10400 strain was grown on Brain Heart Infusion agar (BHI agar, Merck, Germany). All strains were incubated under anaerobic conditions (10% H2, 5% CO2, and 85% N2, Whitley A35 Anaerobic Workstation, Don Whitley Scientific Ltd, UK) at 37 °C for 4-5 days.

Postbiotics preparation

A characteristic colony of the previously activated *L. plantarum* EIR/IF-1 strain on MRS agar was taken and suspended in 20 mL of MRS broth (MRS broth, Merck, Germany). After an overnight incubation at 37 °C, this bacterial culture (≈108 CFU/ml) was used to inoculate 1 L MRS broth with an inoculation ratio of 2% (v/v). After another overnight incubation at 37 °C, the culture broth was centrifuged at 15,000 g for 20 min at room temperature and the supernatant (spent culture medium) was obtained. The supernatant was sterilized using membrane filters (0.22 µm pore size, Sartorius, France) (Mohammedsaeed et al., 2014). The filtered samples were then freeze-dried and powdered (freezing conditions of -20 ºC, a vacuum pressure of 0.120 mB, and a condenser temperature of -58 ºC; Christ freeze dryer, Germany) Approximately 10 g of dried powder was obtained from 1 L culture supernatant.

High performance liquid chromatography analyses

Powdered postbiotics samples were suspended in sterile distilled water to a concentration of 250 mg/mL. The stocks were stored at -20 ºC for further use and HPLC analyses. High performance liquid chromatography (HPLC) technique with LabSolution software (Shimadzu, Japan) was used to detect organic acids in postbiotics (Ankara University, Faculty of Engineering, Department of Food Engineering). HPLC analyses (Shimadzu Prominence Series- LC -20A, Japan) performed with column 87H-3 (250 mm x 4.6 mm, 5 µm; Transgenomic; USA) quantified various types of organic acids like lactic, tartaric, malic, maleic, succinic, formic, acetic, citric, and butyric acids (Merck, Germany). Organic acids were detected using a UV-Visible detector (210_{nm}). Calibration curves were prepared for normalization, calibration, and quantification of the organic acids, which were obtained from references.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) tests

MIC and MBC values for postbiotics were determined by the microdilution method according to Clinical and Laboratory Standards Institute guidelines (Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, M11, CLSI, USA). Briefly, Todd-Hewitt broth (casein peptone 10 g/L, heart infusion 3.1 g/L, sodium carbonate 2.5 g/L, dextrose 2 g/L, sodium chloride 2 g/L, disodium phosphate 0.4 g/L; supplemented with 750 mg/mL cysteine, 5 mg/mL hemin, and 10 mg/mL vitamin K1) containing various concentrations of crude postbiotics or neutralized (pH 7) postbiotics (0-50 mg/mL) were prepared, suspensions were adjusted to 0.5 McFarland standard, and transferred to the microtiter plate wells. Test groups contained postbiotics and inoculum. The wells containing postbiotics without inoculum served as negative controls, while positive controls wells contained only media and inoculum. After 48 h of incubation at 37 ºC under anaerobic conditions, cell density was measured at a wavelength of 490_{nm} in a microplate reader and the first concentration values without visible growth (MIC values) were observed.

After incubation, 100 µL of the culture suspensions were taken from each well and diluted in phosphate buffer solution (PBS, pH 7.4) to perform colony counting. Each dilution was spread on Brucella Blood Agar plates and the plates were incubated at 37 °C under anaerobic conditions for at least 4 days. Colony counts were performed and the log reduction compared to the control groups was calculated. The values corresponding to ≥99.9% log reduction were determined as MBC values.

Antimicrobial screening assay

Culture media (Todd-Hewitt broth) with different concentrations (0, 6.25, 12.5, 25, and 50 mg/mL) of crude postbiotics were prepared and the pH of the media was measured with a pH-meter. The pH values of the media containing crude postbiotics at different concentrations are shown in Table 1. To test the acidity-dependent antimicrobial activities of the postbiotics, pH-adjusted postbiotic free media were used as positive controls. The neutralized postbiotic samples were also prepared using 2 M NaOH to test acid independent antimicrobial activity.

Table 1. pH values of Todd-Hewitt media containing different concentrations of postbiotics

Postbiotics concentrations	Corresponding pH value
6.25 mg/mL	6.01-6.10
12.5 mg/mL	4.99-5.01
25 mg/mL	4.70-4.72
50 mg/mL	4.40-4.42

Colonies from 4-day bacterial pure cultures were harvested and suspended in PBS (pH 7.4). The optical density (OD) of the suspensions was adjusted to 2.0 at 490_{nm}. Colony counting was performed to calculate the CFU/ml values of suspensions and each bacterial suspension was confirmed at ≈10^9 CFU/mL. A 25 µl of these suspensions were inoculated into Todd-Hewitt broth media containing various concentrations of crude and neutralized postbiotics as well as acidified Todd-Hewitt broth media. After inoculation, the culture suspensions were incubated at 37 °C for 48 h under anaerobic conditions. During incubation, 300 µL samples were

taken from each suspension at 6, 24, and 48 time-points and transferred to microcentrifuge tubes. A 100 µL-sample was subjected to pH measurement and 100 µL-sample was used for colony counting. The last 100 µL sample was used for growth recovery. In this context, the collected samples were inoculated in 5 mL of fresh Todd-Hewitt broth and the samples were incubated for additional 4 days. In this way, it was checked whether the growth recovered at the concentrations at which the antimicrobial activity was observed.

Antibiofilm screening assay

To determine the antibiofilm effects of crude postbiotics, polymicrobial biofilm models of *P. denticola*, *F. nucleatum*, and *S. sanguinis* were designed based on their interactions in oral biofilms (Okuda et al., 2012; He et al., 2012).

P. denticola ATCC 33185 + *F. nucleatum* ATCC 25586 + *S. sanguinis* NCTC 10904
P. denticola AHN 32366 + *F. nucleatum* ATCC 25586 + *S. sanguinis* NCTC 10904
P. denticola AHN 32482 + *F. nucleatum* ATCC 25586 + *S. sanguinis* NCTC 10904

The bacterial strains were cultured as described previously. Suspensions of the cultures were prepared by adjusting each of them OD: 2.0 at a wavelength of 490_{nm} (≈10^9 CFU/mL). An equal volume of each bacterial suspension was mixed to prepare different combinations as stated above. Pasteurized human saliva was used to coat the wells of the polystyrene plates as described before (Fteita et al., 2014). A 135 µL of Todd-Hewitt broth adjusted with various concentrations of crude postbiotics was added to the saliva-coated wells (3.12, 6.25, 12.5, and 25 mg/mL postbiotics concentrations). Mixed bacterial suspensions (15 µL) were inoculated to the wells and then plates were incubated at 37 °C for 48 h under anaerobic conditions. The wells containing only inoculum served as positive control groups, while the wells containing only different concentrations of postbiotics without inoculum served as negative groups. After incubation, biofilm determination was conducted with a crystal violet binding assay (OmerOglou et al., 2022).

Statistical analysis

Each experiment was performed in triplicate at least at two independent time points. Data are presented as mean values and standard deviations. Multiple comparisons were performed with the One-Way ANOVA test and the Tukey test was used in post-hoc comparisons. A *p* value of <0.05 was accepted as statistically significant.

Term for 2nd study

Analysis of Chemical Structure and Antibiofilm Properties of Exopolysaccharides from *Lactiplantibacillus plantarum* EIR/IF−1 Postbiotics

Theoretical background and hypothesis:

The interaction of a variety of microorganisms in the oral cavity under the influence of environmental changes provides an extremely dynamic ecosystem in the

mouth. The presence of commensal microorganisms in a healthy mouth suppresses the growth of pathogenic microorganisms. However, this complexity and diversity of the oral microbiota can change in favour of pathogens in oral diseases such as caries and periodontitis (Willis and Gabaldón, 2020). The basis of treatment for these diseases is the removal of bacterial plaques and, if necessary, the use of antibiotics (Haque et al., 2019). Of course, in addition to these approaches, other approaches that can be adjuvant and preventive, such as the use of probiotics, may also be on the agenda. Although probiotics have the potential to be used in intestinal diseases because they compete with pathogenic bacteria, produce some antimicrobial substances and promote the host immune response, there is not yet satisfactory evidence that similar beneficial properties can be achieved in the oral flora (Seminario-Amez et al., 2017). In addition, the use of probiotics for oral health may pose risks for immunocompromised individuals, as probiotics are living cells and the pH reduction may cause demineralisation of enamel due to the ability of probiotic bacteria to ferment various sugars (Laleman et al., 2015; Moraes et al., 2022).

Postbiotics, also known as metabiotics or biogenics, are defined as metabolic by-products released by living bacteria directly into the extracellular environment or through bacterial lysis, which may have beneficial properties for the host (Aguilar-Toalá et al., 2018). Postbiotics consist of metabolites of live probiotic bacteria such as organic acids, released exopolysaccharides, short-chain fatty acids, amino acids, flavonoids, terpenoids, phenolic compounds, and inactivated or dead cells or their lysed parts such as cell surface proteins, peptidoglycan, cell-bound polysaccharides, and teichoic acids (Shenderov et al., 2013; Nataraj et al., 2020). The use of postbiotics derived from probiotics as cellular components or metabolites has several advantages, including better stability than live microorganisms and concentration-dependent precise formulation (Żółkiewicz et al., 2020; Salminen et al., 2021).

Lactic acid bacteria (LAB) are the subject of many areas of research because they have various biological properties that may be beneficial, and in particular because they contain probiotic species. Although LAB species are generally recognised as safe (GRAS), they can be used in various fields due to metabolites such as antimicrobial peptides, bacteriocins, aromatic compounds, organic acids, fatty acids, and exopolysaccharides (EPS) that these bacteria can produce (Oleksy and Klewicka, 2018). All of these metabolites, including EPS, also fall into the realm of postbiotics.

LAB-derived EPSs are of interest for industrial use in food, pharmaceuticals, and nutraceuticals due to their potential health-promoting effects and rheological properties (Abdalla et al., 2021). Many LAB species, particularly those in the genus Lactobacillus, are capable of producing EPS, and some of the EPSs identified to date by LAB have been shown to have biological functions, such as free radical scavenging, free cholesterol binding, modulation of the gut microbiota, antitumour, antimicrobial, and antibiofilm properties (Nataraj et al., 2020; Abdalla et al., 2021). LAB-derived EPSs have the ability to inhibit biofilm formation of pathogenic bacteria. The EPSs of LAB may exhibit antibiofilm activity by modifying the cell surfaces of biofilm-producing bacteria, preventing initial adhesion to surfaces, or by acting as signalling molecules and downregulating the expression of genes responsible for biofilm formation (Wang et al., 2015).

In recent years, there have been studies on the use of postbiotics for oral diseases, especially periodontitis. Most of these studies focus on the antimicrobial and antibiofilm effects of postbiotics on oral pathogens. For example, the fermentative culture broths of *Lactobacillus fermentum* and *Lactobacillus salivarius* can significantly suppress the growth of *Streptococcus sanguinis*, *Streptococcus mutans*, and *Porphromonas gingivalis* (Chen et al., 2012). Shin et al. (2018) showed that the spent culture medium of strain *Lactococcus lactis* HY449 exhibited strong bactericidal activity against strains *Fusobacterium nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, and *Treponema denticola* ATCC 35405. Biofilm formation and the production of cytolethal distending toxin and leukotoxins by *Aggregatibacter actinomycetemcomitans*, which often accompany rapidly progressing periodontitis, can be prevented by cell-free pH-neutralised supernatants (CFS) of the strains *Lactobacillus rhamnosus* Lr32, *L. rhamnosus* HN001, *Lactobacillus acidophilus* LA5, and *L. acidophilus* NCFM (Ishikawa et al., 2021). Cell-free culture of supernatant *Lactobacillus reuteri* AN417 reduced growth, intracellular ATP levels, biofilm integrity, and expression of biofilm-associated genes of *P. gingivalis*, *F. nucleatum*, and *S. mutans* (Yang et al., 2021). Yang et al. (2021) have also suggested that the antibiofilm action of the cell-free culture supernatant of *L. reuteri* AN417 is related to fatty acids and sugars. In summary, postbiotics have direct antimicrobial effects and can prevent biofilm formation or reduce the expression of various virulence factors of periodontal pathogens.

P. gingivalis, as a Gram-negative oral anaerobe, is an important aetiological agent associated with the progression of periodontitis (Bodet et al., 2006). *F. nucleatum* can be found in the mouths of humans as a natural component of the oral flora. However, they are often isolated from infected sites, as in periodontitis. *F. nucleatum* plays an essential and supporting role in biofilms that contribute to periodontal disease (Kolenbrander et al., 2010). The genus *Prevotella*, including *Prevotella denticola*, acts on the early and middle steps of oral biofilm formation with cellular adhesion for later colonisers and in particular supports the biofilm formation of *A. actinomycetemcomitans* (Hajishengallis et al., 2012; Tett et al., 2021). *Filifactor alocis*, a fastidious Gram-positive, has been recently introduced as a diagnostic indicator of periodontal disease. Due to its ability to interact with various oral bacteria and show virulence, *F. alocis* has become an important species for periodontal disease progression (Aja et al., 2021).

Oral biofilms consist of many intermicrobial interactions and reflect a complex ecosystem in the oral cavity. In this study, we thought of demonstrating an approach with dual biofilms of the previously highlighted bacteria accompanied by *F. nucleatum*, which may be partially suitable for the multispecies biofilm concept. We also tested the effects of postbiotics of the strain *Lactiplantibacillus plantarum* EIR/IF-1 (formerly *Lactobacillus plantarum*) on the biofilm formation of oral bacteria, taking into account the new concept of postbiotics. *L. plantarum* can have positive effects on humans as an ideal and trendy probiotic (Fidanza et al., 2021). As we explained in our previous study, the antibiofilm activity against *S. mutans* (Omeroglou et al., 2022) and the antibacterial activity on some oral bacteria (unpublished data) of the postbiotics of this strain led to its use as material in the current study. In addition, we tested the postbiotics of this strain directly on oral biofilms and separately evaluated the antibiofilm effects of EPS in the postbiotics. Thus, in contrast to the evaluation of the effect of complex postbiotics on periodontal pathogens in the literature, we

evaluated the antibiofilm properties of a single postbiotic component defined by the purification and characterization of EPS.

Material and methods:

Culture preparation of the L. plantarum EIR/IF-1 strain for obtaining postbiotics

The *L. plantarum* EIR/IF-1 strain (NCBI GenBank Accession Number: MW057714.1) was kindly provided by the Pharmabiotic Technologies Research Laboratory, Department of Biology, Faculty of Science, Ankara University. The strain *L. plantarum* EIR /IF-1 stored at 80 °C in 50% glycerol was cultured for 24 h at 37 °C on De Man, Rogosa, and Sharpe Agar (MRS) (Merck, Germany) under static conditions prior to preparation of the postbiotics. A typical colony of the strain grown on the agar plate was taken with a sterile loop and transferred to 20 mL of MRS broth (Merck, Germany). This suspension was incubated at 37 °C for 18 h. Subsequently, this activated culture (≈10^8 CFU/mL) was used to inoculate 1 L of MRS broth with an inoculation ratio of 2% (v/v). After a final incubation for 18 h at 37 °C (late log phase), the entire culture was centrifuged at 15,000 g for 20 min at room temperature. The culture supernatant (spent culture medium) and pellet were separated and the supernatant was filtered through a membrane with a pore size of 0.22 µm (Sartorius, France) (Mohammedsaeed et al., 2014). The filtered samples were then freeze-dried and powdered (freezing conditions of -20 ºC, a vacuum pressure of 0.120 mB and a condenser temperature of -58 ºC; Christ freeze dryer, Germany). One liter of culture supernatant yielded about 10 grams of powder. The powdered samples were suspended in both sterile dH_2O and Todd-Hewitt Broth at a final concentration of 250 mg/mL. The suspensions were kept at -20 °C for future research.

Extraction of exopolysaccharide fraction from postbiotics of L. plantarum EIR/IF-1 strain

A volume of 200 mL of previously obtained postbiotics (spent culture medium) was used to extract released exopolysaccharides (EPS). Trichloroacetic acid (TCA; Merck, Germany) was added at a final concentration of 20% to filtered spent culture medium (chemical deproteinization). The treated supernatants were incubated at 4 ºC for 2 h. The samples were centrifuged at 4 °C for 20 min after incubation. After centrifugation, a double volume of 95% ethanol (Merck, Germany) was added to supernatant obtained, and the supernatants were incubated overnight at 4 ºC. The samples were then centrifuged at 6000 rpm for 30 min at 4 ºC. The pellets were subjected to a freeze-drying procedure as described previously. Finally, the amount of EPS was determined and dissolved in 2 mL of dH_2O (Tallon et al., 2003). Furthermore, the protein content of the EPS sample was determined using the Bradford assay using bovine serum albumin as a reference according to the manufacturer's recommendations (Bio-Rad Laboratories Inc.).

Determination of total carbohydrates in postbiotics and EPS fraction

The phenol-sulphuric acid method was used to determine EPS concentrations (Dubois et al. 1956). 500 µL of phenol and 5 mL of sulphuric acid were added to 500 µL of EPS suspension and incubated for 10 min at room temperature. After incubation, the samples were mixed well and incubated at 30 ºC for 20 min. The

optical density of the samples was measured using a microplate reader set to 490 nm. The concentration values were calculated based on the results of glucose standards prepared at different concentrations.

Characterization of EPS fraction

Monosaccharide composition analysis

Extracted EPS was hydrolysed with 1 M sulphuric acid at 100 °C for 2 h and then neutralized with 1 N NaOH. The monosaccharide composition was analysed by HPLC (Shimadzu, Prominence 20A Series) using a Shodex Sugar SP0810 250 × 4.6 mm column with a refractive index detector. Deionised water was used as the mobile phase with a flow rate of 0.6 mL/min for 20 min and an injection volume of 20 µL. The temperature of the column oven was set to 80°C. Quantitative measurement was performed by establishing a linear calibration curve using the derivatives of standard sugars (fructose, glucose, sucrose, galactose, xylose, arabinose and mannose). All unknown samples were diluted with deionised water and filtered with Nylon type filter (Merck, Germany). The sugar composition was detected on HPLC-RID and measured against the calibration curve of the free sugar standard mixture.

Fourier Transform Infrared Spectroscopy Analysis

The functional chemical groups of the extracted EPS were analysed by Fourier transform infrared spectroscopy (FT-IR) using the attenuated total reflectance (ATR) method (IR Spirit Infrared Spectrometer, Shimadzu, Japan). The spectra were recorded between 4000 and 600 cm^{-1}, with a spectral resolution of 2 cm^{-1}. The absorption of the background and the samples was measured with 100 scans.

Determination of the molecular weight

The molecular weight of crude EPS was determined by gel permeation/size exclusion chromatography (Shimadzu-Prominence 20A series) using a PSS SUPREMA 5 µm GPC column 250 × 10mm with a refractive index detector. The crude EPS sample and all dextran standard samples are weighed to 10 mg and dissolved in 3 mL of distilled water. Dextran standards ranging from 20,000 to 1,000,000 Da were used for the parabolic standard calibration curve in the GPC software. Deionised water with a flow rate of 1 mL/min for 30 min and an injection volume of 20 µL was used as the mobile phase.

Thermogravimetric analysis (TGA) of the EPS fraction

Thermogravimetric analysis of the EPS fraction was carried out with the Shimadzu TGA 50 and Diamond TGA instruments using 10 mg of the EPS fraction. The TGA curve represents the temperature of the reference material on the -axis against the TGA signal, which is converted to a percentage change in weight on the axis. The EPS was placed in a platinum crucible and heated at a linear rate of 20 °C/min over a temperature range of 40 °C to 720 °C for 34 min under nitrogen, and the corresponding weight loss was determined.

Test bacteria and culture conditions

P. *denticola* ATCC 33185 (type strain), *P*. *denticola* AHN 32366 (clinical strain), *P*. *gingivalis* ATCC 33277 (type strain), *P*. *gingivalis* AHN 24155 (clinical strain), *F. nucleatum* ATCC 25586 (type strain), and *F*. *alocis* ATCC 35896 (type strain) were provided by the culture collections of the Institute of Dentistry, University of Turku, and preferred as test bacteria in all experiments.

All strains stored at -70 ºC in milk were cultured on Brucella Blood Agar medium supplemented with 750 mg/mL cysteine, 5 mg/mL hemin, and 10 mg/mL vitamin K1. All strains were incubated under anaerobic conditions (10% H_2, 5% CO_2, and 85% N_2, Whitley A35 Anaerobic Workstation, Don Whitley Scientific Ltd., UK) at 37 °C for 5 days prior to experiments.

Antibiofilm activity of postbiotics and EPS fraction

Considering interspecies interactions in oral biofilms, dual biofilm models were developed with *F*. *nucleatum* ATCC 25586 to determine the antibiofilm effect of crude postbiotics (He et al., 2012; Okuda et al., 2012).

P. denticola ATCC 33185 + *F. nucleatum* ATCC 25586
P. denticola AHN 32366 + *F. nucleatum* ATCC 25586
P. gingivalis ATCC 33277 + *F. nucleatum* ATCC 25586
P. gingivalis AHN 24155 + *F. nucleatum* ATCC 25586
F. *nucleatum* ATCC 35896 + *F. nucleatum* ATCC 25586

Activated cultures on Brucella Blood Agar were used for all biofilm tests. Typical colonies of each strain were harvested from the plates and suspended in sterile physiological serum (0.9% NaCl) to set an optical density value (OD) of 2.0 for the strains of *P*. *denticola*, *F*. *nucleatum*, and *F*. *alocis* and a value of 2.5 OD for the strains of *P*. *gingivalis* (≈10^9 CFU/mL for each strain) at a wavelength of 490 nm (Shimadzu UV–visible, BioSpec-mini, Kyoto, Japan). In order to prepare different combinations as described above, equal volumes (7.5 µL) of each bacterial suspension were mixed (15 µL in total).

Antibiofilm experiments were conducted using three different approaches: co-treatment, pre-treatment, and post-treatment.

co-treatment assay

96-well polystyrene microtiter plates were used for all biofilm experiments. The plates were coated with pasteurized human saliva according to the method of Fteita et al. (Fteita et al., 2014). In brief, 10 mL of previously collected saliva samples from 4 healthy individuals were pooled and centrifuged at 10,000 rpm for 40 min at 4 °C. The saliva pellet was removed from the supernatant and the supernatant was pasteurized at 60 °C for 30 min. The pasteurized sample was then centrifuged again and clarified. The final sample was streaked onto Brucella Blood Agar plates, and the plates were incubated under both aerobic and anaerobic conditions to verify the sterility of the saliva samples. Each well of the plates was filled with 100 µL of pasteurized saliva, and the plates were then incubated at 37 °C. The plates were then emptied.

Each saliva-coated well was filled with 135 L of Todd-Hewitt Broth (casein peptone 10 g/L, heart infusion 3.1 g/L, sodium carbonate 2.5 g/L, dextrose 2 g/L, sodium chloride 2 g/L, and disodium phosphate 0.4 g/L; supplemented with 750 mg/mL cysteine, 5 mg/mL hemin, and 10 mg/mL vitamin K1) adjusted with different concentrations of crude postbiotics (3.12, 6.25, 12.5, and 25 mg/mL) or EPS fraction (25, 50, 125, 250, and 500 µg/mL). Mixed bacterial suspensions (15 µL) were transferred to each well and then the plates were incubated at 37 °C for 48 h under anaerobic conditions. Wells containing inoculum and medium were considered positive controls, while wells containing only medium with various concentrations of postbiotics or EPS fraction were considered negative controls. After incubation, biofilm measurements were performed using the crystal violet binding assay (Omeroglou et al., 2022).

pre-treatment assay

At this stage, the culture preparations and coating of the plates with saliva were first carried out as previously described. After coating with saliva, the plate wells were emptied and 150 µL of Todd-Hewitt Broth medium containing crude postbiotics (3.12, 6.25, 12.5, and 25 mg/mL) or EPS fraction (25, 50, 125, 250, and 500 µg/mL) was added to each test well. Only the medium was added to the wells of both positive and negative controls. The plates were incubated at 37 °C for 24 h. At the end of incubation, the plates were emptied and 135 µL Todd-Hewitt Broth medium was added to the wells. With the exception of the negative control wells, 15 µL of the mixed suspensions were added to all other wells. The plates were incubated as previously described and subjected to the crystal violet binding assay.

post-treatment assay

First, the plates were coated with saliva, and 135 µL of Todd-Hewitt broth was added to each well and inoculated with 15 µL of the prepared mixed culture suspensions (except for the negative control wells, which received only medium). The plates were incubated for 48 h under the previously established incubation conditions. At the end of incubation, the wells of the plates were emptied and carefully washed twice with PBS solution (pH 7.2). The test wells were filled with 150 µL of Todd-Hewitt Broth containing different concentrations of postbiotics (3.12, 6.25, 12.5, and 25 mg/mL) or EPS fraction (25, 50, 125, 250, and 500 µg/mL). Both positive and negative control wells were filled with media only. The plates were incubated for 24 h as previously described and subjected to the crystal violet binding assay.

Testing postbiotics and EPS fraction on the auto-aggregation and co-aggregation ability of test strains

The crude postbiotics and EPS fraction were used to test their anti-aggregation abilities. The aggregation tests were performed to understand the potential antibiofilm mode of action of postbiotics and EPS. Aggregation assays were performed according to the Kolenbrander's method with modifications (Kolenbrander, 1995). Test strains were cultured on Brucella Blood Agar plates under the conditions as described previously. A few colonies were taken from each activated culture with sterile loops and transferred to 10 mL of Todd-Hewitt Broth. The culture suspensions were incubated overnight under the previously described anaerobic conditions. The

overnight cultures of the strains were centrifuged at 12,000 g for 10 min. After removal of the supernatant, the cells were washed once with phosphate-buffered saline (PBS, pH 7.2). Another centrifugation at 12,000 g for 10 min took place, and after removing the supernatant, the cells were resuspended in the co-aggregation buffer (Tris HCl, pH 8.0, containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 150 mM NaCl, pH 8.0). The suspensions of *P. denticola*, *F. nucleatum*, and *F. alocis* were optically adjusted to 0.7 OD at 660 nm (Shimadzu UV–visible, BioSpec-mini, Kyoto, Japan). These cultures corresponded to approximately 10^8 CFU/mL (validated by colony count on Brucella Blood Agar). The remaining *P. gingivalis* strains were also adjusted to ≈10^8 CFU/mL with an optical density of 1.0 at 660 nm.

For the auto-aggregation experiments, 1-mL culture suspensions of the individual strains without crude postbiotics and EPS fraction were prepared as control groups, and 1-mL culture suspensions with different concentrations of crude postbiotics (0.78, 1.56, and 3.12 mg/mL) and EPS fraction (100, 250, and 500 µg/mL) were prepared as test groups. The preferred concentrations of postbiotics and EPS fraction had no antibacterial effect (checked before aggregation assay). For the co-aggregation experiments, *F. nuclaetum* ATCC 25586 was preferred because of its high aggregation capacity. 500 µL of each suspension was mixed with 500 µL of *F. nucleatum* ATCC 25586 suspension, and crude postbiotics or EPS fraction were added. The final concentrations of the suspensions were adjusted for both crude postbiotics and EPS fractions as indicated above. All suspensions were shaken vigorously with a vortexer for 2 min before optical measurement at 660 nm and measured immediately (t_0). The measurements of the upper phases of suspensions were taken at different time intervals (t_1: 1 h, t_2: 2 h, t_3: 3 h, t_{24}: 24 h). During the time intervals, the test cuvettes were kept covered with paraffin strips at 37 °C. Optical measurements were recorded.

Adhesion to hydrocarbons

The adhesion of hydrocarbons to test strains was evaluated to determine cell surface hydrophobicity, and this experiment was also preferred to understand the potential antibiofilm mode of action of crude postbiotics and EPS fraction. Adhesion to hydrocarbons was carried out using the modified version of the Rosenberg's method (Rosenberg, 1984). Liquid hydrocarbons such as toluene and xylene were used to assess the degree of adherence of bacterial cells under the influence of postbiotics and EPS.

The bacterial suspensions were prepared according to the steps described in the previous section, except that PBS solution (pH 7.2) was used to prepare the final culture suspensions. Different concentrations of crude postbiotics (0.78 and 1.56 mg/mL) and EPS fraction (250 and 500 µg/mL) were added to 1.2 mL of each bacterial suspension. Suspensions without postbiotics and EPS fraction served as control groups. These suspensions were shaken for 2 min at maximum intensity with a vortex mixer. Before the addition of hydrocarbons, these suspensions were measured with a spectrophotometer at a wavelength of 660_{nm} (t_0). These suspensions were transferred to 10 mL glass tubes and 0.5 mL of toluene or xylene was added to each tube. The tubes were shaken under the same conditions, and the tubes were left at room temperature for 30 min. Finally, 1 mL of each suspension was taken from the lower phase and transferred to plastic cuvettes. Optical measurements were performed and the results were recorded again. The formula for

calculating the percent of cell surface hydrophobicity was 100 x [(initial OD-final OD)/(initial OD)].

Statistical analysis

Each experiment was performed in triplicate. The mean and standard deviation of the data were presented. One-Way ANOVA tests were used for the comparisons between the groups, and Tukey's test was used for the post hoc analyses. A *p* value of < 0.05 was accepted as statistically significant. Prism GraphPad 8.0.1 (Graph Software Inc.) was used for the statistical analysis and the creation of the figures.

Term for 3rd study

Determination of The Immunostimulatory Properties of Protein and Exopolysaccharide Fractions Obtained from Postbiotics on Gingival Keratinocyte Cell Lines

Theoretical background and hypothesis:

P. gingivalis is the most important pathogenic bacterium in the development of periodontal disease. With the development of plaque in the oral cavity, the oral mucosa can also be colonized by pathogenic bacteria. *P. gingivalis* is an important bacterium in oral diseases due to its production of fimbriae, its high LPS content, and its ability to produce a large number of proteases, especially proteases. *P. gingivalis* achieves cellular colonization and facilitates its spread by manipulating the host immune response. At the onset of infection, this bacterium suppresses cytokine and chemokine responses. The balance of neutrophil flux is critical to the balance between the host and its microbiome. *P. gingivalis* degrades IL-8, particularly with the gingipain proteases it secretes, disrupting the balance and facilitating its invasion of host tissues. *P. gingivalis* not only paralyzes the immune response, but also targets epithelial, fibroblast, periodontal ligament and bone cells of the gums, causing a persistent inflammatory response in these areas (How et al., 2016).

Beta-defensins are a family of proteins in vertebrates that play a role in epithelial cell resistance to microbial colonization (Yilmaz et al., 2020). In this study, the effect of protein and EPS fractions from *L. plantarum* EIR/IF-1 postbiotics on the expression of beta-defensins, which are the primary line of defence against pathogen colonization in oral epithelial and keratinocyte cells, was investigated. In this context, the changes in the expression of beta-defensin 1 (HBD-1), beta-defensin 2 (HBD-2) and beta-defensin (HBD-3) were observed in gingival fibroblast cells treated with different concentrations of postbiotic ingredients (protein and EPS).

Interleukin 1-β is considered the inflammatory cytokine that plays an important role in inflammation and further resorption of bone. Therefore, interleukin 1-β is considered a very important marker in periodontal pathology. Interleukin 1-β stimulates bone resorption and further accelerates bone resorption (Stashenko et al., 1991). Also in this study, the changes of 1-β response in gingival fibroblast cell lines were followed under the influence of different concentrations of postbiotic proteins and exopolysaccharides.

Material and methods:

***Protein and exopolysaccharides extraction from L. plantarum* EIR/IF-1 postbiotics**

Previously extracted EPS was used in this study.

200 mL of previously obtained postbiotics (spent culture medium) were used to extract large volume of proteins. Ammonium sulphate precipitation was carried out and an 80% saturation of ammonium sulphate was slowly added to the culture supernatant with gentle stirring for 16 h at 4 ºC. The suspension was buffered with 1 M Tris- HCL solution to prevent acidification. After this step, the suspension was transferred to 50 mL tubes and centrifuged at 25,000 x g for 20 min at 4 ºC. The supernatant was removed and loose protein pellets were collected. And resuspended the pellet in PBS solution (pH 7.2). Desalting procedure was performed with Amicon® Ultra-4 Centrifugal Filter Unit (#UFC8003, Merck Millipore) according to the manufacturer's recommendation. Protein concentration final sample was determined by the Bradford method (Bio-Rad Laboratories Inc.).

Cell line

Human gingival keratinocytes (HMK cell line), originally obtained from a healthy human gingival biopsy, were cultured in serum-free keratinocyte medium containing human recombinant epidermal growth factor, bovine pituitary extract (17.005.075, Gibco, Paisley, Scotland) and antibiotics (100 IU/mL penicillin and 100 µg/ml streptomycin) (15,140-122, Gibco, Bethesda, Maryland, USA) at 37°C and 5% CO_2. Culture media were changed three times per week and cells were passaged weekly until they reached 80-90% confluence.

Cell line media were suspended with different concentrations of postbiotics, and postbiotics protein or exopolysaccharides.

Culture media were prepared (6 mL for each group) in 15 mL polystyrene tubes as described below.

1) HMK media only
2) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS
3) Media with 0.5 mg/mL postbiotics
4) Media with 2.5 mg/mL postbiotics
5) Media with 0.05 mg/mL postbiotics exopolysaccharide
6) Media with 0.25 mg/mL postbiotics exopolysaccharide
7) Media with 0.1 mg/mL postbiotics protein
8) Media with 0.5 mg/mL postbiotics protein
9) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 0.5 mg/mL postbiotics
10) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 2.5 mg/mL postbiotic
11) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 0.05 mg/mL postbiotics exopolysaccharide
12) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 0.25 mg/mL postbiotic exopolysaccharide

13) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 0.1 mg/mL postbiotic protein
14) Media with 1 µg/mL *Porphyromonas gingivalis*-LPS and with 0.5 mg/mL postbiotic protein

$3x10^5$ HMK cells were seed in tissue culture plate wells (14 groups in triplicate) and the plates were incubated overnight. Media were poured and the wells were washed. The wells were again filled with 2 mL of culture media containing different concentrations of postbiotics, protein and exopolysaccharides. After overnight incubation, media were collected and placed to -70 °C for further ELISA assay.

ELISA assay for human beta defensins and interleukin 1-β

ELISA assays were run according to the manufacturer's recommendations.

Human BD-1 Mini ABTS ELISA development kit (Catalog#900-M202).

Human BD-2 Mini ABTS ELISA development kit (Catalog#900-M172).

Human BD-3 Mini ABTS ELISA development kit (Catalog#900-M210).

Bio-Plex Pro Human Bio-Rad Cytokine IL-1β Set (Catalog#171B5001M).

Term for 4th study

Effects of Postbiotics and Postbiotic Proteins on Cell Membranes of *P. gingivalis* and *F. nucleatum* and Some Virulence Properties of *P. gingivalis*

Theoretical background and hypothesis:

The discovery of new antimicrobial agents with different chemical structures and mechanisms of action is urgently needed in view of the increase in new and re-emerging infectious diseases. In addition, the occurrence of undesirable side effects of certain antibiotics and the increasing resistance to antibiotics currently in clinical use are also of concern. Bacterial cell membranes are a potential target for the development of new antibacterial drugs because membrane-based efflux pump systems play an important role in bacterial pathogenicity and antimicrobial resistance (Chitemerere et al., 2014).

It is thought that gingipains, which are cysteine proteases, are important virulence factors of *P. gingivalis* that mediate the interaction between the bacteria and their hosts. Rgp and Kgp are two types of gingipains: arginine-dependent and lysine-dependent. *P. gingivalis* gingipains can accomplish 85% of the proteolysis outside of the cell when both types of gingipains are combined (de Diego et al., 2014). In order for *P. gingivalis* to obtain proteinaceous nutrition from the mixed-species environment of the subgingival sulcus, exopeptidases, such as dipeptidyl peptidases (DPPs), tripeptidyl peptidases (TPPs), and acyl peptidyl oligopeptidases (AOP), are thought to be important (Nemoto et al., 2021).

Material and methods:

Membrane integrity assay

This assay is based on the ability of bacteria to take up N-phenyl-1-naphthylamine (NPN) and measure the corresponding changes in the bacterial membrane (Loh and Hanccock, 1984; Ellison and Champlin, 2007). After cultures of *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, and *F. nucleatum* ATCC 25586 were prepared, the stock NPN solution prepared in pure ethanol was diluted in 5 mM HEPES buffer to a final concentration of 20 mM. 100 µL each of the culture suspension and the intermediate NPN solution were added to the wells of the microtiter plate. The final components were modified with different concentrations of postbiotics and proteins as mentioned earlier. Colony counts were also performed from the remaining initial suspensions that were not included in the assay. Plates were read immediately using a fluorometer (BIOTEK Synergy HT, BioTek, Highland Park, VT, US) at 360_{nm} excitation and 460_{nm} emission.

Effect of postbiotics and postbiotic proteins on dipeptidyl peptidase IV (DPPIV) enzyme activity of *P. gingivalis*

In the related study, the effects of a postbiotic mediator on the activity of the DPPIV enzyme of *P. gingivalis* were investigated. Biofilm samples of *P. gingivalis* ATCC 33277 and *P. gingivalis* AHN 24155 were collected on nitrocellulose membranes prior to determination of enzyme activity. The prepared bacterial cultures were adjusted to an optical density of 0.7 at $OD490_{nm}$ and resuspended in Todd-Hewitt broth containing various concentrations of postbiotics and protein. 8 µL of the prepared suspensions were withdrawn and transferred separately to sterile nitrocellulose membranes (6 mm). Membranes were transferred to Brucella blood agar surfaces and incubated at 37°C for 24 h under anaerobic conditions (Merritt et al., 2005; Gursoy et al., 2012).

After biofilm sampling, the determination of enzyme activity was started. The membranes with the biofilm samples on them were transferred to the wells of the microtiter plate. To dissolve the enzymes in the biofilm samples, 50 µL of Tris-HCl (pH 8.0) buffer containing 0.05% Triton X-100 was added to the wells. Immediately after this treatment, 1 mM H-Ala-Pro-7-amido-4-trifluoromethylcoumarin (H-Ala-Pro-AFC; Bachem, Bubendorf, Switzerland) prepared in 100 mM Tris-HCl (pH 8.0) buffer was added to the wells. 50 µL of each fluorogenic substrate was transferred. After starting the reaction, the contents of the wells were immediately read on the fluorometer at intervals of 0-20 minutes (1 measurement/minute). The last measurement was taken after 1 h to confirm the end of the reaction. The measurement of the amido-4-trifluoromethylcoumarin product was performed at 380 nm. During the measurement, the plate was incubated at 37 °C. Possible reductions in enzyme activity in the test groups compared to the control groups were calculated.

Effects of postbiotics and postbiotic proteins on gingipain activity of P. gingivalis

Culture preparation of *P. gingivalis* ATCC 33277 and *P. gingivalis* AHN 24155 was performed as previously described. The culture suspensions were modified with different concentrations of postbiotics and proteins. Culture suspensions without active substances were created as a control group. After these procedures, an additional 18-h incubation was performed under ideal conditions for the strains.

After the cultures were centrifuged at the end of the incubation, the supernatants were collected for determination of enzyme activities. FITC-Ahx-(L) Arg-(L)Arg-KDbc), a fluorogenic substrate reactive with gingipain, was used to determine gingipain activity (Galassi et al., 2012; Kaman et al., 2012; Özdemir et al., 2020).

Term for 5th study

This review is a comprehensive study of the possible role of postbiotics in Nrf2 activation. Nuclear Factor Erythroid-Related Factor 2 is a transcription factor that occurs in humans and is responsible for the expression of proteins involved in the alleviation of problems caused by oxidative damage. The pleotropic role of Nrf2 is effective not only in regulating the oxidative stress response, but also in many cellular processes such as the inflammatory response, autophagy, mitochondrial physiology, and immune response.

In this review, due to the lack of studies on the effects of postbiotics on the regulation of Nrf2 response and the possible effects of various functional components based on the rich content of postbiotics, the possible mechanisms of action of all types of lactic acid bacteria-derived components that can be included in the definition of postbiotics were discussed and a study on their use in periodontal diseases was conducted. future perspective was discussed.

3. RESEARCH RESULTS

Results for 1st study

Postbiotics of the *Lactiplantibacillus plantarum* EIR/IF-1 strain show antimicrobial activity against oral microorganisms with pH adaptation capability

Organic acid composition of postbiotics

Of organic acids in the composition of *L. plantarum* EIR/IF-1 postbiotics, lactic acid was interpreted as the highest concentration (35.82 mg/mL), and the concentrations of formic and maleic acids were also relatively high (3.55 and 2.47 mg/mL, respectively). Malic acid, succinic acid, butyric acid, acetic acid, and tartaric acid were also detected with the concentrations of 0.18, 0.20, 0.22, 0.98, and 1.62 mg/mL, respectively.

MIC and MBC tests

The MIC and MBC values of all test strains are presented in Table 2. The MIC and MBC values of the crude postbiotics for the clinical strains of *P. denticola* and the type strain of *S. sanguinis* were 12.5 mg/mL and 25 mg/mL, respectively, and for the type strains of *F. nucleatum* and *P. denticola*, both MIC and MBC values were 12.5 mg/mL. At the tested concentrations of the neutralized postbiotics, no MIC and MBC values were determined for both clinical strains of *P. denticola* AHN 33266 and 32482 and the type strain of *S. sanguinis*. While the MIC and MBC values of the neutralized postbiotics for *F. nucleatum* ATCC 25586 were 50 mg/mL, the concentration value for the MIC and MBC values in the case of *P. denticola* ATCC 33185 was 3.12 mg/mL.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of test strains.

	Postbiotics		Neutralized postbiotics	
	MIC value (mg/mL)	**MBC value (mg/mL)**	**MIC value (mg/mL)**	**MBC value (mg/mL)**
P. denticola ATCC 33185	12.5	12.5	3.12	3.12
P. denticola AHN 32366	12.5	25	*nd*	*nd*
P. denticola AHN 32482	12.5	25	*nd*	*nd*
F. nucleatum ATCC 25586	12.5	12.5	50	50
S. sanguinis NCTC 10904	12.5	25	*nd*	*nd*

nd; not determined among tested concentrations

Antimicrobial screening

P. denticola ATCC 33185

The concentrations of 25 and 50 mg/mL, and after 48 h also the concentration of 12.5 mg/mL, completely inhibited the microbial growth of *P. denticola* ATCC 33185. After 6 and 24 h of incubation, no viability was detected in the culture media containing 25 and 50 mg/mL of postbiotics. Compared with the control group, a significant increase in viability was observed in the media containing 6.25 mg/mL postbiotics at the end of 6h- and 24h-incubation, whereas a 3-log decrease was observed at the concentration of 12.5 mg/mL. Moreover, the decrease in pH of the culture media after 48 h was quite drastic in the media containing 6.25 mg/mL postbiotics compared with the control group (without postbiotics). No significant pH changes were observed at both media containing 12.5, 25, and 50 mg/mL concentrations of postbiotics and pH-adjusted media with HCl. The pH changes were lower in media containing neutralized postbiotics (Supplementary File Figure S1, Figure 1a).

The microbial growth and inhibition observed in HCl-acidified media showed a similar trend as in media with postbiotics. Similarly, an increase in cell viability was observed at the end of 24 h compared to the control group. This indicates that just a pH of 6.01, corresponding to 6.25 mg/mL postbiotics, provides an ideal environment for the growth of strain *P. denticola* ATCC 33185 (Figure 1 b).
Although the neutralized postbiotics (pH 7.0) were not as effective as the crude postbiotics after 6 h, antimicrobial activity was observed at increasing concentrations and longer incubation times. (Figure 1c).

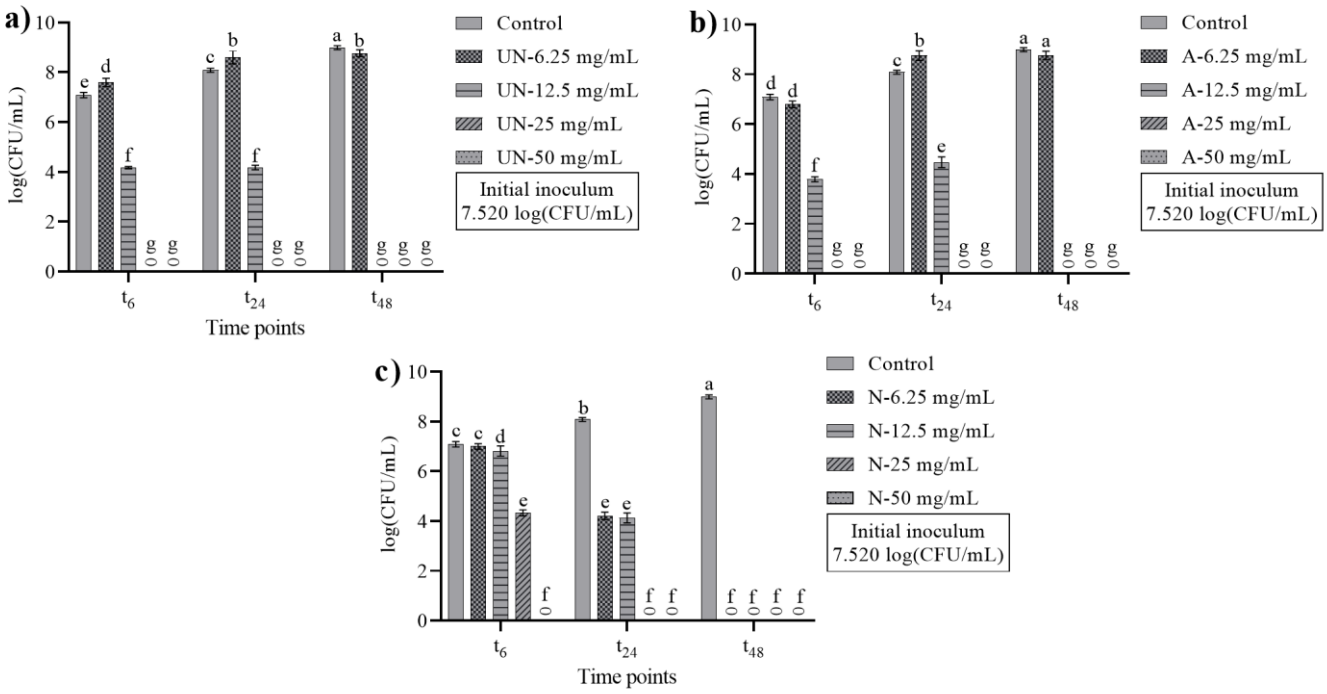

Figure 1. Microbial growth of *P. denticola* ATCC 33185 under the influence of crude postbiotics, acidified media and neutralized postbiotics. C; unneutralized postbiotics in their crude form. Colony counting at indicated time points **(a)**, A; acidified media with HCl. Colony counting and pH of the media at indicated time points **(b)**, N; neutralized postbiotics (pH 7). Colony counting and pH of the media at indicated time points **(c)**. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$). t_6; 6 h, t_{24}; 24 h, t_{48}; 48 h.

Clinical strains of P. denticola

The antimicrobial activities of postbiotics, acidified media, and neutralized postbiotics were quite similar in both clinical strains of P. denticola (AHN 32366 and AHN 32482). Unlike from the type strain of *P. denticola* ATCC 33185, there was no difference in the media containing 6.25 mg/mL postbiotics at the end of 6h-incubation for both clinical strains, compared to the control groups. However, in contrast to the type strain, the antimicrobial activity under the influence of the neutralized postbiotics was observed only at the end of the short-term incubation (6 h), whereas this effect disappeared after 24 and 48 h and even a slight increase in viability was observed compared with the control group (Figure 2). The trends of pH changes were very similar in media containing postbiotics and pH-adjusted media with HCl (Supplementary File, Figure S1). Since the results of *P. denticola* AHN 32482 and AHN 33266 were very similar (Figure 3).

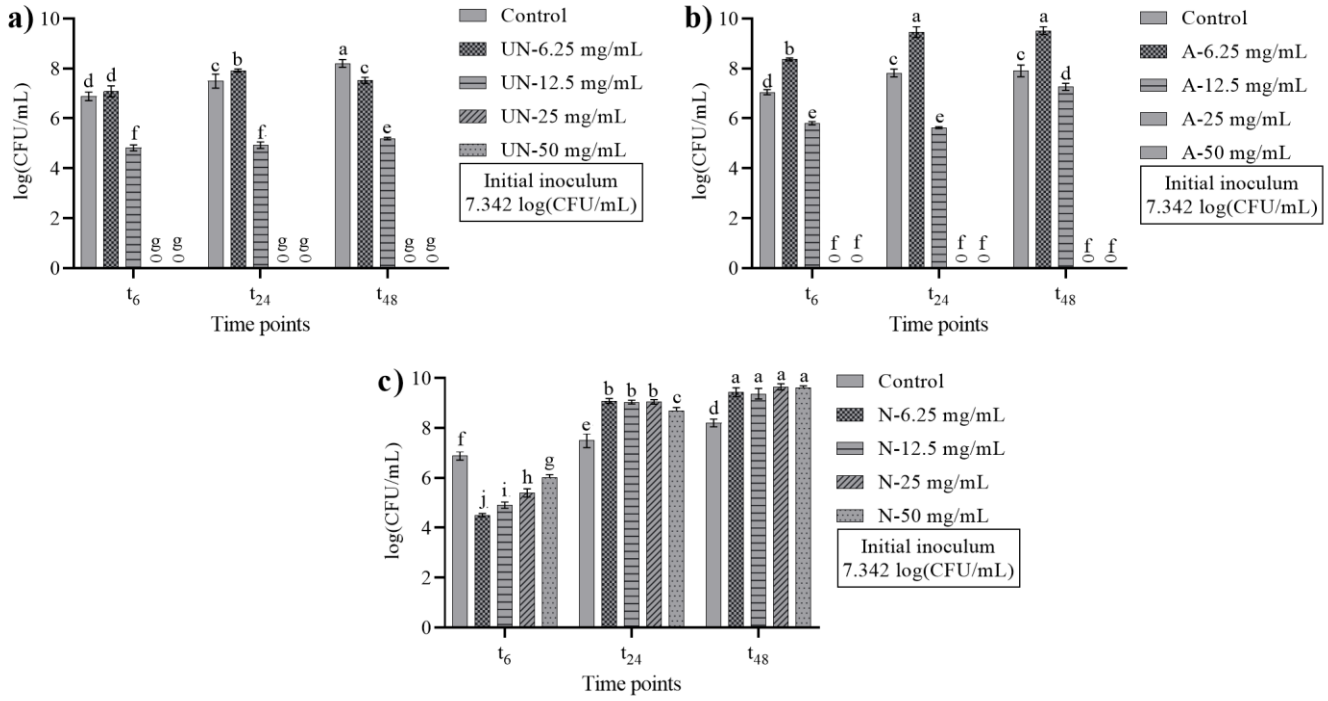

Figure 2. Microbial growth of *P. denticola* AHN 32366 under the influence of crude postbiotics, acidified media and neutralized postbiotics. C; unneutralized postbiotics in their crude form. Colony counting at indicated time points for AHN 33266 **(a)**, A; acidified media with HCl. Colony counting and pH measurement results at indicated time points for AHN 33266 **(b)**, N; neutralized postbiotics (pH 7). Colony counting and pH measurement results at indicated time points for AHN 33266 **(c)**. C; unneutralized postbiotics in their crude form. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$). t_6; 6 h, t_{24}; 24 h, t_{48}; 48 h.

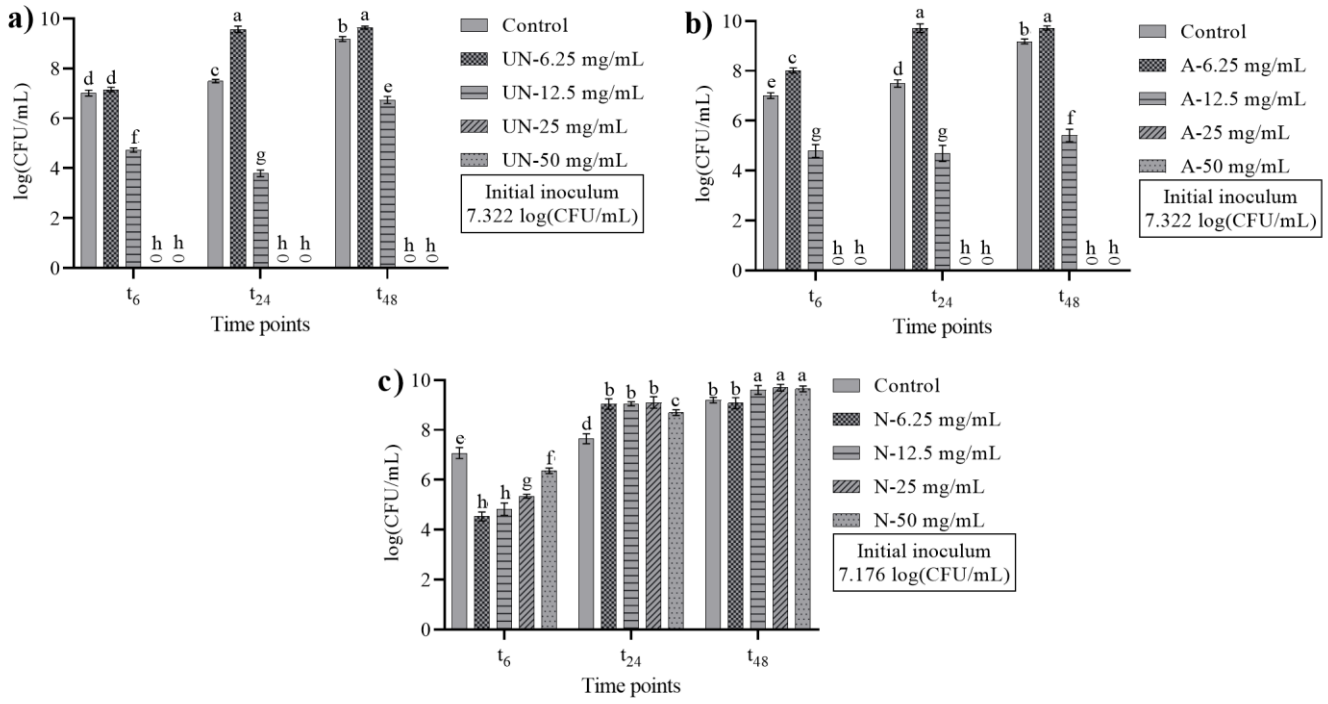

Figure 3. Microbial growth of *P. denticola* AHN 32482 under the influence of crude postbiotics, acidified media and neutralized postbiotics. C; unneutralized postbiotics in their crude form. Colony counting at indicated time points for AHN 32482 **(a)**, A; acidified media with HCl. Colony counting and pH measurement results at indicated time points for AHN 33266 **(b)**, N; neutralized postbiotics (pH 7). Colony counting and pH measurement results at indicated time points for AHN 33266 **(c)**. C; unneutralized postbiotics in their crude form. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$). t_6; 6 h, t_{24}; 24 h, t_{48}; 48 h.

F. nucleatum ATCC 25586

At all time-points, bacterial viability was drastically inhibited at 12.5, 25, and 50 mg/mL postbiotics concentrations in both groups containing different concentrations of postbiotics and HCl-acidified media. In the acidified media groups, bacterial viability was still present after 6 h at a pH value corresponding to 12.5 mg/mL postbiotics. In contrast to the strains of *P. denticola*, no significant increase in microbial growth at lower concentrations of postbiotics was observed in *F. nucleatum*. Considering the antimicrobial activity of the neutralized postbiotics, a decrease in microbial growth was observed only at 25 and 50 mg/mL. Although the increase in microbial growth at high concentrations was remarkable, especially at long incubation periods (24 and 48 h), it has been observed that neutralized postbiotics were more effective in the early incubation period. (Figure 4). When pH changes were considered in the case of *F. nucleatum* ATCC 25586, it was found that even lower concentrations of postbiotics (6.25 mg/mL) had buffering capacity. However, a drastic decrease was observed in the pH-adjusted media with a pH of 6.25 mg/mL postbiotics (Supplementary File, Figure S1).

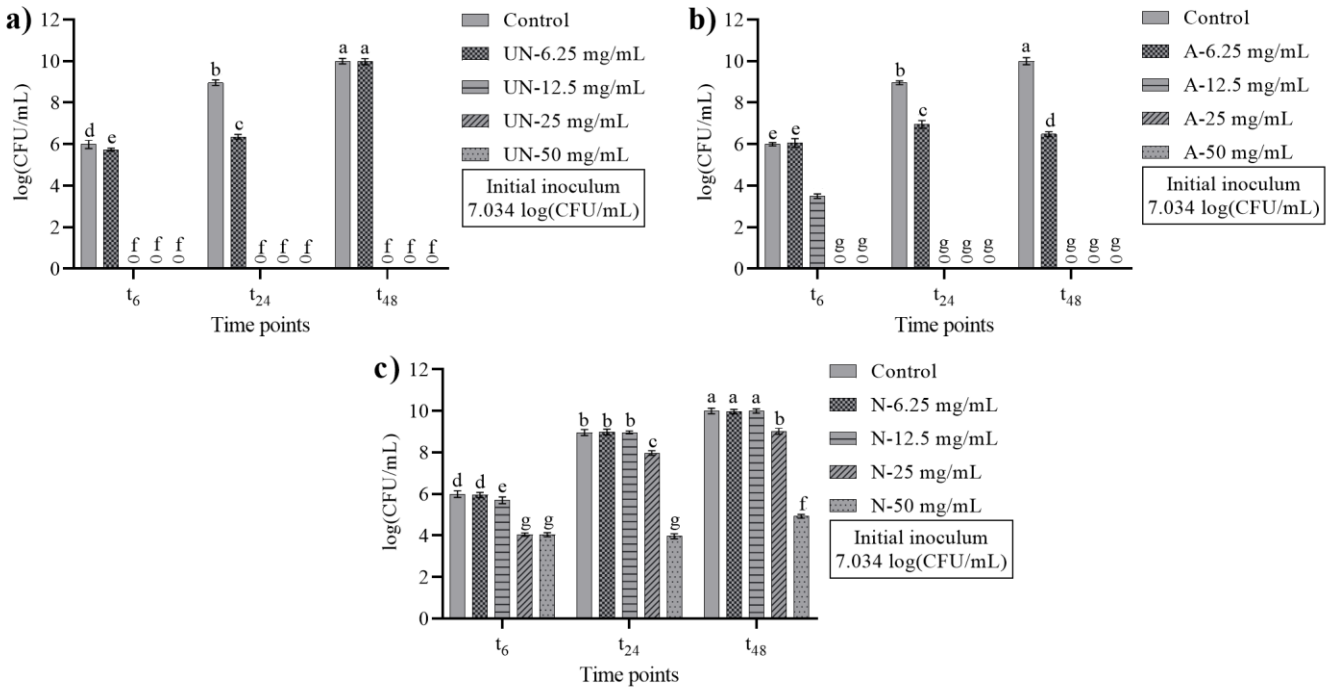

Figure 4. Microbial growth of *F. nucleatum* ATCC 25586 under the influence of crude postbiotics, acidified media and neutralized postbiotics. C; unneutralized postbiotics in their crude form. Colony counting at indicated time points **(a)**, A; acidified media with HCl. Colony counting and pH of the media at indicated time points **(b)**, N; neutralized postbiotics (pH 7). Colony counting and pH of the media at

indicated time points **(c)**. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$). t_6; 6 h, t_{24}; 24 h, t_{48}; 48 h.

S. sanguinis NCTC 10904

At the end of 48 h, viability was present only in the media containing 6.25 mg/mL of postbiotics. However, in the acidified media, microbial growth was also present at a pH equal to the pH of the 12.5 mg/mL postbiotics. 25 and 50 mg/mL postbiotics and their pH equivalents in manipulated media with HCl were found to be completely inhibitory. In the neutralized postbiotics, a small reduction in growth was observed only at a concentration of 50 mg/mL at the end of all incubation periods (Figure 5). The trends of pH changes were very similar in media containing postbiotics and pH-adjusted media with HCl (Supplementary File, Figure S1).

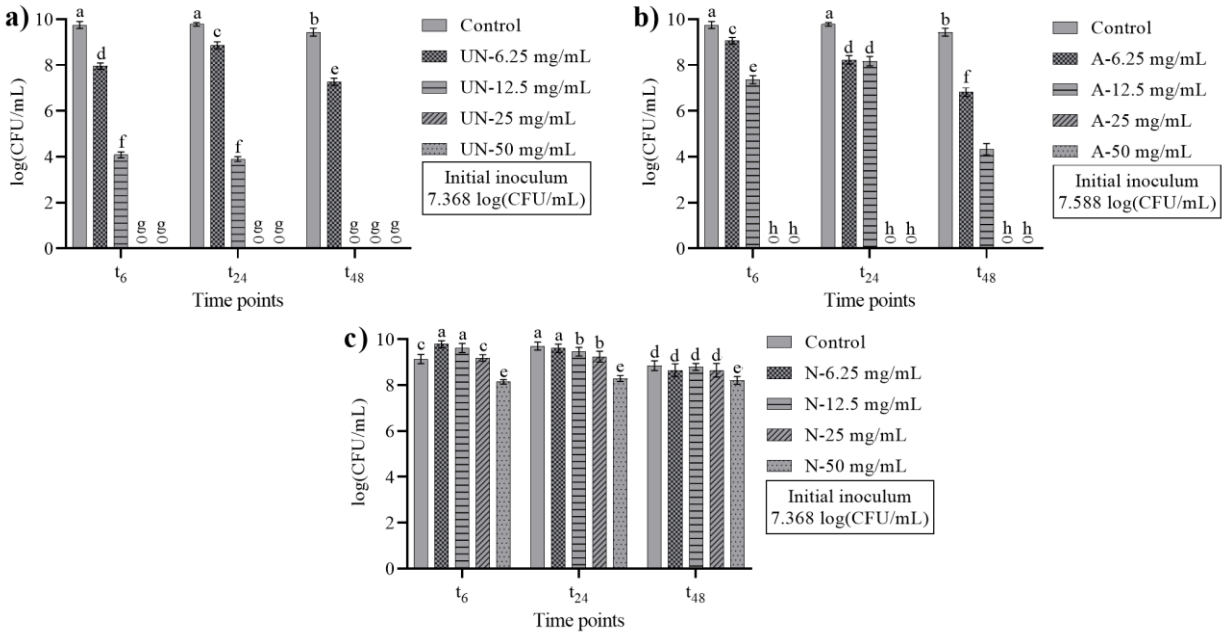

Figure 5. Microbial growth of *S. sanguinis* NCTC 10904 under the influence of crude postbiotics, acidified media and neutralized postbiotics. C; unneutralized postbiotics in their crude form. Colony counting at indicated time points **(a)**, A; acidified media with HCl. Colony counting and pH of the media at indicated time points **(b)**, N; neutralized postbiotics (pH 7). Colony counting and pH of the media at indicated time points **(c)**. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$). t_6; 6 h, t_{24}; 24 h, t_{48}; 48 h.

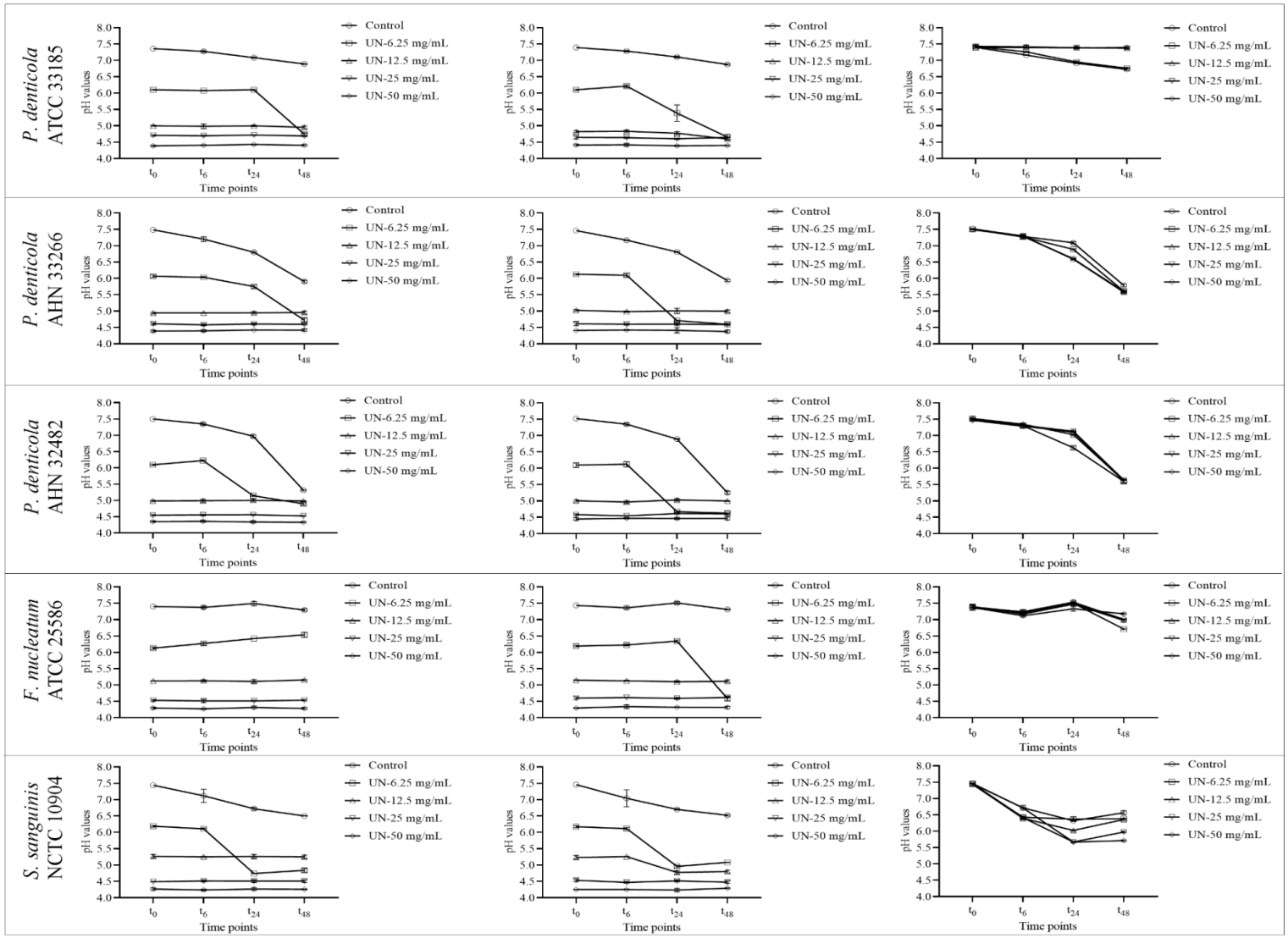

Figure S1. pH changes in culture media including different concentrations of postbiotic, neutralized postbiotics or acidified media with HCl. UN; unneutralized postbiotics, A; acidified media with HCl, N; neutralized postbiotics. Each row represents different strain.

Growth recovery after treatment

After the treatments to determine antimicrobial activity, samples were taken from the cultures and inoculated into fresh media. In this way, it was confirmed whether the applied contents or conditions (postbiotics in crude form, neutralized postbiotics, or acidified media) resulted in permanent inhibition of microbial viability. At postbiotics concentrations and time points, in which no bacterial viability was detected during the experimental process, no bacterial growth recovery was observed, except for *F. nucleatum* and 12.5 mg/mL of postbiotics at 6 h where growth was observed after the recovery process (Supplementary file, Table S1).

Table S1. Microbial growth after recovery

		***P. denticola* ATCC 33185**		
Time points	**6.25 mg/mL**	**12.50 mg/mL**	**25 mg/mL**	**50 mg/mL**
t6[a]-C[d]	+	+	-	-
t6-N[e]	+	+	+	-
t6-A[f]	+	+	-	-
t24[b]-C	+	+	-	-
t24-N	+	+	-	-
t24-A	+	+	-	-
t48[c]-C	+	-	-	-
t48-N	-	-	-	-
t48-A	+	-	-	-
		***P. denticola* AHN 32366**		
Time points	**6.25 mg/mL**	**12.50 mg/mL**	**25 mg/mL**	**50 mg/mL**
t6[a]-C[d]	+	+	-	-
t6-N[e]	+	+	+	+
t6-A[f]	+	+	-	-
t24[b]-C	+	+	-	-
t24-N	+	+	+	+
t24-A	+	+	-	-
t48[c]-C	+	+	-	-
t48-N	+	+	+	+
t48-A	+	+	-	-
		***P. denticola* AHN 32482**		
Time points	**6.25 mg/mL**	**12.50 mg/mL**	**25 mg/mL**	**50 mg/mL**
t6[a]-C[d]	+	+	-	-
t6-N[e]	+	+	+	+
t6-A[f]	+	+	-	-
t24[b]-C	+	+	-	-
t24-N	+	+	+	+
t24-A	+	+	-	-
t48[c]-C	+	+	-	-
t48-N	+	+	+	+
t48-A	+	+	-	-
		***F. nucleatum* ATCC 25586**		
Time points	**6.25 mg/mL**	**12.50 mg/mL**	**25 mg/mL**	**50 mg/mL**
t6[a]-C[d]	+	+	-	-
t6-N[e]	+	+	+	+
t6-A[f]	+	+	-	-
t24[b]-C	+	+	-	-
t24-N	+	+	+	+
t24-A	+	-	-	-
t48[c]-C	+	-	-	-
t48-N	+	+	+	+
t48-A	+	-	-	-
		***S. sanguinis* NCTC 10904**		

Time points	6.25 mg/mL	12.50 mg/mL	25 mg/mL	50 mg/mL
t6[a]-C[d]	+	+	-	-
t6-N[e]	+	+	+	+
t6-A[f]	+	+	-	-
t24[b]-C	+	+	-	-
t24-N	+	+	+	+
t24-A	+	+	-	-
t48[c]-C	+	-	-	-
t48-N	+	+	+	+
t48-A	+	+	-	-

Legends: [a]6th h; [b]24th h; [c]48th h; [d]crude postbiotics; [e]neutralized postbiotics; [f]acidified media; "+" growth after recovery; "-" no growth after recovery

Antibiofilm effects of postbiotics on polymicrobial biofilm formation

In the biofilm assay, three different polymicrobial consortia were tested, all containing type strains of *S. sanguinis* and *F. nucleatum*, but a different *P. denticola* strain. Values below the MIC concentrations were also tested (3.12 mg/mL and 6.25 mg/mL). The postbiotics showed significant antibiofilm activity even at non-bactericidal concentrations in the polymicrobial consortia containing the *P. denticola* AHN 32366 strain. In polymicrobial consortia containing *P. denticola* ATCC 33185 and *P. denticola* AHN 34482, only the two highest concentrations reduced biofilm formation (Figure 6).

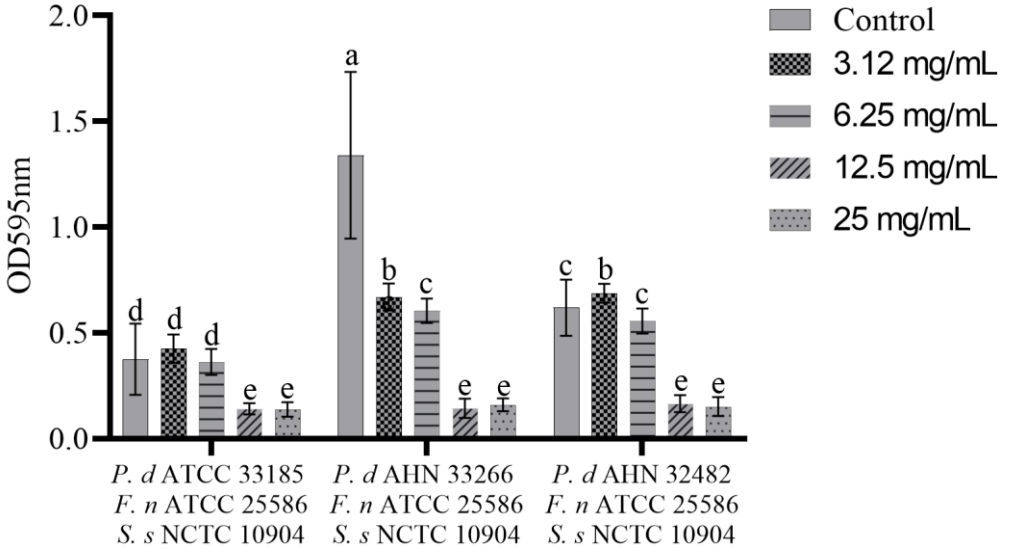

Figure 6. Antibiofilm activities of postbiotics against polymicrobial biofilm formation. Wells of a microtiter plate were coated with saliva and equal amounts of indicated bacteria were added into media containing postbiotics. After 48 h the amount of accumulated biofilm was measured by crystal violet staining. Bars indicate standard deviation, n=3. Different letters indicate the significant differences between groups (One-Way ANOVA, Tukey's Test, $p < 0.05$).

Work packages associated with 1st study:

Work package 1:

As indicated in the first work package, the antimicrobial activities of postbiotics of the strain *L. plantarum* EIR/IF-1 on periodontal pathogens were tested. Co-aggregation and determination of changes in protein and exopolysaccharide

components in the biofilm matrix were performed in later studies. In this study, *Prevotella denticola* was preferred instead of the strain of *Treponema denticola* indicated in the project proposal because of its important role in the periodontitis in recent years. In addition, *Streptococcus sanguinis*, a more virulent *Streptococcus* species, was preferred instead of *Streptococcus mutans* mentioned in the project proposal.

Results for 2nd study

Analysis of Chemical Structure and Antibiofilm Properties of Exopolysaccharides from *Lactiplantibacillus plantarum* EIR/IF−1 Postbiotics

Characterization of EPS

Monosaccharide Composition Analysis

The results were evaluated with respect to the mono- and disaccharides in EPS. The HPLC chromatogram showed that glucose was the highest saccharide with a concentration of 5.674 g/100 g. Galactose and fructose were found in EPS at 4.476 g/100 g and 0.23 g/100 g, respectively. Overall, all saccharides were detected in statistically significant amounts in EPS (Figure 1a). The presence of various monosaccharides indicates that the EPS is a heteropolysaccharide.

Fourier Transform Infrared Spectroscopy Analysis

A FTIR spectrum was recorded in absorption mode from 4,000 to 400 cm^{-1} to investigate the functional groups of EPS (Figure 1b). Polysaccharides have a large number of hydroxyl groups that exhibit a strong broad stretching peak at 3307 cm^{-1}. Due to the hydrogen bonds formed between the different hydroxyl groups, the absorption in this region exhibits the round shape which is typical of most O-H stretching modes, indicating that the molecule is EPS. Two weak C-H stretching peaks at 2966 and 2936 cm^{-1} in the FTIR spectra of *L. plantarum* EIR-IF-1 EPS show both methyl groups and methylene groups. Strong absorption at 1652 cm^{-1}, corresponding to amide I > C=O stretching and C-N bending of protein and peptide amines. Another weak peak at 1540 cm^{-1} can be considered as N-H bending of amides II of proteins. The peak at 1450 cm^{-1} was assigned to C-H bending in CH3 groups or aromatic -C=C stretching vibrations in proteins. A medium peak at 1400 cm^{-1} was considered as a >C=O stretching of COO- groups and a C-O bending of COO- groups. The peak at 1219 cm^{-1} could be assigned to C-O stretching in ether or alcohol groups. A strong and broad peak at 1000-1200 cm^{-1} indicates that the analyzed molecule is a carbohydrate, since C-O-C and C-O are present. FTIR spectroscopy can characterize the sample as a whole molecule below the 1500 cm^{-1} region and the peak at 1070 cm^{-1} indicates that the molecule is a polysaccharide. The peak at 836 cm^{-1} is characteristic of α-D-glucan (Figure 1b).

Molecular Weight Estimation of EPS

In size exclusion chromatography or gel permeation chromatography, analytes are separated based on their molecular size. A calibration curve was constructed using the logarithm of molecular weight as a function of retention time with the dextran standards. The chromatogram of EPS appeared as a symmetrical sharp peak at 20.60 min and the chromatogram of EPS showed a single symmetrical narrow peak, confirming the homogeneity of the purified EPS sample (Figure 1c).

Two fractions with different molecular weights were obtained: 51 kDa and 841 kDa (Figure 1d).

Thermogravimetric Analysis (TGA) of the EPS

Thermogravimetric analysis of the EPS was performed dynamically between temperature and weight loss. When the EPS is exposed to different temperatures, the first temperature increase leads primarily to gelatinization and swelling. Further increase in temperature leads to dehydration and pyrolysis of the exopolysaccharide. A two-stage weight loss due to dehydration was observed during thermogravimetric analysis of EPS (Figure S1, Supplementary file). The EPS showed an initial weight loss between 50 °C and 130 °C. This initial weight loss could be due to the loss of moisture by the carboxyl groups, which are present in large amounts and bound to water molecules. Thus, the initial weight loss of EPS was due to the presence of a high content of carboxyl groups. The second weight loss was observed at a degradation temperature of 129.20 °C.

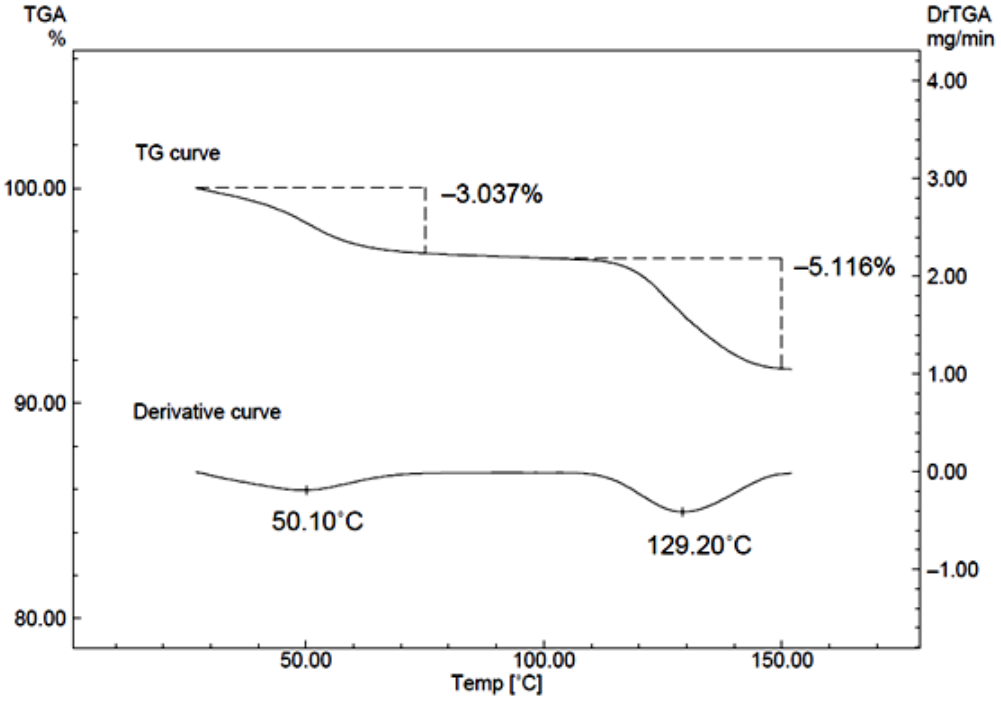

Figure S1. TG curves of *L. plantarum* EIR/IF–1 EPS fraction

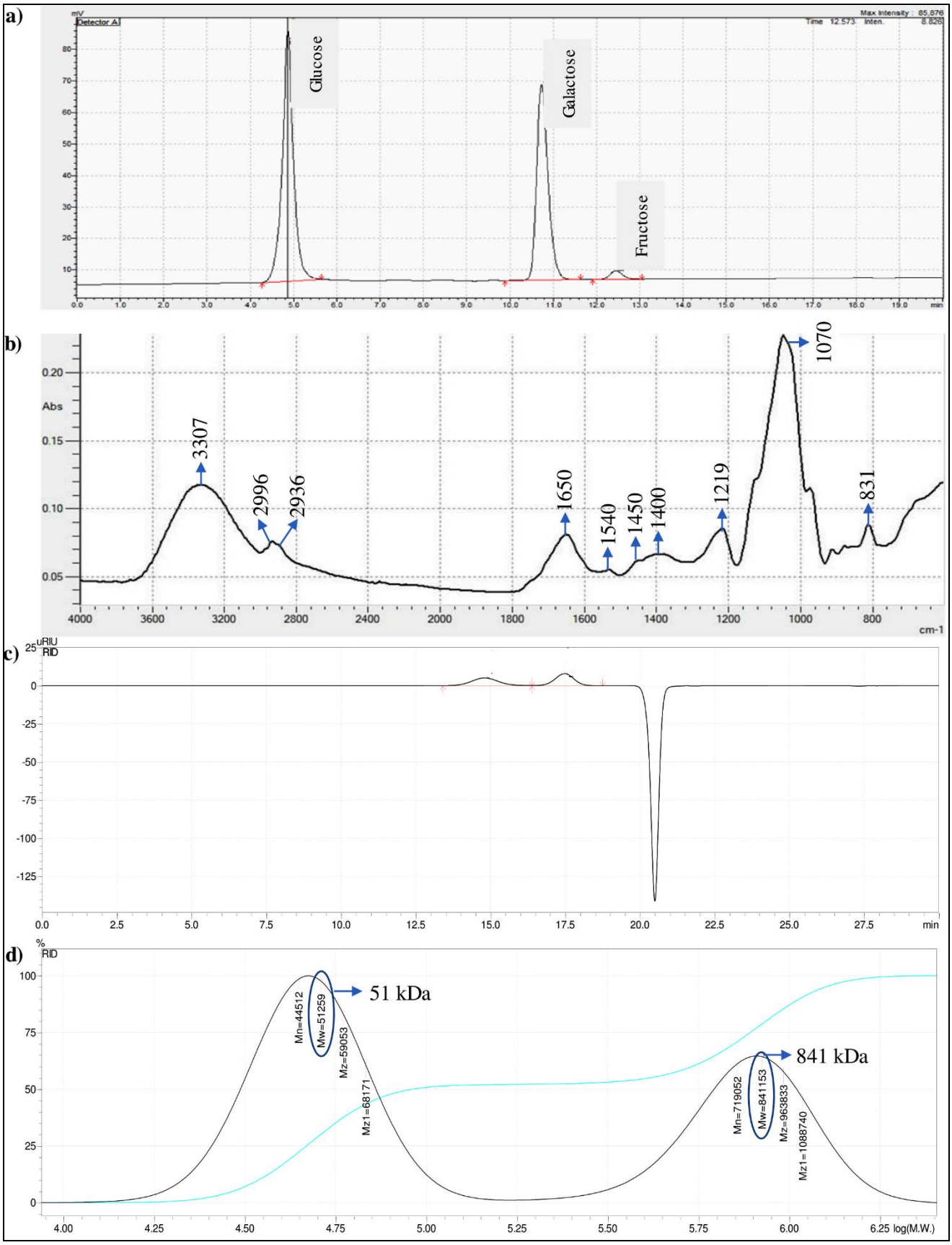

Figure 1. (a) Hydrolyzed EPS from *L. plantarum* EIR/IF-1 **(b)** FTIR spectrum of the EPS **(c)** Gel permeation chromatogram of EPS **(d)** Molar Mass Distribution (MMD) pattern. Molecular weight of two fractions in EPS were indicated in blue circles. Other molecular weights represent the standards.

Antibiofilm Activity of Postbiotics and EPS

Postbiotics

In evaluating the antibiofilm activities of the postbiotics, it was found that the co-treatment application, in which the postbiotics were introduced into the medium, was much more effective than the pre-treatment and post-treatment applications. For all other test strains involved in biofilm formation with *F. nucleatum* ATCC 25586, antibiofilm activity was observed with co-treatment, even at postbiotics concentrations (3.12 and 6.25 mg/mL). In summary, the use of low concentrations of postbiotics had significant anti-biofilm activity (Figure 2a) ($p<0.0001$).

There was no significant difference in the biofilm production of *P. denticola* ATCC 33185 + *F. n* ATCC 25586 (*P. d*; *P. dentiocla*, *F. n*; *F. nucleatum*) and *P. d* AHN 32366 + *F. n* ATCC 25586 compared to the control groups in the pre-treatment assay where the polystyrene surfaces were coated with different concentrations of postbiotics before biofilm sampling. For *P. g* ATCC 33277 + *F. n* ATCC 25586 (*P. g*; *P. gingivalis*) ($p=0.03$) and *P. g* AHN 24155 + *F. n* ATCC 25586 ($p=0.025$), only treatment with 12.5 and 25 mg/mL postbiotics improved the antibiofilm response, while no antibiofilm activity was observed at lower concentrations. In contrast, for *F. a* ATCC 35896 + *F. n* ATCC 25586 (*F. a*; *Fil. alocis*), the antibiofilm effect of all applied postbiotics concentrations proved effective and no difference was observed between concentrations ($p=0.0004$) (Figure 2b).

While no change was observed in the concentrations of all postbiotics applied to the established biofilms of *P. d* ATCC 33185 + *F. n* ATCC 25586, there was a decrease in postbiotics concentrations with an increasing trend in the established biofilms of *P. d* AHN 32366 + *F. n* ATCC 25586 ($p=0.0123$). On the other hand, the different concentrations applied to *P. g* ATCC 33277 + *F. n* ATCC 25586 and *P. g* AHN 24155 + *F. n* ATCC 25586 resulted in a decrease in the amount of established biofilms, although the difference between the groups that differed from the control group was not considered significant ($p<0.0008$ and $p<0.022$, respectively). In the mature biofilms of *F. a* ATCC 35896 + F. n ATCC 25586, 6.25, 12.5, and 25 mg/mL postbiotics applications showed a significant eradication in biofilm (Figure 2c).

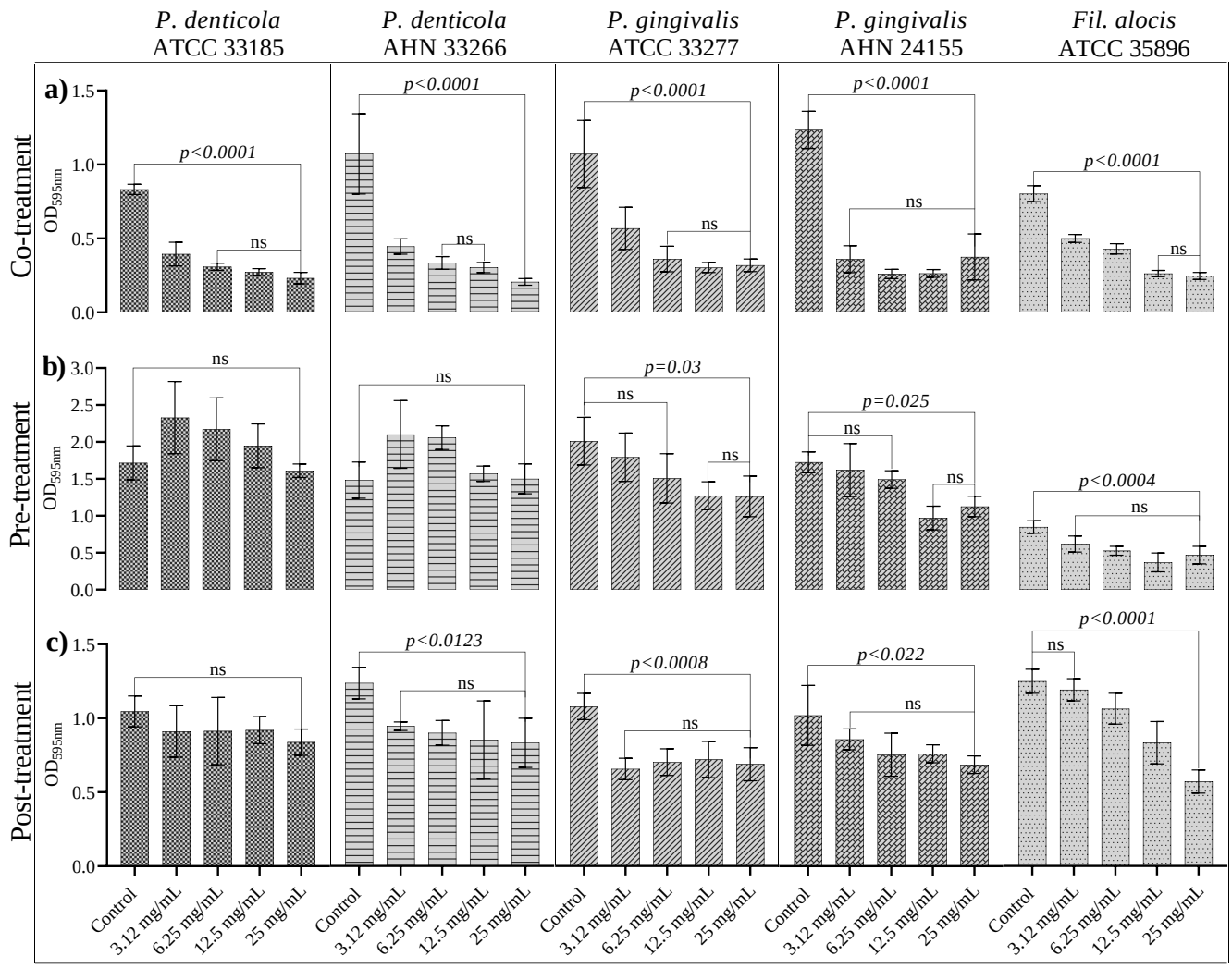

Figure 2. Antibiofilm activity of postbiotics. All biofilms are dual-species biofilms including *F. nucleatum* ATCC 25586. Co-treatment assay: Biofilm formation in culture media adjusted with different concentrations of postbiotics **(a)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row a. Pre-treatment assay: Biofilm formation on treated surfaces with different concentrations of postbiotics **(b)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row b. Post-treatment assay: Postbiotics treatment with different concentrations on established biofilms **(c)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row c. ns; not significant. Bars include standard deviation. Y-axis was equalized in each row.

EPS

To determine whether the extracted EPS affected the biofilms of the test bacteria, co-treatment, pre-treatment, and post-treatment experiments were conducted. As shown in the co-treatment experiments (Figure 3a), all concentrations of EPS proved sufficient to inhibit biofilm formation (*p<0.0001* for *P. d* ATCC 33185 + *F. n* ATCC 25586, *P. d* AHN 33266 + *F. n* ATCC 25586, *P. g* ATCC 33277 + *F. n* ATCC 25586, *P. g* AHN 24155 + *F. n* ATCC 25586, and *p<0.0002* for *F. a* ATCC 35896 + F. n ATCC 25586). Although the EPS concentrations tested did not exhibit antimicrobial activity, very effective inhibition of biofilm formation was observed for all test strains. Testing of EPS on the growth of the test strains showed no antimicrobial activity. The test strains were cultured in 96-well polystyrene plates for 48 h at 37 °C

under anaerobic conditions with the indicated EPS concentrations in biofilm experiments (Supplementary File, Figure S2).

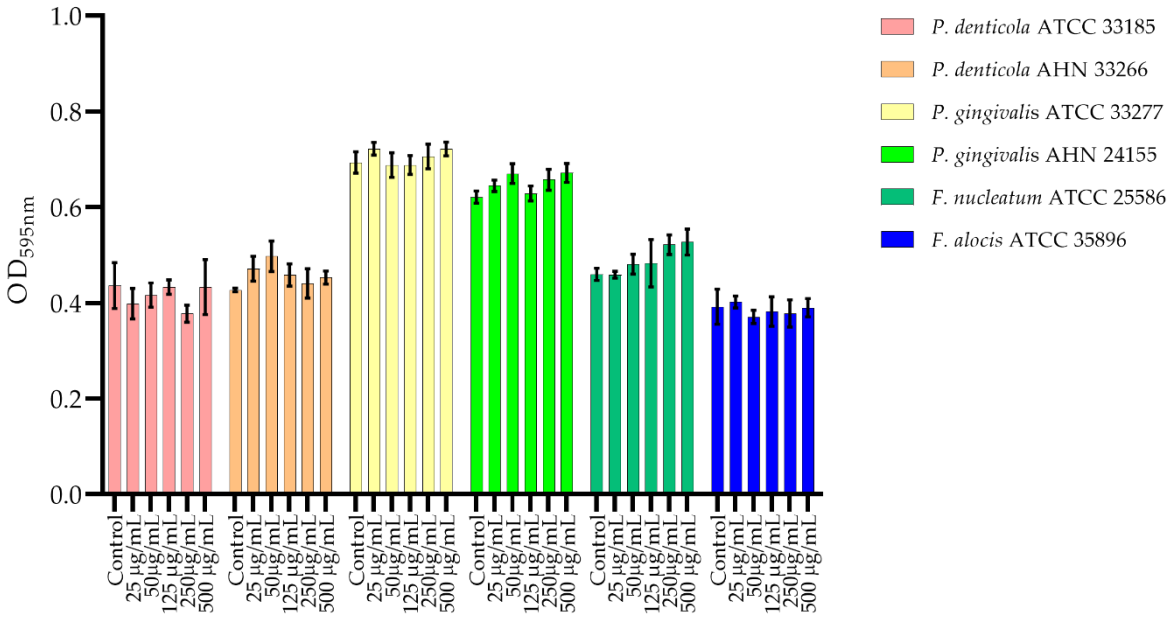

Figure S2. Testing of EPS fractions for growth of test strains. No antimicrobial activity was observed. Test strains were cultured in 96-well polystyrene plates for 48 h at 37 °C under anaerobic conditions with the indicated EPS concentrations in biofilm experiments.

In contrast to postbiotic pretreatment, a decrease in biofilm production was observed in all strains, including *P. d* ATCC 33185 + *F. n* ATCC 25586, due to increasing EPS concentrations compared to postbiotic pre-treatment (*p<0.0001*). While EPS at a concentration of 25 µg/mL was found to be ineffective in *P. d* ATCC 33185 + *F. n* ATCC 25586, the extent of biofilm production in *P. d* AHN 33266 + *F. n* ATCC 25586 under the influence of 25 and 50 µg/mL concentrations did not differ from the control group. Moreover, higher EPS concentrations were very effective for pre-treatment of these biofilms (*p<0.0001*). For *P. g* ATCC 33277 + *F. n* ATCC 25586, only the 25 µg/mL EPS concentration proved ineffective, while for *P. g* AHN 24155 + *F. n* ATCC 25586, a significant decrease in biofilm production was observed only at 250 and 500 µg/mL EPS concentrations (*p<0.0001* and *p<0.0002*, respectively). For *F. a* ATCC 35896 + *F. n* ATCC 25586, a significant decrease in biofilm formation was observed at all EPS concentrations in an increasing trend (*p<0.0001*) (Figure 3b).

Again, application of the EPS to mature biofilms gave a much better result than post-treatment application of postbiotics. All tested concentrations of the EPS were effective in eradicating mature biofilms (Figure 3c) (*p<0.0001* for all groups).

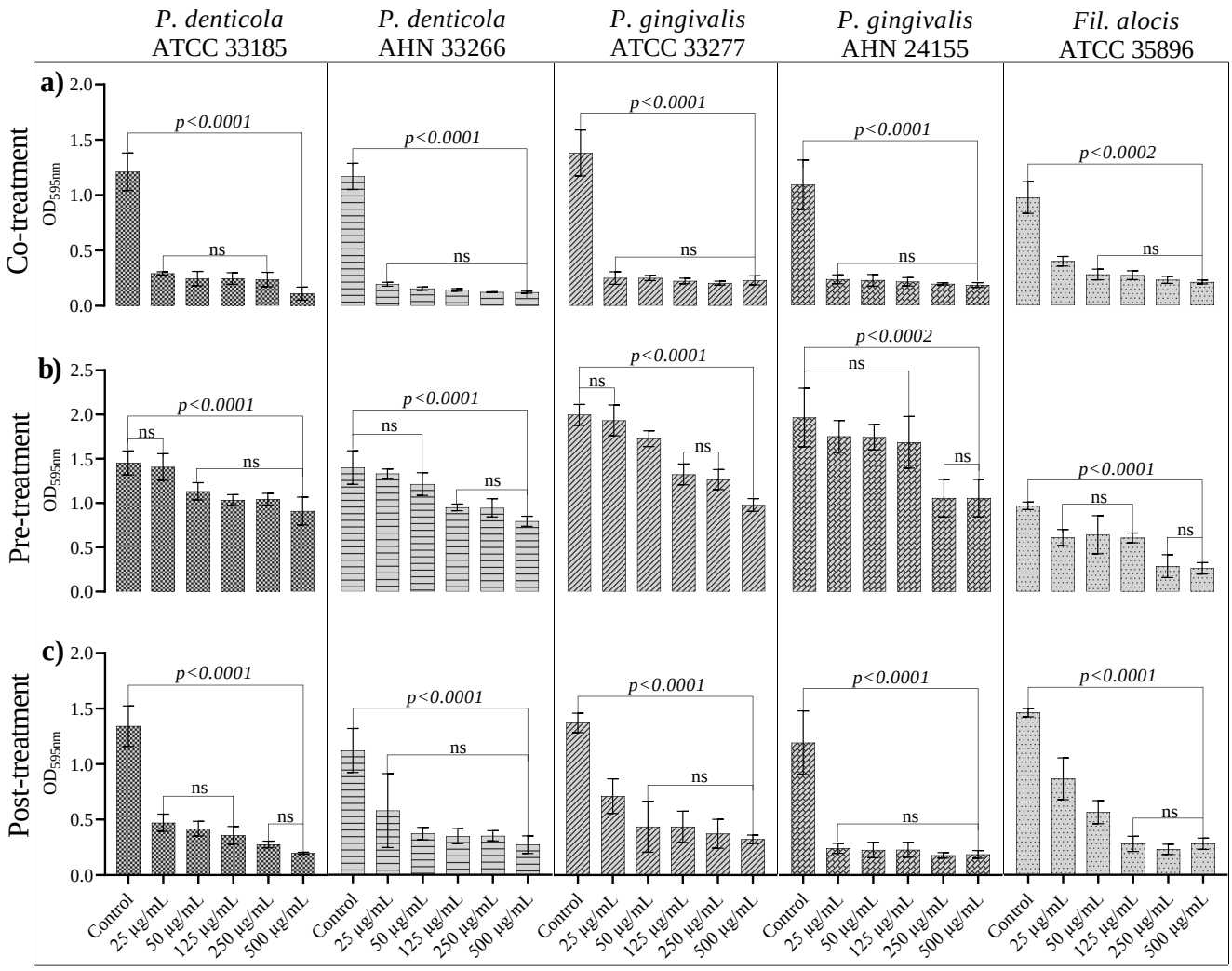

Figure 3. Antibiofilm activity of EPS. All biofilms are dual-species biofilms including *F. nucleatum* ATCC 25586. Co-treatment assay: Biofilm formation in culture media adjusted with different concentrations of postbiotics **(a)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row a. Pre-treatment assay: Biofilm formation on treated surfaces with different concentrations of postbiotics **(b)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row b. Post-treatment assay: Postbiotics treatment with different concentrations on established biofilms **(c)** *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* ATCC 33277, *P. gingivalis* AHN 24155, *Fil. alocis* ATCC 35896, respectively in the row c. ns; not significant. Bars include standard deviation. Y-axis was equalized in each row.

Investigation of Antibiofilm Activity of Postbiotics and EPS in terms of Hydrophobicity and cell-cell interactions

Testing Postbiotics and EPS on Auto-aggregation and Co-aggregation Ability of Test Strains

Postbiotics and EPS had inhibitory effects both on the auto-aggregation and co-aggregation levels of all strains tested. The auto-aggregation (Figure 4) and co-aggregation (Figure 5) results of postbiotics and EPS are given within same figures to allow comparison. The effects of both postbiotics and EPS on delaying auto-aggregation generally showed a similar trend. At the end of the 24-h incubation period, the effects of the tested postbiotics and EPS concentrations on the auto-

aggregation rates of *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 had almost disappeared, whereas the effects within the first three hours were partially preserved. Only at the highest EPS concentration (500 µg/mL) a partial effect remained (Figures 4c and 4f). In *P. denticola* ATCC 33185, *P. denticola* AHN 33266, *P. gingivalis* AHN 24155, and *Fil. alocis* ATCC 35896, both postbiotics and EPS significantly inhibited auto-aggregation rates at all incubation times (Figures 4a, 4b, 4d, and 4e).

The efficacy of postbiotics and EPS on coaggregation rates again showed a very similar trend. For all dual cultures, the change in optical density varied as a function of the different concentrations, i.e., higher concentrations were found to be more effective. Even after 24 h, the test groups of *P. denticola* strains had higher optical values than the control group at all concentrations tested (Figure 5a and 5b). A rapid decrease in optical density of dual culture with *P. gingivalis* ATCC 33277 was observed, similar to the auto-aggregation rate, even under the influence of postbiotics and EPS (Figure 5c). While no remarkable results were found regarding the effects of postbiotics and EPS on co-aggregation rates in *P. gingivalis* AHN 24155, EPS proved to be more effective in *Fil. alocis* ATCC 35896.

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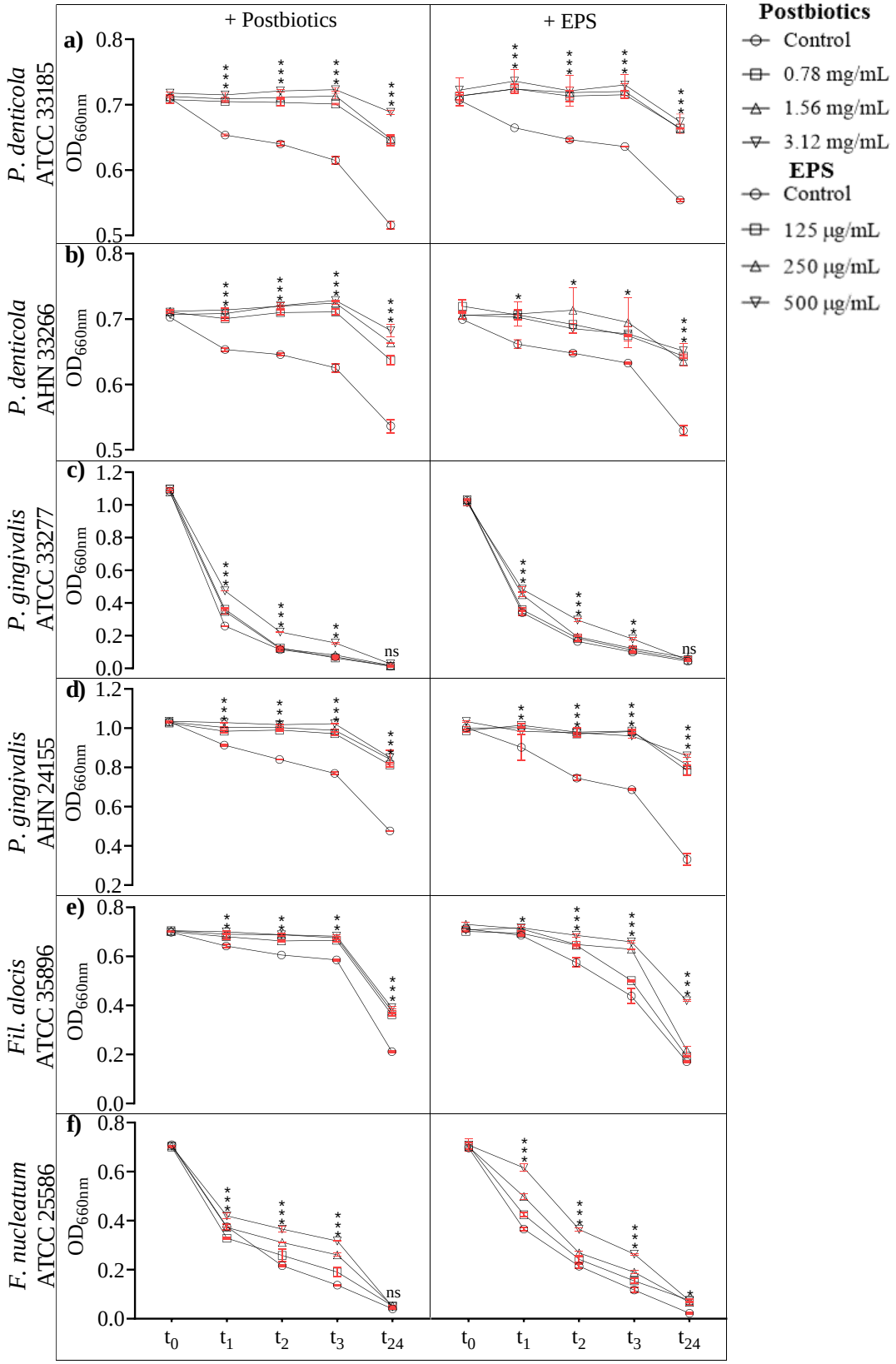

Figure 4. Effects of postbiotics and EPS on auto-aggregation. **(a)** *P. denticola* ATCC **(b)** *P. denticola* AHN 33266 **(c)** *P. gingivalis* ATCC 33277 **(d)** *P. gingivalis* AHN 24155 **(e)** *Fil. alocis* ATCC 35896 **(f)** *F. nucleatum* ATCC 25586. ns; not significant, ***; $p<0.001$; **; $p<0.01$; *; $p<0.05$. Dots include standard deviation. Y-axis was equalized in each row.

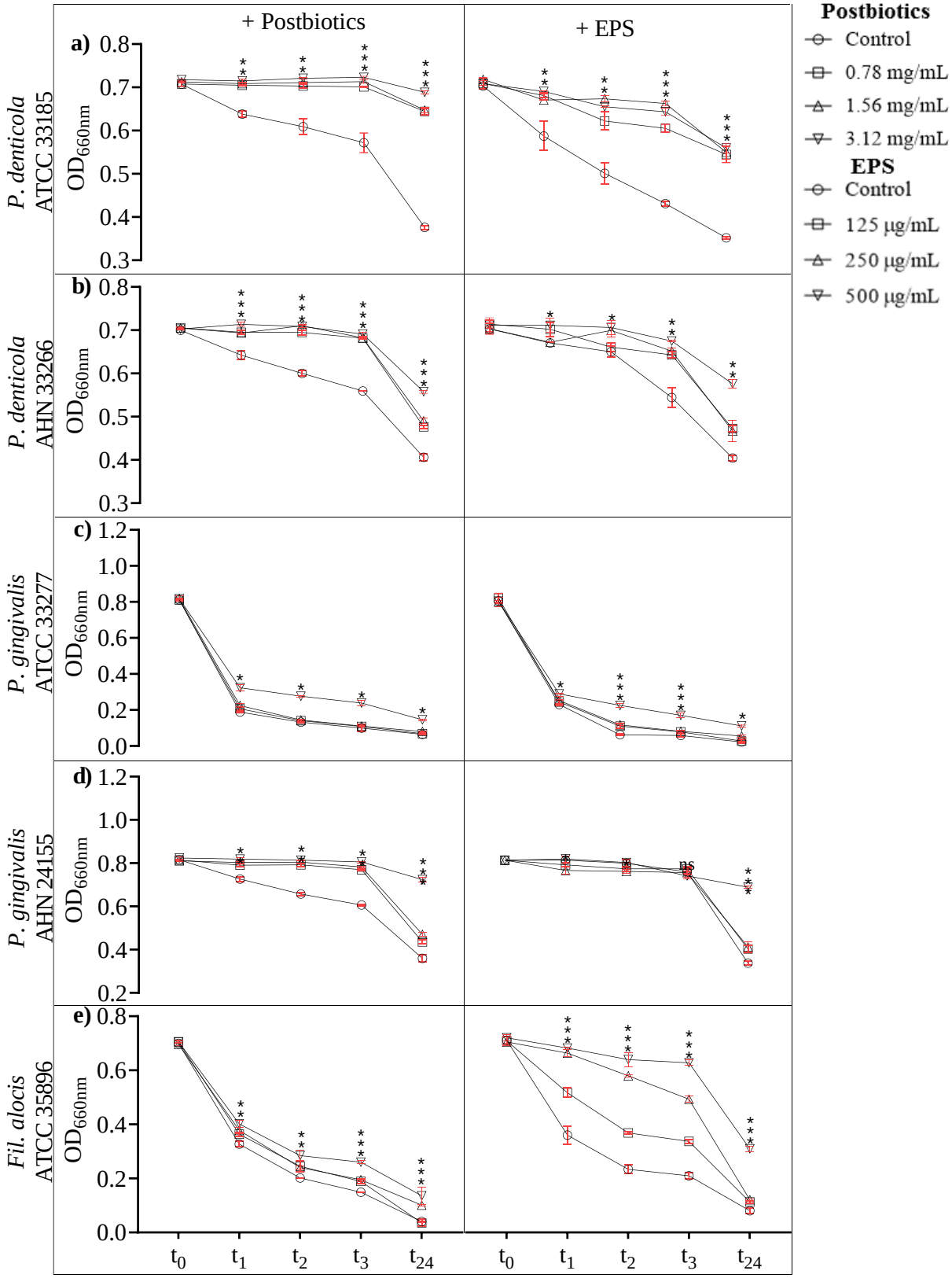

Figure 5. Effects of postbiotics and EPS on co-aggregation. **(a)** *P. denticola* ATCC **(b)** *P. denticola* AHN 33266 **(c)** *P. gingivalis* ATCC 33277 **(d)** *P. gingivalis* AHN 24155 **(e)** *Fil. alocis* ATCC 35896 **(f)** *F. nucleatum* ATCC 25586. ns; not significant, ***; $p<0.001$; **; $p<0.01$; *; $p<0.05$. Dots include standard deviation. Y-axis was equalized in each row.

Adhesion to Hydrocarbons

The adhesion percentages of test strains under the effects of postbiotics and EPS are shown in Figure 6. The hydrophobicity of all strains except *P. gingivalis* ATCC 33277 was 0% at the concentration of 1.56 mg/mL postbiotics. The effect of the EPS on hydrophobicity showed a similar trend to the postbiotics. Adhesion to xylene and toluene varied between the test strains. *F. nucleatum* ATCC 25585 and *P. gingivalis* ATCC 33277 showed a higher percentage of hydrophobicity to hydrocarbons, while *P. denticola* ATCC 33185 and *P. denticola* AHN 33266 and *Fil. alocis* ATCC 35896 showed the lowest hydrophobicity under the effects of both postbiotics and EPS.

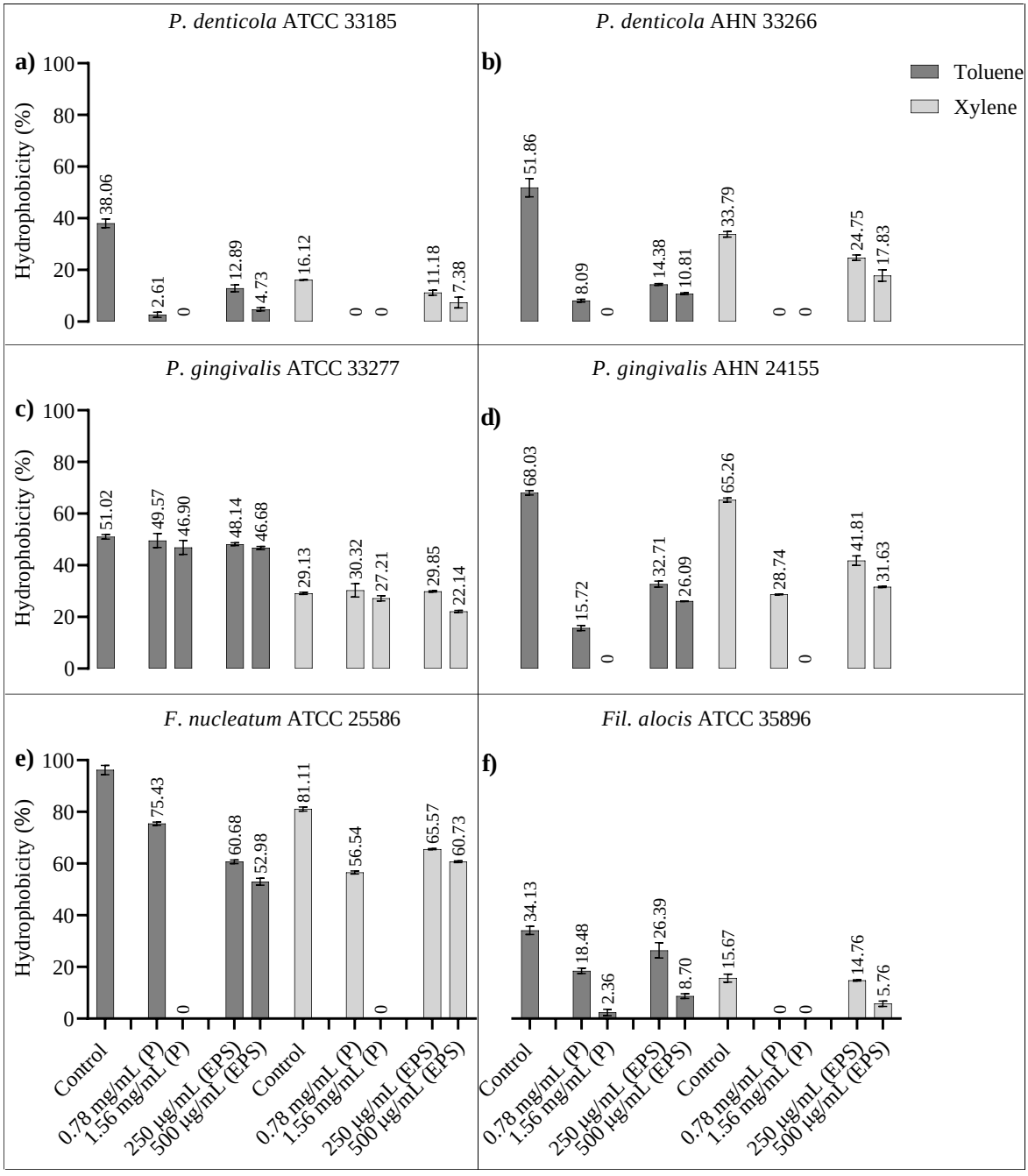

Figure 6. (a) *P. denticola* ATCC 33185 **(b)** *P. denticola* AHN 33266 **(c)** *P. gingivalis* ATCC 33277 **(d)** *P. gingivalis* AHN 24155 **(e)** *F. nucleatum* ATCC 25586 **(f)** *Fil. alocis* ATCC 35896. Bars include standard deviation. P; postbiotics, EPS; exopolysaccharide. Bars include standard deviation.

Work packages associated with 2nd study:

Work package 1 and 3:

As indicated in the third work package, the antibiofilm activities of postbiotics of the strain *L. plantarum* EIR/IF-1 on periodontal pathogens were tested. Furthermore, in contrast to the project proposal, the specific exopolysaccharide was purified in postbiotics and an extended physicochemical characterization was performed. This part was studied in a different way in addition to the project proposal. The exopolysaccharide was purified and characterized to test its antibiofilm activity on biofilms of periodontal pathogens. Co-aggregation and auto-aggregation were performed in these studies. In this study, *Filifactor alocis* was also preferred as another Gram-positive model because of its important role in the periodontitis in recent years. The postbiotics and exopolysaccharides we purified were tested on polystyrene surfaces on multispecies biofilm structures of periodontal pathogens. It was not deemed necessary to perform the same work on the hydroxyapatite and glass surfaces mentioned in the project proposal within this work package. This is because the characterization of the exopolysaccharides took a lot of time.

Results for 3rd study

Determination of The Immunostimulatory Properties of Protein and Exopolysaccharide Fractions Obtained from *Lactiplantibacillus plantarum* EIR/IF-1 Postbiotics on Gingival Keratinocyte Cell Lines

Human beta defesin responses under the effects of postbiotics and their protein and EPS components

Human beta defensin responses were given in figures 1, 2, and 3, respectively. Considering the effects of the tested postbiotics and their constituents on human beta-defensin, significant differences were found only for human beta-defensin 2. An increase in this defensin response was observed under the influence of all tested ingredients (Figure 2).

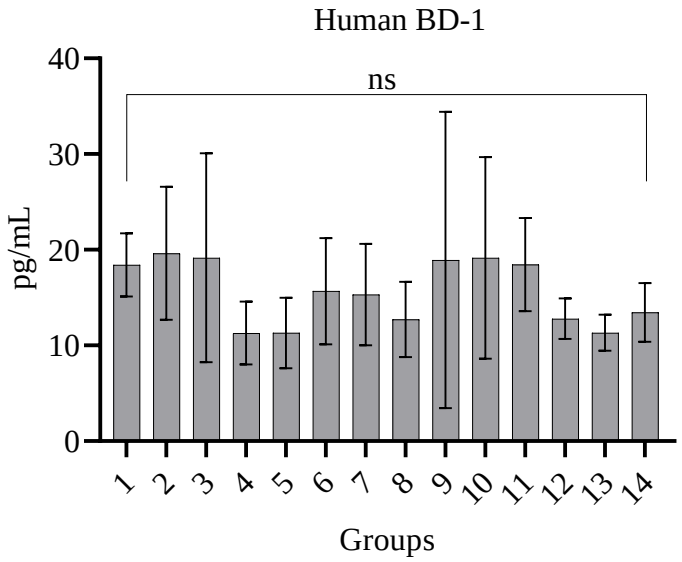

Figure 1. Human beta defensin-1 responses under the effects of postbiotics and their constituents. ns: statistically not significant. One-way ANOVA Test, Tukey's Test. Group numbers from 1 to 14 were explained in material and methods section.

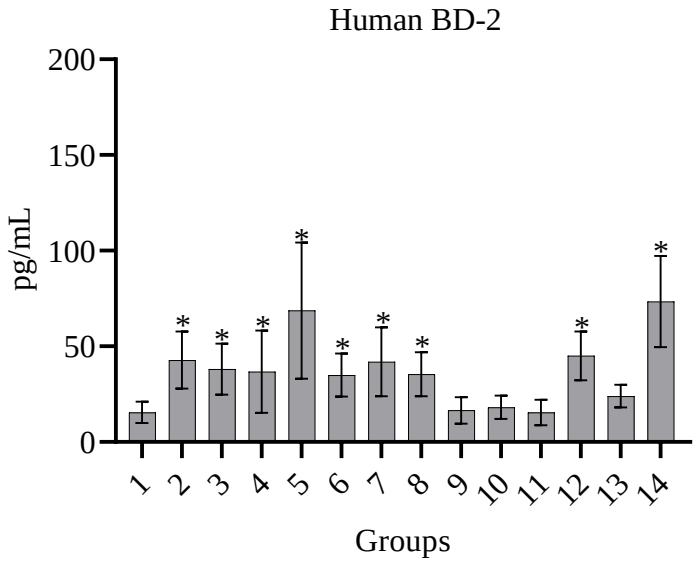

Figure 2. Human beta defensin-2 responses under the effects of postbiotics and their constituents. *: statistically significant, $p < 0.05$. One-way ANOVA Test, Tukey's Test. Group numbers from 1 to 14 were explained in material and methods section.

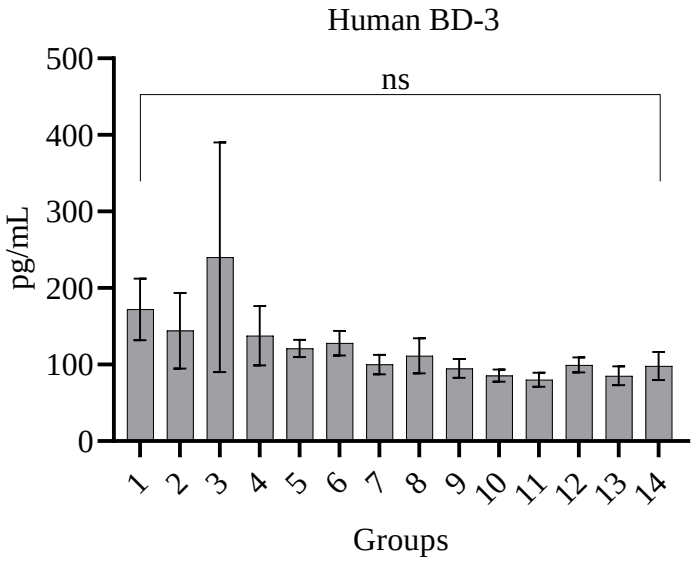

Figure 3. Human beta defensin-3 responses under the effects of postbiotics and their constituents. ns: statistically not significant. One-way ANOVA Test, Tukey's Test. Group numbers from 1 to 14 were explained in material and methods section.

Interleukin 1-β beta response under the effects of postbiotics and their protein and EPS components

There were no differences between test and control groups in terms of interleukin 1-β response (Figure 4).

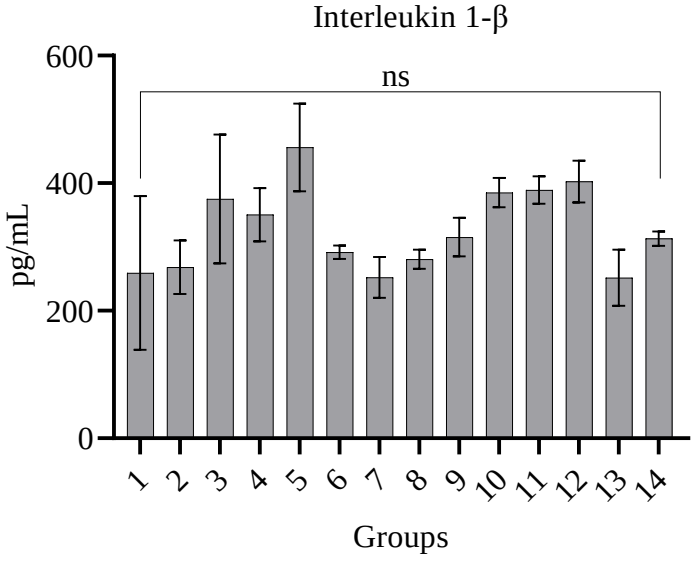

Figure 4. Interleukin 1-β response responses under the effects of postbiotics and their constituents. ns: statistically not significant. One-way ANOVA Test, Tukey's Test. Group numbers from 1 to 14 were explained in material and methods section.

Work packages associated with 3rd study:

Although this study is unrelated to any of the work packages identified in the project proposal, it is an additional study. The purpose of this study was to investigate the potential effects of postbiotics, which are a potential agent against periodontitis, and their components on immune regulation in oral tissues, as well as the demonstrated antimicrobial and antibiofilm activities against periodontal pathogens.

Results for 4th study

Effects of Postbiotics and Postbiotic Proteins on Cell Membranes of *P. gingivalis* and *F. nucleatum* and Some Virulence Properties of P. gingivalis

Membrane integrity assay

To understand the effects of postbiotics and proteins on membrane stability of *P. gingivalis* and *F. nucleatum*, membrane integrity tests were performed at concentrations below the nonlethal minimum inhibitory concentrations of both postbiotics and proteins. The protein content of postbiotics and postbiotics increased membrane permeability on both *P. gingivalis* strains at all concentrations (Figure 1 and Figure 2). However, only postbiotics were found to be effective in increasing membrane permeability of *F. nucleatum* ATCC 25586 (Figure 3). The increase in membrane permeability is evidenced by increasing fluorescence intensity. The membrane-stabilizing effect of postbiotic proteins only in *P. gingivalis* suggests the possibility of an antimicrobial peptide specific to *P. gingivalis*.

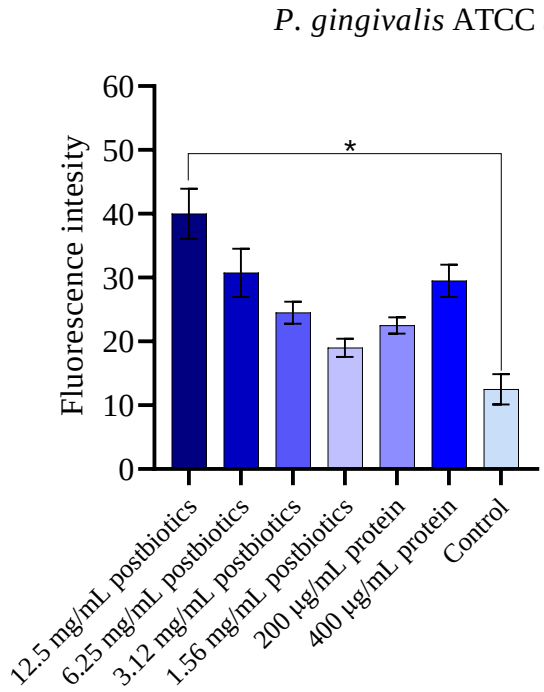

Figure 1. Effects of postbiotics and proteins on membrane integrity of *P. gingivalis* ATCC 33277. * indicates statistically difference. One-Way ANOVA Test, Tukey's Test, $p < 0.05$.

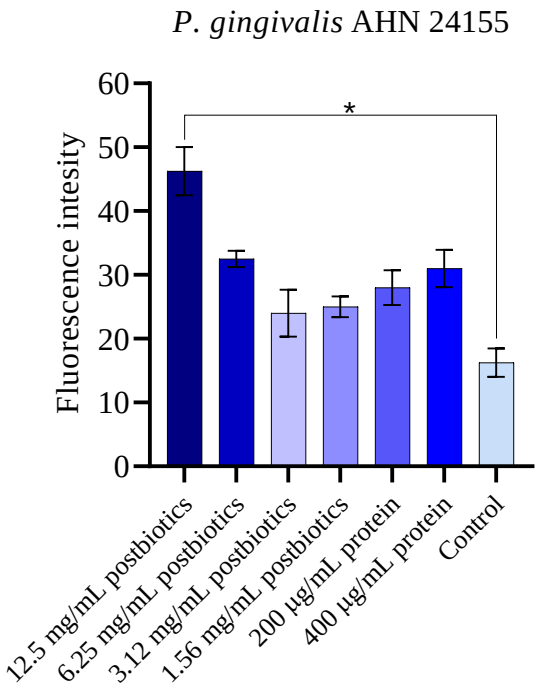

Figure 2. Effects of postbiotics and proteins on membrane integrity of *P. gingivalis* AHN 24155. * indicates statistically difference. One-Way ANOVA Test, Tukey's Test, $p < 0.05$.

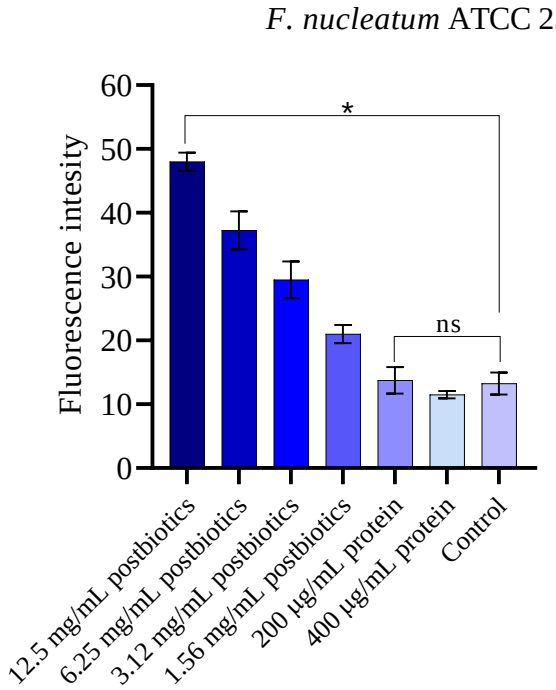

Figure 3. Effects of postbiotics and proteins on membrane integrity of *F. nucleatum* ATCC 25586. * indicates statistically difference. One-Way ANOVA Test, Tukey's Test, $p < 0.05$.

Effect of postbiotics and postbiotic proteins on dipeptidyl peptidase IV (DPPIV) enzyme activity of *P. gingivalis*

There was no significant effect of the evaluated postbiotics and postbiotics proteins on the dipeptidyl peptidase activity of *P. gingivalis*.

Effects of postbiotics and postbiotic proteins on gingipain activity of P. gingivalis

There was no significant effect of the evaluated postbiotics and postbiotics proteins on the gingipain activity of *P. gingivalis*.

Work packages associated with 4th study:

Work package 2.

4. CONCLUSION AND COMMENTS

- 1st study: Although the antimicrobial activity of *L. plantarum* EIR/IF-1 postbiotics against *F. nucleatum*, *S. sanguinis*, and *P. denticola* is mainly dependent on its acidity, the antimicrobial activity in neutral postbiotics suggests the presence of other antimicrobial agents distinct from the dominant acidic components. Further characterization of the antimicrobial and antibiofilm activity of functional components in postbiotics is needed. Finally, considering the complexity of oral biofilms in both health and disease, the use of postbiotics can be evaluated with a more holistic approach.

- 2nd study: In the context of this in vitro study, it can be concluded that the EPS of *L. plantarum* EIR/IF-1 from postbiotics consisting of two fractions of 51 and 841 kDa have antibiofilm activity against oral bacteria, which can be explained by the inhibitory effect of EPS on bacterial auto-aggregation, co-aggregation, and hydrocarbon binding. Although EPS ap-pears to be the major antibiofilm component of the *L. plantarum* EIR/IF-1 postbiotics, other components such as proteins and short-chain fatty acids may also have beneficial effects on symbiotic host-bacteria interactions. Therefore, further studies are needed to function-ally characterize the postbiotics of *L. plantarum* EIR/IF-1 and investigate other possible functions against biofilms. For EPS derived from LAB could also act as an agent against biofilms by down-regulating adhesin production of some bacteria or inhibiting the quorum sensing mechanism.

- 3rd study: *L. plantarum* EIR/IF-1 postbiotics and their protein EPS fractions caused a significant increase in the expression of human beta-defensin 2. In addition to the antimicrobial and antibiofilm activities of the postbiotics, it was predicted that they would increase the expression of beta-defensin 2, a natural antimicrobial peptide that plays a role in primary defence, when administered intraorally.

- 4th study: It has been determined that *L. plantarum* EIR/IF-1 postbiotics and proteins obtained from postbiotics increase the membrane permeability of periodontal pathogens even under lethal concentrations.

Comments on some mishaps in the work process

1) Due to the severe COVID-19 situation I had in December 2021, I could not work actively for about a month. This is because my existing heart condition worsened.

2) In July 2022, I had to return to Turkey for fifteen days due to a compelling and special reason.

3) The fourth work package could not be implemented. This was due to the fact that the dental simulator, which was the subject of the study, belonged to a company with which the Institute of Dentistry collaborated. This company stopped most of its work due to the difficult conditions after the pandemic.

5. OUTPUTS (PUBLICATIONS, PRESENTATIONS, ETC.)

Publications

Karaca, B., Haliscelik, O., Gursoy, M., Kiran, F., Loimaranta, V., Söderling, E., & Gursoy, U. K. (2022). Analysis of Chemical Structure and Antibiofilm Properties of Exopolysaccharides from *Lactiplantibacillus plantarum* EIR/IF-1 Postbiotics. Microorganisms, 10(11), 2200.

Karaca, B., Yilmaz, M., & Gursoy, U. K. (2022). Targeting Nrf2 with Probiotics and Postbiotics in the Treatment of Periodontitis. Biomolecules, 12(5), 729.

As an original research article, the study titled “Postbiotics of the *Lactiplantibacillus plantarum* EIR/IF-1 strain show antimicrobial activity against oral microorganisms with pH adaptation capability” is in the process of peer review.

The study titled “Determination of The Immunostimulatory Properties of Protein and Exopolysaccharide Fractions Obtained from *Lactiplantibacillus plantarum* EIR/IF-1 Postbiotics on Gingival Keratinocyte Cell Lines” is being prepared as an original research article.

Presentation

Effects of lactic acid bacteria derived postbiotics against oral microorganisms. 2nd International Dental Oral Infections, Sakarya, TURKEY,2022. Oral presentation.

Seminar

From prebiotics to postbiotics in the FINDOS-Turku Doctoral Programme seminar series on 24 November 2021.

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