

Research Article**A Comparative Analysis of Biofilm Characteristics of Dual-Species Periodontopathogenic Biofilm based on *Fusobacterium nucleatum* and the Dual-Species Biofilm Response in the Presence of Antimicrobial Peptide**

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Abstract: *Fusobacterium nucleatum* (*F.nucleatum*), which acts as a linking periodontopathogenic bacteria, has the ability of coaggregation with early and late colonization species. It is the most intense gram-negative bacteria found in periodontal health and increases in periodontal diseased areas numerically. In this study, comparative analyses of dual and mono-biofilm structural characteristics formed by *Porphyromonas gingivalis* and *Prevotella intermedia* with *F. nucleatum* have been evaluated. In all results, more biofilm formation was observed in dual-species compared to mono-species biofilms. Biofilm formation of *F. nucleatum-P.intermedia* has increased in the presence of Human Neutrophilic Peptide (HNP-1). In this study it is concluded that dual-periodontopathogenic coaggregations based on *F. nucleatum* could produce more intense biofilm structure than single-species ones, and the increase in dual-species biofilm mass in the presence of low doses of HNP-1 may be a defense mechanism of dual periodontopathogenic biofilm.

Keywords: *Periodontitis; subgingival; biofilm; antimicrobial; peptide; gram-negative bacteria; defensin; inflammation*

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1. Introduction

Bacteria within the oral cavity are organized as multi-species biofilms. These structures containing around 500-700 species play an important role in the development of caries and periodontal diseases. Periodontitis is a biofilm induced bacterial inflammatory disease and characterized by progressive destruction of tooth supporting tissues. It is known that bacteria in biofilm have different characteristics compared to their planktonic life (Walker et al., 2007). There are studies showing that bacteria in biofilm can possess a more resistant structure against variables such as antimicrobial agents, physical forces, and pH changes (Shaddox et al., 2010). It has also been reported that multi-species biofilm structure may exhibit more

pathogenicity than single-species bacterial infections (Ebersole et al., 2017; Lamont et al., 2015; Lamont et al., 2018). Gram-negative bacteria are etiologically the most common species in the development of periodontitis. Among these, *F. nucleatum* plays a key role in the formation of dental biofilms and is involved in the association of early and late colonization types, including *P. gingivalis* and *P. intermedia*, which play important role in the progression of periodontitis (Bolstad et al., 1996; Kolenbrander et al., 1993).

The Cationic Antimicrobial Peptides (CAMPs), important elements of the host defense system against the presence of infectious agents, belong to two families in human, namely defensins and cathelicidins. CAMPs act as a broad spectrum antimicrobial and also involve in immune system stimulation. HNP-1 has been shown to be the most abundant one among the 4 types of defensins isolated from neutrophilic granules (Keskin et al., 2014; Lundy et al., 2008). Bacteria and defensins are constantly present in the mouth. According to study of Keskin M. et al. (2014) it was concluded that *F. nucleatum* could develop some adaptation responses to the presence of defensins. The aim of this study is to investigate the dual-species biofilm characteristics of *F. nucleatum* with *P. gingivalis* and *P. intermedia* and also the dual-species biofilm characteristics in the presence of low concentration of HNP-1.

2. Materials and Methods

2.1. Bacterial Species and Cultures

In all laboratory experiments, type strains of *F. nucleatum*, *P. gingivalis* and *P. intermedia* (*F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611) were used. All bacteria were incubated in hemin (5 mg/l) and vitamin K1 (10 mg/l) supplemented Brucella agar at 37°C in 10% H₂, 5% CO₂, and 85% N₂ anaerobic atmosphere (Whitley A35 Anaerobic Workstation, Don Whitley Scientific Ltd., West Yorkshire, UK) for 72 hours. Mature colonies were collected with sterile cotton swabs and transferred to KH₂PO₄ (final concentration: 25 mM), MgSO₄ (final concentration: 4 mM) and saccharose (final concentration: 1%) supplemented Bactoeryptone specific broths and incubated for 48 hours under anaerobic conditions. Optical densities were standardized to 0.6_{OD} at 490 nm just before the experiments.

2.2. Experimental Groups

The following seven different groups were designed;

Group A: *F. nucleatum* alone: 180 µl bacterial growth media + 20 µl *F.nucleatum*

Group B: *P. gingivalis* alone: 180 µl bacterial growth media + 20 µl *P.gingivalis*

Group C: *P. intermedia* alone: 180 µl media + 20 µl *P.intermedia*

Group D: *F.nucleatum* +*P.gingivalis* (Dual): 180 µl media + 10 µl *F.nucleatum* + 10 µl *P.gingivalis*

Group E: *F.nucleatum* + *P. intermedia* (Dual): 180 µl media + 10 µl *F.nucleatum* + 10 µl *P.intermedia*

Group F: *F.nucleatum* + *P. gingivalis*. (Dual+HNP): 180 µl media + 10 µl *F.nucleatum* + 10 µl *P.gingivalis*. + 5 µg/ml HNP-1)

Group G: *F.nucleatum* + *P. intermedia* (Dual+HNP): 180 µl media + 10 µl *F.nucleatum* + 10 µl *P.gingivalis* + 5 µg/ml HNP-1)

All experiments were performed in triplicate wells for each condition and repeated at least twice. Samples were taken from all the wells and placed on Brucella agar for contamination control.

2.3. Preparation of Defensin Suspension

HNP-1 was purchased commercially (HNP-1/Code: 4271-s Lot: 610505, Peptide institute, Japan) and stored at -20°C until use. HNP-1 was dissolved in bacterial growth media on the day of the experiment and prepared as 5 µg/ml concentration.

2.4. Preparation of Saliva Coated 96-well Plates

Clarified Saliva were prepared and stocked before all experiments. Salivas were collected from healthy male and female volunteers to 50 ml propylene tubes and centrifuged at 12,000 g at 4°C for 40 minutes. The supernatants were collected and divided into sterile glass tubes. The tubes were incubated for 30 minutes at 60°C for pasteurization and then centrifuged again at 12,000 g and stored at +4°C until use. 20 µl of each supernatant was collected and placed on Brucella agar, then incubated under aerobic and anaerobic conditions for 4 days and sterilization controls were performed. 50 µl of saliva was added to all the wells and plates were incubated for 1 hour at 37°C. All wells were then dried without washing and prepared for the experiment.

2.5. Analysis of Biofilm Mass Formation

Biofilm mass formation tests were performed by Crystal Violet Staining Method in all groups (Keskin et al., 2014). All wells were stained with 0.1% Crystal Violet. After incubation at room conditions for 15 minutes, the wells were washed twice with PBS (Phosphate Buffer Saline) to remove excess dye. 200 µl of acetic acid was added to each well and incubated for 10 minutes to allow homogenous distribution of Crystal Violet dye. Afterwards, spectrometric analysis (570 nm) was performed on all plates.

2.6. Analysis of Biofilm Protein Content

The incubated plates were washed with PBS and dried. At the end of these washing and drying processes, wells free from planktonic bacteria and liquid medium were filled with 100 µl of 0.2 N NaOH. 2 sec/ 80 W sonication (dr hielscher UP50H homogenizer) was applied to each well to ensure homogeneous distribution of the present biofilms at the bottom (Lu et al., 2007). Afterwards, the plates were transferred to a microwave oven operated at 600 W for 20 seconds (Akins et al., 1995). Then, BIO-RAD protein assay dye was added to each well at a rate of 1/4 of the total volume (25 µl). Following the instructions for the

BIO-RAD kit manual, plates were incubated for 5 minutes at room temperature. Then absorbances were measured at 595 nm by spectrophotometer (Thermo Labsystems x-355) and the OD value was recorded.

2.7. Analysis of Biofilm Polysaccharide Quantity

FITC-labeled Concanavalin A (FITC-Con A) fluorescent dye was used for fluorometric analysis of biofilm polysaccharide content. Washing and drying of 96-well plates was carried out for twice with PBS. Each well was filled with 100 µl of FITC-Con A fluorescent dye (concentration of 50 µg/ml) and incubated at room temperature in a dark room for 5 minutes. After incubation, plates were washed twice with PBS and dried. The 96-well plates were analyzed and recorded by using the BIOTEK Synergy HT Fluorometer Instrument (Excitation=485, Emission=528).

2.8. Statistical Analysis

In dual-species biofilm tests, single bacterial values were accepted as constant, and each of different dual-species biofilm structures was accepted as a variable. Paired-sample parametric t-test was used to evaluate whether there was a difference in the biofilm structures of any group. P values <0.05 were considered statistically significant. In defensin tests, dual-species biofilms with HNP-1 free media were accepted as constant value and the wells with HNP-1 were accepted as variable and paired-sample parametric t test was applied for statistical analysis.

3. Results

P. gingivalis-*F. nucleatum* dual-species biofilms produced more biofilm mass, protein and polysaccharide synthesis compared to all mono-species biofilms (Figure 1, 2 and 3) ($p < 0.05$). Similar results have been observed in the dual-species biofilms of *P. intermedia* and *F. nucleatum* (Figure 1, 2 and 3) ($p < 0.05$). Statistically significant polysaccharide synthesis was found in *P. gingivalis*-*F. nucleatum* and *P. intermedia*-*F. nucleatum* dual-species biofilm structures compared to mono-species biofilms (Figure 3) ($p < 0.05$). It was observed that *F. nucleatum*-*P. intermedia* dual-species biofilms were able to produce statistically significant amounts of biofilms in the presence of HNP-1 compared to HNP-1 free media (Figure 4) ($p < 0.05$). All other dual-species biofilm tests with HNP-1 showed no statistically significant difference (Figure 5 and 6).

Figure 1. Dual-Biofilm Formation (Crystal Violet)

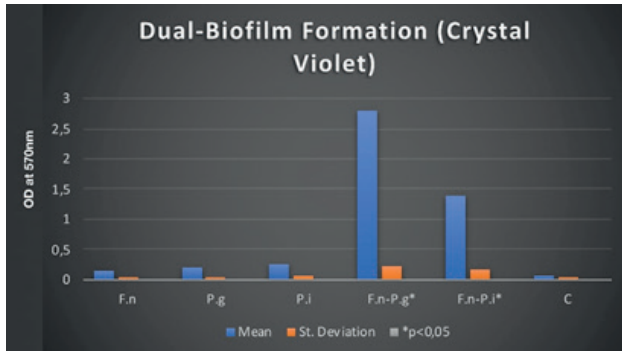


Figure 2. Dual-Biofilm Formation (Protein)

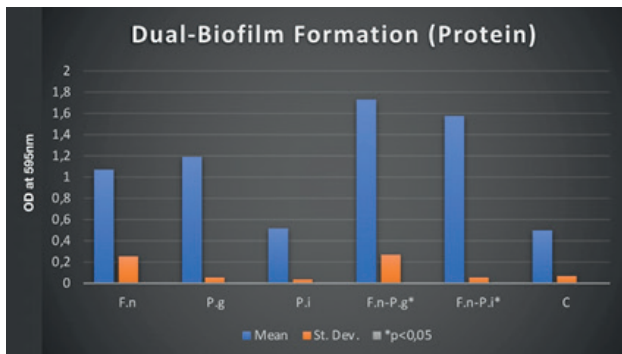


Figure 3. Dual-Biofilm Polysaccharide Production

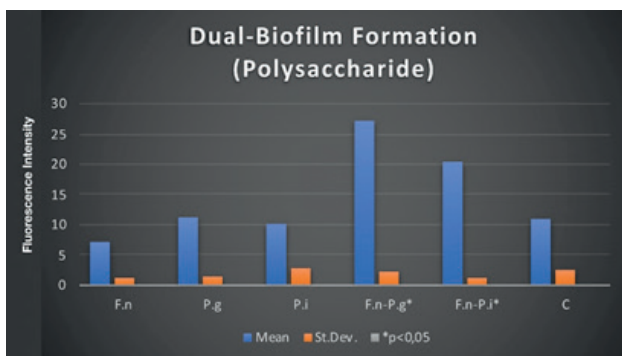


Figure 4. Dual-Biofilm + HNP-1 (Crystal Violet)

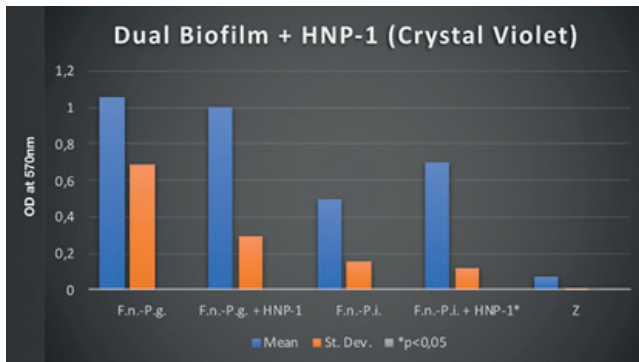


Figure 5. Dual-Biofilm + HNP-1 (Protein)

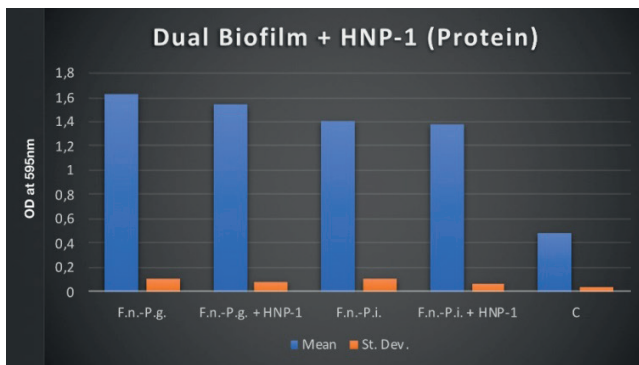
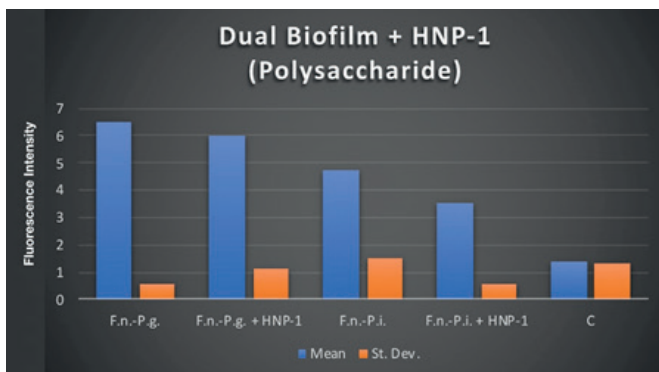


Figure 6. Dual-Biofilm + HNP-1 (Polysaccharide Production)



4. Discussion

Gram-negative, anaerobic bacilli types are most closely associated with periodontitis. Among these, *F. nucleatum*, *P. intermedia*, *P. gingivalis* are the most common bacterial species and play a key role in the progression of the disease (Socransky et al., 1998; Teles et al., 2010).

In this study, characteristics of dual-species biofilms formed by *P. gingivalis* and *P. intermedia* with *F. nucleatum* were investigated. In order to grow them, incubation time of the bacteria in anaerobic conditions was determined as 48 hours for biofilm formation experiments. In previous studies it was shown that anaerobic incubation period of 48-72 hours could provide the appropriate time for the biofilm formation of *F. nucleatum* (Jang et al., 2013; Saito et al., 2008). The Crystal Violet technique was used in the analysis of biofilm mass after incubation. Because it is known as a reliable method and has been used in many studies (Merritt et al., 1998; Pitts et al., 2003; Stepanovic et al., 2000). Bradford Protein Analysis was also included for comparative analysis of total biofilm mass and biofilm protein values. While performing Bradford Protein Analysis, sonication was applied to all the wells to ensure homogeneous distribution of the biofilm mass (Bradford et al., 1976; Lu et al., 2007). FITC - Con A fluorescent dye was used to determine the amount of biofilm polysaccharide. FITC labeled Concanavalin A is a fluorescent fluorophore substance that binds with polysaccharide and is frequently used in *in vitro* polysaccharide studies (Roth et al., 1978; Sato et al., 2006; Yang et al., 2006).

The *P. gingivalis* and *P. intermedia* dual-species biofilms formed by *F. nucleatum* showed statistically significant increase in biofilm mass, polysaccharide synthesis and protein ratios when compared with mono-species biofilms. All these results show that *P. gingivalis* and *P. intermedia* dual-species biofilms formed with *F. nucleatum* are able to produce intense biofilms compared to single-species biofilms synergistically. Based on previous studies, it was observed that bacterial coaggregation plays an important role in biofilm formation. Similar to the results of this study, there are many other studies showing that *F. nucleatum* could play an important role in binding of late colonization species within the biofilm (Kolenbrander et al., 1989; Kolenbrander et al., 1993; Rickard et al., 2003).

In this study, dual periodontopathogenic biofilm characteristics were analyzed in the presence of HNP-1 comparatively. Other studies show that the HNP-1 concentration in the oral cavity can be found in a wide range of values from 0.40 µg/ml to 100 µg/ml (Dale et al., 2006; Fanali et al., 2008; Goebel et al., 2000; Puklo et al., 2008). However, there is a finding which indicates no lethal effect at low concentration levels of HNP-1 on *F. nucleatum* (Miyasaki et al., 1998). Therefore, the concentration of HNP-1 (5 µg/ml), which can be found in oral cavity continuously, was studied to evaluate the defensive response of dual-species biofilms to HNP-1 challenge. It was observed that *F. nucleatum* - *P. gingivalis* dual-species biofilm did not show any significant change of biofilm production in the presence of HNP-1 compared to defensin-free media. Additionally, it was seen that the presence of HNP-1 did not lead to statistically significant difference in polysaccharide production at any biofilm group. It was formerly reported that dual-bacterial biofilms form an intense biofilm structure than single bacteria biofilms (Okuda et al., 2012; Saito et al., 2007).

Although *P. intermedia* - *F. nucleatum* dual-species biofilm showed a statistically significant increase in biofilm mass in the presence of HNP-1, the same increase was not observed in polysaccharide

values. Flemming et al. reported that the matrix structure of the biofilm occurs in products other than polysaccharides such as proteins, lipids and nucleic acids, as well (Flemming et al., 2010). Consequently, dual-periodontopathogenic coaggregations based on *F. nucleatum* could produce more intense biofilm structure than single-species ones, and the increase in dual-species biofilm mass in the presence of low doses of HNP-1 could act as a defense mechanism of *F. nucleatum*-*P. intermedia* dual-species biofilm. More enlightening studies are needed over this subject.

Conflict of interest

Author declares no conflict of interests.

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