

Biofilm and planktonic lifestyles of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

Proteomic analysis of bacteria grown as planktonic cells, mono- and dual species biofilm, and characterization of the biofilm extracellular polymeric matrix'

Marwan Mansoor Ali Mohammed

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2018

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Abbreviations

ATP	Adenosine triphosphate
Bap	Biofilm associated protein
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CLSM	Confocal Laser Scanning Microscopy
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic Acid
eDNA	Extracellular Deoxyribonucleic Acid
EPH	Ecological Plaque Hypothesis
EPM	Extracellular Polymeric Matrix
EPS	Extracellular Polymeric Substances
FAA	Fastidious Anaerobic Agar
FASP	Filter Aided Sample Preparation
LC-MS	Liquid chromatography-mass spectrometry
LFQ	Label-Free Quantification
LPS	Lipopolysaccharide
NSPH	Non-Specific Plaque Hypothesis
OD	Optical Density
OMVs	Outer Membrane Vesicles
PBS	Phosphate Buffered Saline
PSD	Polymicrobial Synergy and Dysbiosis
SPH	Specific Plaque Hypothesis

Scientific environment

The following doctoral work including all laboratory work in this study were conducted at the Faculty of Medicine, University of Bergen, Bergen, Norway.

Laboratory experiments

Section for Microbiology and Immunology

Department of Clinical Science

Proteomics

The Proteomics Unit at the University of Bergen (PROBE)

The Department of Biomedicine

Course and administrative work

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Bergen, December 18, 2017

Summary

Periodontitis is one of the most prevalent infectious diseases affecting humans. Periodontitis leads to the destruction of the dental support tissues, which in the terminal stage causes loss of teeth. Periodontitis is biofilm related, a situation where several bacterial species are organized as a community whose resident species differ in many respects from their planktonic (free-living) counterparts. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are among the subgingival bacterial species that play a major role in the dental biofilm formation. *F. nucleatum* acts as a bridge between early and late colonizers in the dental biofilm and coaggregates with almost all the species that are considered putative periodontal pathogens. *P. gingivalis* harbors many virulence factors that facilitate colonization and invasion of the periodontal epithelial lining. The main aim of this project was to study in depth and characterize *in vitro* a dual species biofilm composed of *F. nucleatum* and *P. gingivalis* using molecular imaging techniques and proteomics. Furthermore, we wanted to explore the extracellular polymeric substances in the biofilm matrix of the dual and mono-species biofilm, followed by protein identification and analysis of their differential expression.

Our results show that proteins and carbohydrates are the major components of the biofilm matrix, and that extracellular (eDNA) is also present. The matrix components are also shown to vary among the species. Proteinase K enzyme showed no effect on the concentration of the eDNA or carbohydrate isolated from the treated matrices. DNase I and proteinase K enzymes had no significant effect on biofilm formation or on mature biofilms under the conditions studied. In the flow-cell biofilm model, *F. nucleatum* was able to grow in partially oxygenated conditions while *P. gingivalis* failed to form a biofilm alone under similar conditions but it can grow with *F. nucleatum* as a dual species biofilm.

We identified 542, 93 and 280 proteins from the matrices of *F. nucleatum*, *P. gingivalis*, and the dual-species biofilms, respectively. Nearly 70% of all matrix

proteins in the dual-species biofilm originated from *F. nucleatum*, and a majority of these were cytoplasmic proteins, suggesting enhanced lysis of *F. nucleatum* cells. The proteomic analysis also indicated an interaction between the two species: 22 *F. nucleatum* proteins showed differential levels between the mono and dual-species extracellular polymeric matrices (EPMs), and 11 proteins (8 and 3 from *F. nucleatum* and *P. gingivalis*, respectively) were exclusively detected in the dual-species EPM. Oxidoreductases and chaperones were among the most abundant proteins identified in all three EPMs. The biofilm matrices also contained several known and hypothetical virulence proteins, which can mediate adhesion to the host cells and disintegration of the periodontal tissues.

Comparisons between the protein profiles for the two bacterial species grown as a biofilm or in the planktonic state, and when grown as a mono- or dual-species biofilm, showed significant differences between each setting examined. The most abundant proteins have function such as oxidoreductases, acyltransferases, outer membrane proteins and proteases. Several virulence factors were among the most abundant proteins in both biofilm and planktonic growth conditions. Vitamin B biosynthesis proteins were increased in the biofilm setting compared to the planktonic. When grown in dual species, *P. gingivalis* showed reduced protein levels in many functions including vitamin biosynthesis, nucleotide biosynthesis, lipid or fatty acid biosynthesis and translation and ribosomal process. These results indicated how growing in a community provides a favorable environment to *P. gingivalis* and reduces its stress.

List of publications

I. **Mohammed MMA**, Nerland AH, Al-Haroni M, Bakken V. Characterization of extracellular polymeric matrix, and treatment of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNase I and proteinase K. **J Oral Microbiol.** 2013;5: 20015 - <http://dx.doi.org/10.3402/jom.v5i0.20015>.

II. **Mohammed MMA**, Pettersen VK, Nerland AH, Wiker HG, Bakken V. Quantitative proteomic analysis of extracellular matrix extracted from mono- and dual-species biofilms of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. **Anaerobe.** 2017;44:133-42.

III. **Mohammed MMA**, Pettersen VK, Nerland AH, Wiker HG, Bakken V. Proteomic analysis of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms and planktonic cultures when grown as mono- and dual-species models. **In manuscript.** 2017; to be submitted for publication.

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1. Introduction

1.1 Bacterial biofilm

1.1.1 Biofilm mode of growth

A biofilm has been defined as “an organized community of surface adherent microorganisms embedded in an external polymeric matrix” or as a “matrix-enclosed bacterial population adherent to each other and/or to surface or interface”. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media (1). Biofilms occur in a variety of places, from pipelines and ship bottoms to teeth. Biofilms develop in a four-stage process; the initial stage includes the attachment of planktonic microorganisms to the substratum (Fig. 1). This is followed by bacterial growth, and cell division which leads to the colonization of the surrounding area (irreversible attachment), followed by external matrix production and formation of the biofilm (maturation) (2). These three stages are followed by the final stage of biofilm development which is the detachment of cells from the biofilm and their dispersal into the environment (3). Bacteria do not act individually to form biofilms, but co-aggregate to help initiate the early stages of biofilm formation. The regulation of gene expression in response to this local accumulation of large numbers of bacteria is recognized as quorum sensing (4). With quorum sensing, a population of unicellular organisms can synchronize the production of virulence factors for shared defense, or of colonization factors for symbiotic interaction with the host (4). Following the initial adhesion, adherent cells begin to change their original pattern of gene expression to their biofilm phenotype, and the secretion of polysaccharides and other matrix components transform their physical connection to the surface and to each other. Surfaces may in turn influence the resultant microbial communities, if they contain insoluble nutrients (e.g., cellulose) or reduced metal salts, because the biofilms will produce high local concentrations of enzymes and shuttle molecules to mobilize this energy (5).

The mature biofilm is a complex heterogeneous structure of dormant and actively growing bacterial colonies along with further enzymes, excretory products and small channels forming part of the overall structure. The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) and a wide range of physical, metabolic and chemical heterogeneities (6).

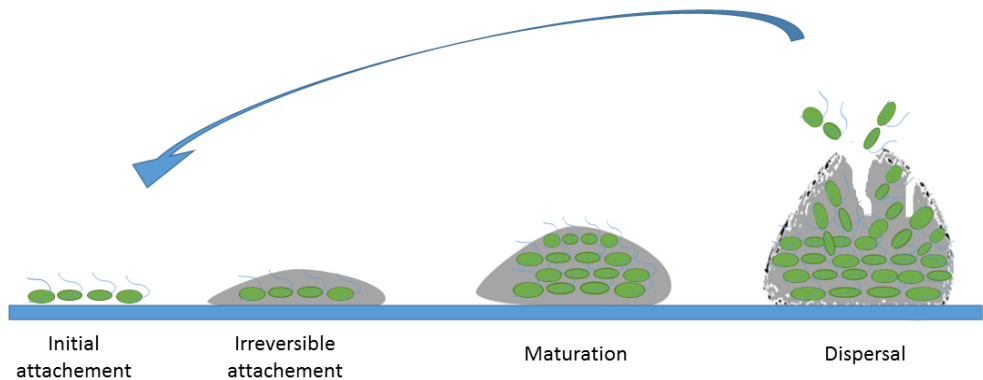


Figure 1. Diagram showing the development of a biofilm as a four-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of the extracellular polymeric substance. Stage 3: maturation of biofilm architecture. Stage 4: dispersion of single cells from the biofilm. Detached cells disseminate and adhere elsewhere to start new biofilm if conditions are suitable. Adopted from (7).

It is now recognized that biofilm formation is an important aspect of many diseases, including endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients (8). According to the CDC, 65% of all infections in developed countries are caused by microbial biofilms (9). Biofilms can tolerate antimicrobial agents at concentrations of 10–1000 times more than that needed to eradicate genetically equivalent planktonic bacteria (10, 11). They are also very resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts (9).

1.1.2 Extracellular polymeric substances

All biofilms share several common features including the production of EPS, which makes up the major constituent of biofilm other than the bacterial cells. In general, it is estimated that the microorganisms account for less than 10% of the dry weight of the biofilms, whereas the matrix can account for more than 90% (12). EPS are hydrated biopolymers secreted by bacteria that surround and immobilize microbial aggregates, leading to the macroscopic appearance of biofilms, which are frequently referred to as 'slime'(12). The matrix increases resistance to host defenses and antimicrobial agents, compared with the more vulnerable; free-floating cells, and it forms a hydrated barrier between cells and their external environment. The functions of the matrix include adhesion, aggregation of microbial cells, cohesion of biofilm, retention of water, absorption of organic and inorganic material, enzymatic activity, nutrient source, exchange of genetic information, and export of cell components (12). The EPS are chemically complex and can vary significantly between biofilms, depending on the microorganisms present, the shear forces experienced, the temperature and the accessibility of nutrients. EPS were initially called 'extracellular polysaccharides' but were renamed, as it became clear that the matrix also contains proteins (Fig. 2), nucleic acids, lipids and other biopolymers such as humic substances (12).

Biofilms of different origins have been found to contain extracellular DNA (eDNA), but it was reported to occur in particularly large amounts in waste-water biofilms and recent studies indicate that eDNA plays an important role in the establishment of *S. aureus* biofilm structure (12-14).

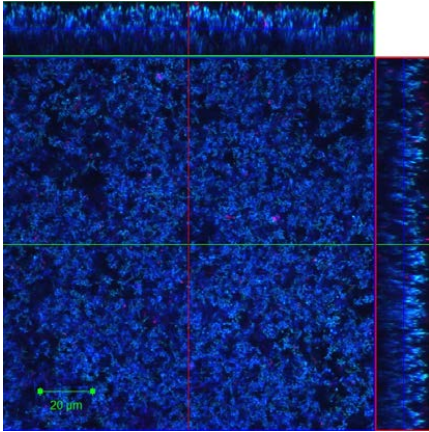


Figure 2. Representative CLSM image showing proteins in EPM of 24 h dual species biofilm (*Fusobacterium nucleatum* ATCC 25586 and *Porphyromonas gingivalis* W50) grown in flow cells. The proteins were stained with SYPRO® Ruby stain (blue). (Source: Marwan M A Mohammed).

1.2 Biofilm dispersion

The extracellular polymeric substances can be considered as a house for the biofilm cells (15). Biofilm dispersal can be defined as a mode of biofilm detachment with mechanisms that cause individual cells to separate from the biofilm and return to planktonic life (16). The mechanisms of biofilm dispersal can be active or passive (3). Active dispersal refers to mechanisms that are initiated by the bacteria, whereas passive dispersal refers to biofilm cell detachment that is mediated by external factors such as fluid shear, abrasion, predator grazing, and human intervention (3). Promoting detachment by the use of substances to induce biofilm removal directly by destroying the physical integrity of the biofilm matrix became an alternative for both medical and industrial applications where complete biofilm removal is essential (17). These substances (enzymes) can be also used in research that deals with the extraction of EPS components, enabling good separation for the components of the EPS to facilitate further investigations on these molecules (18).

1.2.1 Biofilm matrix-dispersing enzymes

The increase in the prevalence of antibiotic resistance has made the use of antimicrobial enzymes in the disruption of bacterial biofilm formation an area of intense exploration (19). Production of extracellular enzymes that degrade adhesive components in the biofilm matrix is the basic mechanism of biofilm dispersal and the enzymes implicated in active biofilm dispersal include glycosidases, proteases, and deoxyribonucleases (DNase) (3) as shown in Table 1.

It has been shown that eDNA is important for biofilm formation, and for providing adhesive support and protection of microbial cells in the biofilm (13, 20-22). Targeting eDNA in the biofilm matrix with enzymatic treatment therefore became an area of interest for many researchers, and a number of studies have now confirmed that different DNase enzymes can inhibit the formation of biofilms, or can disperse preformed biofilms, of many bacteria and fungi (23). Treatment of *Escherichia coli* and *Staphylococcus aureus* biofilms with DNase I displayed reduced biofilm biomass, total bacterial biomass, decreased the viability of bacteria, and decreased tolerance to antibiotics (24). Comparison has also been made of *Acinetobacter baumannii*, *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus* and *Streptococcus pyogenes* treated with DNase I alone and combined with antibiotics. The use of antibiotics combined with DNase I resulted in a significant decrease in the established biofilm biomass compared to the reduction of biomass when each antibiotic or DNase I was used alone (25). Clinically, Dornase alfa (Pulmozyme, recombinant human DNase 1, rhDNase) is an enzyme based product that has become one of the most commonly used medications to treat cystic fibrosis in the lung (26).

However, there are also several examples of biofilms that contain significant quantities of eDNA but are not dispersed by DNase enzymes (27-29). *F. nucleatum* and *P. gingivalis* biofilms are examples of biofilms that contain eDNA in their matrix but show no significant response when treated with DNase I (30).

Proteases also show anti-biofilm activity, because they degrade proteinaceous adhesins such as pili, fimbriae, and surface adhesins that are required for bacterial

Table 1. Examples of the enzymes that can be used to disperse bacterial biofilms grouped according to the targeted structural components of the EPS. The table was prepared depending on these references (19, 31, 32).

Enzyme type	Examples
Proteolytic enzymes	Subtilisins, lysostaphin, bacteriophage lysins, proteinase K, protease A, papain, serratiopeptidase
Polysaccharide-degrading enzymes	Lysozymes, pectin methylesterase, alginate lysases, Dispersin B, amylases, N-glycanases, hyaluronidase
DNA-degrading enzymes	DNase I, restriction endonucleases, nuclease NucB, Dornase alpha
Oxidative enzymes	Glucose oxidase, hydrogen peroxide-responsive enzymes, lactoperoxidase
Anti-quorum sensing enzymes	Lactonase, acylase I, paraoxonase

cell-to-cell and cell-to-surface interactions (19). Proteinase K enzyme showed dispersal effect on *S. aureus* (33, 34). This effect was targeted to biofilm-associated protein (Bap), which has been reported to have a crucial role in the early stages of *S. aureus* biofilm development (34). On the other hand, oral bacterial biofilms of *F. nucleatum* and *P. gingivalis* were resistant to detachment by proteinase K even when tested at high concentrations (30). Interestingly, *Rhodococcus ruber* C208 bacteria respond with enhanced biofilm formation when treated with proteinase K, and the heat inactivated enzyme produces no effect (35). It may be hypothesized that in this bacteria, proteinase K degrades the self-secreted extracellular proteases responsible for the detachment process, suggesting that it may be necessary to tailor treatment specifically for different species or microorganisms (35).

Among the polysaccharide hydrolyzing enzymes; lysozymes, alginate lysases, Dispersin B and amylases are by far the most commonly used enzymes (19). One well-studied biofilm-matrix-degrading enzyme is Dispersin B, which is a 42-kDa bacterial glycoside hydrolase produced by the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (36). Dispersin B degrades poly-N-acetylglucosamine (PNAG), a biofilm matrix polysaccharide that facilitates attachment of *A. actinomycetemcomitans* to abiotic surfaces (3). Several studies showed the efficacy of this enzyme alone or combined with other materials in dispersing bacterial biofilms (37-40).

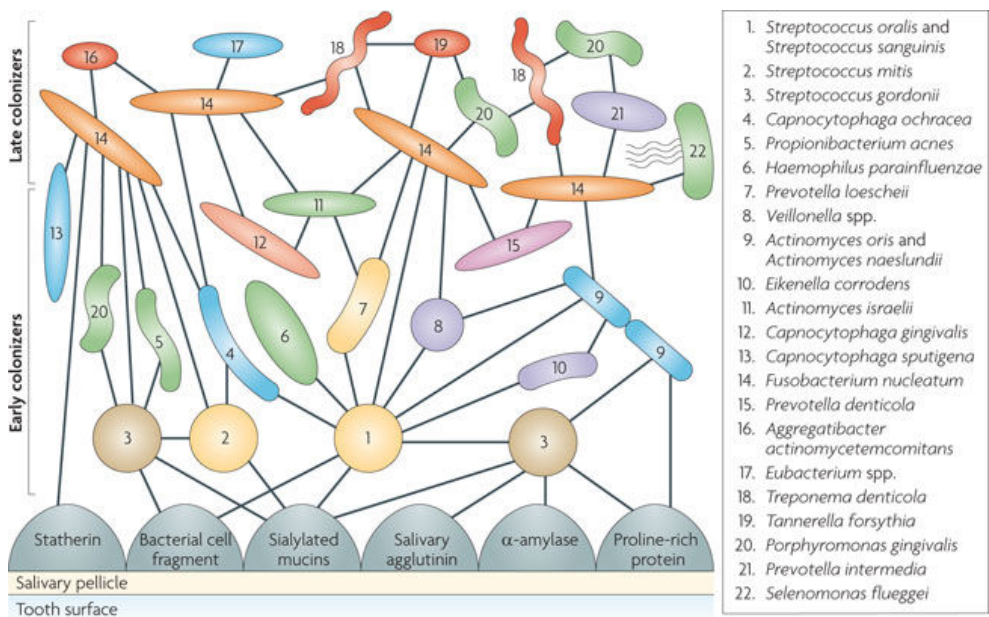
Most dispersal studies have been done *in vitro* with mono-species biofilms. It is extremely difficult to generalize these results to any environmental biofilm, especially to a complex biofilm community like dental biofilm. While several potential dispersal-inducing agents have been identified, it remains to be seen whether any of these agents will have clinical significance (3).

1.3 Oral biofilm

1.3.1 Definition, structure and formation

Dental plaque is a complex microbial biofilm (Fig. 3), and it is the key factor associated with the two main dental and oral diseases, dental caries and periodontal disease (41). Dental plaque was the earliest biofilm studied: it was explored in the seventeenth century by Antonie van Leeuwenhoek when he reported the diversity and high number of ‘animalcules’ present in ‘scrapings’ taken from around human teeth (42). It is defined clinically as the soft, tenacious deposit that forms on tooth surfaces that is not readily removed by rinsing with water (43). Microbiologically, it can be defined as the diverse community of microorganisms found on a tooth surface as a biofilm, embedded in an extracellular matrix of polymers from the host, and is of microbial origin (41).

Biofilm development in the oral cavity starts with the formation of acquired pellicle, which is a thin coating of salivary proteins that attach to the tooth surface within minutes after a professional cleaning. Microorganisms are then transported passively by salivary flow and attach to the outer surface of the pellicle by reversible and weak physicochemical forces (Van der Waals and electrostatic energy). The attachment becomes irreversible when the adhesins on the microbial surfaces interact with receptors on the acquired pellicle (adhesin-receptor interaction).



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Figure 3. Spatiotemporal model of oral bacterial colonization, representing the initial colonizers binding to the complementary salivary receptors in the acquired pellicle, and the late colonizers and the bridging bacteria in-between. The model was proposed by Kolenbrander and London (44-46). Reprinted by permission of Nature Publishing Group.

At this stage, the tooth's surface is colonized predominantly by Gram positive facultative cocci, primarily streptococcal species, followed by coaggregation/coadhesion and microbial succession to form the mature biofilm with excessive

diversity and a slower mode of growth. Detachment from surfaces begins due to shear forces and can be attached or colonize elsewhere (47).

1.3.2 Microbial interactions in biofilms

The close proximity of the cells within a biofilm offers an ideal environment for cell-to-cell interactions. These interactions occur through metabolic communication, which can be synergistic and thus beneficial to the involved population, or antagonistic. For example, the excretion of a metabolite by one organism can be used as a nutrient by a different organism, or the breakdown of a substrate by extracellular enzymatic activity of one organism may create biologically available substrates for different organisms (45). The exchange and metabolism of oxygen within the biofilm is another form of communication between different aerobic and obligate anaerobic species and plays an especially significant role for the survival of obligate anaerobes (48).

Coaggregation is the physical interaction between bacteria of different species. It is not random among oral bacteria; each species binds specifically to other bacteria. Coaggregation interactions are believed to contribute to the development of biofilms by two routes. The first route is by single cells in suspension specifically recognizing and adhering to genetically distinct cells in the developing biofilm. The second route is by the prior coaggregation in suspension of secondary colonizers followed by the subsequent adhesion of this coaggregate to the developing biofilm. In both cases, bacterial cells in suspension (planktonic cells) specifically adhere to cells in the biofilm in a process known as coadhesion (49, 50).

Another form of communication among oral bacteria in dental biofilm is cell-cell signaling whereby individual cells are able to communicate with, and respond to, neighboring cells by means of small, diffusible, effector molecules such as cell density dependent growth (quorum sensing) (47). The close proximity of the cells in the biofilm may also offer an excellent milieu for DNA exchange (gene transfer), as

the cells are in close juxtaposition and DNA can be trapped within the extracellular matrix (46, 47).

In summary, the oral biofilm is associated with some of the most frequent chronic infections in humans (51) and it is among the first and most thoroughly studied biofilm causing infectious diseases. However, the diversity, complexity and multispecies nature of the oral biofilm makes further research imperative (52, 53).

1.4 Periodontal diseases

The periodontal diseases are a group of diseases characterized by inflammatory responses in the periodontium to bacterial accumulation on teeth adjacent to the gingiva (54). According to the periodontal diseases classification that resulted from a 1999 international workshop (55), diseases of the periodontium contains a long list of conditions involving the supporting structures of the tooth. The two most common and most investigated periodontal diseases are dental plaque-induced gingivitis and chronic periodontitis. Gingivitis is the simplest and reversible form of periodontal disease characterized by inflammation of the gingiva without destruction of the supporting tissues, while periodontitis is characterized by loss of the collagen periodontal attachment, loss of supporting alveolar bone and formation of deep periodontal pockets. Periodontal diseases are highly prevalent and can affect up to 90% of the worldwide population and are considered to be the main cause of tooth loss in adults. Recently, there has been increasing interest in the relationship of periodontal disease to important systemic diseases such as cardiovascular disease, stroke, diabetes mellitus, pulmonary disease and complications in pregnancy (54, 56).

The last 10 to 15 years have seen the emergence of several important new findings and concepts regarding the pathogenesis of periodontal diseases. These findings include the recognition of dental bacterial plaque as a biofilm, identification and characterization of genetic defects that predispose individuals to periodontitis, host-

defense mechanisms implicated in periodontal tissue destruction, and the interaction of risk factors with the host defenses and bacterial plaque (57).

1.5 Microbiology of periodontal disease

There is wide agreement that microorganisms are the primary etiologic agents of various forms of periodontal disease. Particularly convincing data to support this came from the demonstrations by L oe and co-workers that removal of dental plaque by rigorous plaque control procedures or antiseptic agents could prevent or reverse clinical gingivitis in human volunteers (58-60).

The search for the etiological agents of destructive periodontal disease has been in progress for over 100 years. However, until recently, there has been on-going controversy as to which bacteria within the biofilm are involved in the causation of these diseases. Two main hypotheses exist: the non-specific and specific plaque hypotheses (NSPH and SPH, respectively), first described by Loesche (1976). The NSPH considers the entire plaque flora as a producer of irritant products that, if exceeding the host detoxification threshold, result in slow tissue destruction (61). Consequently, treatment based on this hypothesis relies upon mechanical debridement of dental biofilm from the tooth surfaces for treatment and prevention; this non-specific plaque mass reduction has been the paradigm of dental care for more than 100 years (61, 62), but the NSPH failed to explain why certain individuals with longstanding plaque and gingivitis do not develop periodontitis. While the NSPH focuses on quantitative changes, the SPH focuses on qualitative changes, and states that only plaque with certain pathogens and/or a relative increase in levels of given indigenous plaque organisms causes infections. It was proposed that the treatment should be aimed at the diagnosis and then elimination of causative organisms, usually with an antimicrobial component. While there is evidence to support effectiveness of this approach from selective suppression of the microflora by chemotherapy using

both human and animal models, the current treatment paradigm dictated by the NSPH still predominates (63, 64).

Some of the reasons for the uncertainty in defining periodontal pathogens were determined and described by Haffajee and Socransky in 1994, including: the complexity and diversity of the subgingival microbiota, difficulty obtaining a representative sample, difficulties in cultivation, characterization and identification of microorganisms in subgingival plaque, mixed infections, and opportunistic microbial species that may grow as a result of the disease, taking advantages of the conditions produced by the true pathogen, and periodicity of disease activity. Periodontal disease appears to progress with periods of exacerbation and remission. Ideally, a plaque sample should be taken at the peak of disease activity. Failure to detect the peak of activity may lead to an underestimate of the contribution of a pathogen(s) to a given lesion. Multiple periodontal diseases in different subjects that might not be differentiated on a clinical basis, thus, disease types may be misclassified and inappropriately pooled. Differences observed in clinical symptoms in different parts of the mouth may be explained by differences in levels of the pathogen or the stage of the destructive process. Disease might have occurred in shallow lesions due to one species and in deepening lesions by a succession of other species. Disease occurring in one site in the mouth could be due to an agent that is different from the one inducing destruction at a second site at the same time. Pathogens may be carried in low numbers in mouths that are free of destructive periodontal diseases (the so-called carrier state), making their role in disease more difficult to evaluate. Strains of putative pathogens may differ in virulence. A virulent clonal type might be detected in periodontally healthy subjects, whereas non-virulent clonal types might be present in subjects with periodontal disease. An inability to distinguish virulent from non-virulent clonal types would impede understanding. It has been suggested that more virulent strains may harbour bacteriophages or plasmids. Bacterial plasmids are known to code for several virulence factors like invasiveness, adherence, and antimicrobial resistance as well as the production of toxins and noxious products (64). In light of these issues and after reviewing the literature, Haffajee and Socransky

pointed out some candidates as etiological factors of periodontal diseases (64). They later came up with the color-coded system reflecting the cluster analysis, and they described them as microbial complexes (65). The red complex was the species that were strongly associated with periodontitis, followed to a lesser extent by organisms in the orange complex. The rest of the complexes show no association with periodontitis (65).

The ecological plaque hypothesis (EPH) was proposed by Marsh in 1994. According to this hypothesis, the periodontal diseases are opportunistic endogenous infections resulting from a shift in the ecology of the plaque biofilm from a predominantly Gram positive facultatively anaerobic microflora to a Gram negative obligate anaerobic or micro-aerophilic flora, creating an anaerobic environment which helps their growth (66). Thus, any species in the dental biofilm may be pathogenic since ecological changes in the environment may favour the pathogenicity and virulence mechanisms for that particular organism (66, 67). Disease may thus be prevented by interruption of the environmental factors responsible for the ecological shifts as well as elimination of the putative pathogen (68, 69).

Recently the concept of “Polymicrobial Synergy and Dysbiosis (PSD)” was proposed by Hajishengallis et al. (2012), which describes periodontitis initiation by a synergistic and dysbiotic microbiota, within which different members or specific gene combinations fulfill distinct roles that converge to shape and stabilize a disease provoking bacteria (70). The PSD concept was based on the keystone-pathogen hypothesis that states how low-abundance keystone species can disturb the tissue homeostasis through quantitative and qualitative changes to the commensal microbiota and orchestrate the inflammatory disease by remodelling a normal microbiota into a dysbiotic one (71). In a study on mice, it has been shown that *P. gingivalis* can impair innate immunity in ways that enhance the growth of the periodontal microbiota and change its composition (72). The keystone-pathogen *P. gingivalis* was present at low concentration levels (<0.01% of the total microbiota)

and still had the ability to remodel the symbiotic community into dysbiotic state that triggered inflammatory bone loss (72, 73).

1.5.1 *Porphyromonas gingivalis*

P. gingivalis is classified in the genus *Porphyromonas*, family *Porphyromonadaceae*, order Bacteroidales, class Bacteroides, phylum Bacteroidetes (74). The bacterium is non-motile, Gram negative, rod-shaped, anaerobic, asaccharolytic and highly proteolytic. *P. gingivalis*, which is often found in deep periodontal pockets of humans, produces a broad array of potential virulence factors involved in tissue colonization and destruction as well as host defense perturbation (75).

After it was mentioned as member of the red complex (a group of three species including *P.gingivalis*, *Trepomema denticola* and *Tannerella forsythia*, which was strongly associated with each other and with periodontal disease site) and because it was the easiest of the three to grow and genetically manipulated, it became the most widely studied periodontal bacterium (70).

P. gingivalis can locally invade the periodontal tissues and evade the host defense system by utilizing a panel of virulence factors that cause disruption in the immune and inflammatory reactions. The potential virulence factors of *P. gingivalis* have been extensively described in several reviews (75-78). These virulence properties include:

- Ability to adhere to host cells followed by invasion or internalization via lipid rafts (79). This asaccharolytic pathogen can survive and replicate within a vacuole utilizing the host proteins derived by autophagy.
- Lipopolysaccharide (LPS) of *P. gingivalis* is a key factor in the development of periodontitis. It induces pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-6, and IL-8, which induce periodontal tissue destruction and disrupt the bone-remodeling process (80).

- Fimbriae in *P. gingivalis* seem to participate in many interactions between the bacterium and the host, as well as with other bacteria. There are two main types of fimbriae that can be expressed by this pathogen, the major fimbria (FimA) and the minor fimbria (Mfa) (81).
- Hemagglutinins, which are involved in non-fimbrial adhesion of the microorganism to host cells and aid hemin acquisition, which is necessary for bacterial growth, from erythrocytes (82).
- Proteinases, especially cysteine proteases are known to be the most important virulence factors since they are able to degrade the periodontal tissue and at the same time disrupt host defence mechanisms (83). Gingipain is the term describing the cysteine proteases of *P. gingivalis*. They are classified as either Arg-gingipain or Lys-gingipain according to where they cleave the polypeptide (either after arginine or lysine residues) (78).
- Outer membrane vesicles (OMV) are usually involved in bacterial adherence, defense against host factors, and the delivery of a wide range of toxins (84, 85).

The extensive research on *P. gingivalis* leads lastly to consider it as a keystone-pathogen in the periodontal biofilm, since even when available in low abundance it plays a major supporting role for an entire ecological community (71). By destabilizing innate immune signaling including the crosstalk between complement and Toll-like receptors (TLR), *P. gingivalis* can impair host defenses in ways that alter the growth and development of the entire microbial community (86), thereby triggering a destructive change in the normally homeostatic relation with the host. Therefore, *P. gingivalis* orchestrates rather than directly causes inflammatory bone loss, which is largely mediated by commensals that under conditions of disrupted homeostasis have the potential to cause deregulated inflammation and disease (87).

1.5.2 *Fusobacterium nucleatum*

F. nucleatum is the type species of the genus *Fusobacterium*, which belongs to the family *Bacteroidaceae*. Among the 13 species in this genus (88), *F. nucleatum* species are most frequently isolated from the oral cavity. The bacterium is an anaerobic, non-spore forming, non-motile Gram negative rod bacterium with fused ends (89). The heterogeneity of *F. nucleatum* is well known and four (or five) different subspecies of *F. nucleatum* have been proposed (90-93). The five described *F. nucleatum* subspecies are: *nucleatum*, *vincentii*, *polymorphum*, *fusifforme* and *animalis* (90, 91, 93). The taxonomy of *F. nucleatum* subspecies is still an open discussion, as Kook et al. (94) recently proposed that *F. nucleatum* subsp. *fusifforme* and *F. nucleatum* subsp. *vincentii* could be classified as a single subspecies. *F. nucleatum* subsp. *vincentii* was an early published name; therefore, *F. nucleatum* subsp. *fusifforme* proposed by Gharbia and Shah can be regarded as a later synonym of *F. nucleatum* subsp. *vincentii* proposed by Dzink et al. (94).

F. nucleatum is typically considered a strict anaerobe, but it can tolerate up to 6% oxygen atmosphere (89), and it responds by physiological changes and increased pathogenicity to oxidative stress (95, 96). In addition, *F. nucleatum* shows more tolerance to aerobic conditions in a biofilm than in planktonic form (30, 97). Figure 4 shows *F. nucleatum* grown in a flow-cell biofilm model in partially oxygenated condition. This capacity enables *F. nucleatum* to play a protective role to the obligate anaerobic species in both biofilm and planktonic phases of aerated, mixed cultures of oral bacteria. It has been proposed that this co-aggregation is the mechanism by which strict anaerobes, such as *P. gingivalis*, survive under aerobic conditions, due to the formation of microenvironments in which the facultative organisms mediated reducing conditions (98, 99).

F. nucleatum has an excellent co-aggregating capacity with many bacterial species in the oral cavity (100). This capability allows *F. nucleatum* to work as a bridge or a central species in physical interaction between Gram positive partners which represent the early colonizers on the teeth surfaces and Gram negative partners which

are the late colonizers and mostly obligate anaerobic species (46). These strict anaerobes can also benefit from the capacity of *F. nucleatum* to adapt to and reduce an oxygenated environment as mentioned earlier (45, 99).

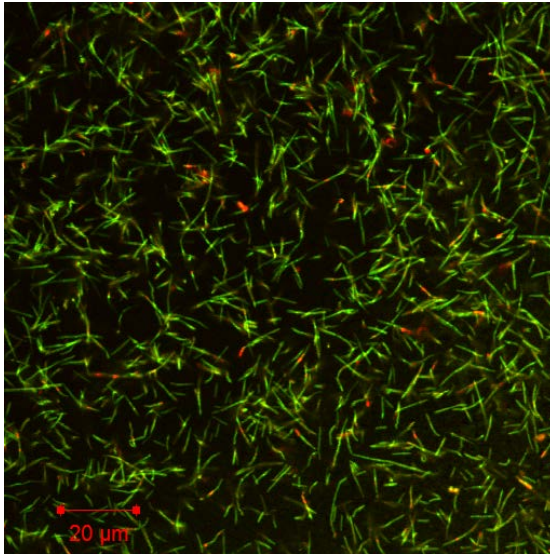


Figure 4. 36 h old *F. nucleatum* biofilm grown in the flow-cell biofilm model and stained with Live/dead stain. (Source: Marwan M A Mohammed).

In addition to the ability to coaggregate with other bacterial cells, *F. nucleatum* can also adhere and invade cells, e.g. human gingival epithelial cells (HGEC), leading to increased production of the pro-inflammatory chemokine interleukin-8 (IL-8) by these cells (101). The bacteria also show the ability to enter other types of oral cells like gingival fibroblasts and periodontal ligament fibroblasts *in vitro* (102). This ability to adhere to and invade host cells has been demonstrated both *in vitro* and *in vivo* (101-103).

F. nucleatum can affect the host immune response of the host by adhering to lymphocytes and inducing apoptosis by Fap 2 outer membrane protein (104). Also,

the *F. nucleatum* immunosuppressive protein (FIP) is capable of suppressing human B- and T-cell responsiveness (105).

Other potential virulence factors include endotoxins (89), stimulation of matrix metalloproteinase production (106) and outer membrane proteins (89).

F. nucleatum isolates have a higher proportion and greater number in individuals with compromised periodontal tissues; in general, it continues to maintain its proportion in the periodontal flora as gingivitis progresses and as periodontitis develops. The cell mass of *F. nucleatum* increases as much as 10,000-fold, making it one of the most abundant anaerobic species in the disease sites (107). However, the definite role of *F. nucleatum* in periodontal disease pathogenesis is probably masked because the bacterium is also a common isolate in healthy individuals (108). In addition, virulence factors of *F. nucleatum* are less studied than those in other bacteria known to be etiological agents of periodontal diseases.

F. nucleatum is also common in clinical infections of other body sites, including brain, lung, liver, pelvic, ovarian and kidney abscesses, blood, spinal fluid and intrauterine device infections and pleurisy (107). The pathogenic role of *F. nucleatum* in otitis media, orofacial and skin infections, tonsillar abscesses, septic arthritis, and bacterial endocarditis has been documented (89, 109), and it has been recovered from a variety of infections in children (110).

Recently, accumulated studies show that *F. nucleatum* is associated with colorectal carcinoma (CRC) (111, 112), and is involved in CRC pathogenesis of promoting cellular proliferation and invasion in human epithelium and CRC cell lines and to enhance the progression of OSCC and CRC in animal models (113-117). *F. nucleatum* protein FadA modulates E-cadherin and activates b-catenin signaling, leading to increased expression of transcription factors, oncogenes, Wnt genes, and inflammatory genes, as well as growth stimulation of CRC cells (113). A recent study on the association between bacteria and oral squamous cell carcinoma (OSCC) showed that *F. nucleatum* was the most significantly overrepresented species in the tumors followed by *P. aeruginosa* (118). Bacterial-cancer association may be a

promising approach for the early detection of cancer by the assessment of immune response to antigens of tumor-associated microbe (119). Antibody-based serological testing against cancer-associated microorganisms including Epstein–Barr virus, human papillomavirus and *Helicobacter pylori* has been used in the diagnosis of the infection and tumor screening (120, 121).

1.5.3 Dual species interaction between *P. gingivalis* and *F. nucleatum*

F. nucleatum and *P. gingivalis* work synergistically during growth, as *P. gingivalis* stimulates *F. nucleatum* biofilm formation (122), and *F. nucleatum* supports the growth of *P. gingivalis* in aerated and CO₂ depleted environment (99). The effect of the presence or absence of *F. nucleatum* on anaerobe survival was tested on both planktonic and biofilm lifestyles in a complex community of oral bacteria grown in a partially aerated chemostat system. *P. gingivalis* number was significantly reduced in the absence of *F. nucleatum* and coaggregation-mediated interaction facilitated the survival of the obligate anaerobes (98).

The coaggregation between the two species is mediated by a galactoside moiety on the *P. gingivalis* surface and a lectin on the *F. nucleatum* and this coaggregation has been shown to be inhibited by lactose, galactose and other related sugars (123). *F. nucleatum* significantly enhances the adherence of *P. gingivalis* to hydroxyapatite discs (124) and also to flow-cell glass biofilms (Fig. 5) when they are grown together (30). The virulence of *P. gingivalis* LPS was shown to be enhanced by co-culture with *F. nucleatum* compared to the virulence of LPS from *P. gingivalis* cultured alone (125).

The dual species model composed of *F. nucleatum* and *P. gingivalis* was also used on human cell lines *in vitro* to study the inflammatory effect and the invasion ability of the bacteria to the cells (126-129). Studies showed an enhancement in the attachment of *P. gingivalis* to human fibroblast and this was mediated by *F. nucleatum* (128). The invasion of the gingival epithelial cells by *P. gingivalis* was shown to be enhanced by

co-infection of these two species, and this cell entry was modulated by *F. nucleatum* and dependent on lipid rafts (126). Mixed infection also appears to significantly provoke the inflammatory response in epithelial cells (KB cells), as higher levels of interleukins 6 and 8 were detected when *F. nucleatum* ATCC25586 and *P. gingivalis* ATCC33277 were co-cultured with KB cells (127).

The dual species model composed of *F. nucleatum* and *P. gingivalis* was also used *in vivo* with mouse models (130-133). Infection of mice with a combination of *P. gingivalis* and *F. nucleatum* elicited a significantly greater lesion (abscess) size ($P < 0.001$) and lethality compared with *P. gingivalis* alone (132) and synergistic pathogenicity was also shown in the mouse subcutaneous chamber model (131).

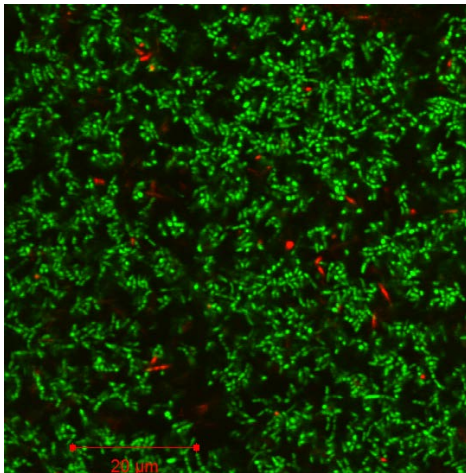


Figure 5. 24 h old dual species biofilm composed of *F. nucleatum* and *P. gingivalis*, grown in the flow-cell biofilm model and stained with Live/dead stain. (Source: Marwan M A Mohammed).

Experimental periodontitis was induced by *F. nucleatum* and *P. gingivalis* in a mouse model and the mice showed significantly more bone loss compared with that of mono-infected mice (130). Increased levels of inflammatory mediators (TNF- α and IL-1 β) were also demonstrated, compared with the levels in the mono-infected group (130). Vaccinated mice with either bacteria (heat killed whole bacteria) were also

challenged in a subcutaneous chamber model and in an experimental periodontitis (oral infection) model (134). The mice immunized against either bacteria showed decreased TNF- α but not IL-1 β , compared to non-immunized mice (134). The level of bone loss induced by the infection with dual species showed no change with vaccination even though the antibody titers were still high (134).

Materials and substances with potential antibacterial properties were also tested in the dual species model composed of *F. nucleatum* and *P. gingivalis* which include cranberry polyphenol (135), povidone-iodine (136) and ruthenium based sensitizer (137). When tested with povidone –iodine the dual species biofilm showed an approximately 200-fold increase in the viable count compared with mono-microbial biofilm (136). This indicates how these two species can support each other during stress.

The extracellular polymeric substances of the dual species biofilm have been explored and shown to be rich in proteins, carbohydrates and nucleic acids (30). The matrix proteins of the dual species biofilm have been identified, quantified and compared with mono-species biofilm matrix. Several proteins were recognized as oxidoreductases and chaperons have been shown to be among the most abundant proteins (138).

F. nucleatum and *P. gingivalis* are also members of the 10-species subgingival Zurich biofilm model (139) that is composed of frequently studied plaque bacteria representing early, intermediate and late colonizers of the subgingival biofilm (139, 140). *F. nucleatum* and *P. gingivalis* have also been included in a three species biofilm model of oral microbial community where *Streptococcus gordonii* was the third species and represented the early colonizers. This model was used to study the proteomics of each bacterium in the microbial community (141-143).

1.6 Methods used to study bacterial biofilms

Numerous approaches have been used to study biofilm formation, but as yet there is no single ideal model system (144). Selection of model systems depends on many factors including the type and characteristics of the targeted bacteria, the aim of the investigation, the preferences of the investigator and other more objective criteria (144, 145).

There are two practical models for studying biofilms, static systems that are more suitable to exploring early events in biofilm formation, and continuous flow or chemostat systems, which are preferable for mature biofilm studies (144, 146, 147). One example of each system will be described in this overview.

The microtiter plate biofilm assay is a popular static model used to assess bacterial attachment by measuring the adherent biomass. Also known as the 96-well plate assay and first mentioned by Christensen et al. (148), and the protocol was modified and promoted in the 1990s (146, 149, 150). Being user friendly with high-throughput capacity makes it among the most frequently used biofilm models, with the advantage of low cost as it uses small amount of reagents. The system has good versatility with ability to grow biofilm on the bottom of the wells or on a coupon made of different materials placed in the well. Another variation is to grow biofilm on pegs attached to the plate-lid, as in the system developed by Ceri et al. and then patented and marketed as the Calgary Biofilm Device and later as the MBEC Device by Innovotech (151).

The microtiter plate biofilm model can be used in many applications. It was used in testing the ability of the bacterial strains to form biofilm (152), screening for the antimicrobial and anti-biofilm effect of different substances (153) and examining the effect of different modifications in the growth environment including coating, growth media, temperature, humidity, etc. (154).

The flow cell biofilm model is an example of growing biofilm in hydrodynamic conditions (Figure. 6) (147). This method allows a good microscopic visualization for developing biofilm (145). As the biofilm has a three-dimensional structure, confocal

laser scanning microscopy (CLSM) with appropriate molecular staining can help to obtain a spatiotemporal follow up of biofilm formation (147, 155).

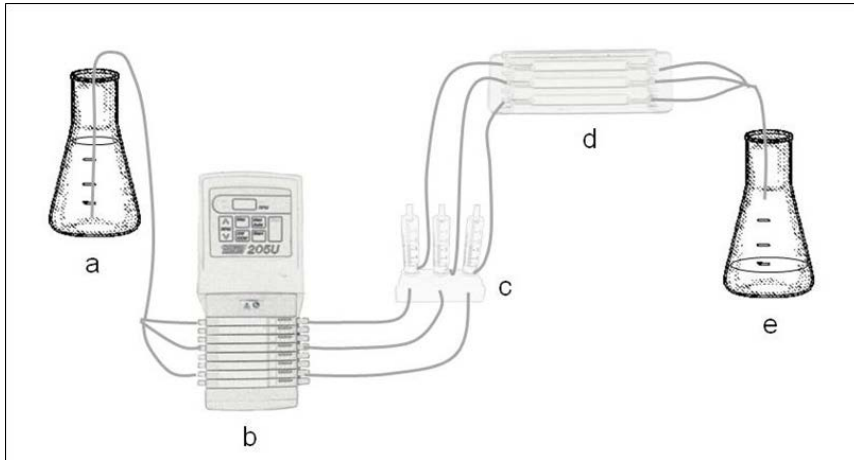


Figure 6. Schematic illustration of the flow cell system, medium bottle (a) the pump (b) the bubble trap (c) the flow cell (d) and the effluent bottle (e). Adopted from (147)

With CLSM it is possible to view live biofilm samples of fluorescent labeled bacteria that have not subjected to any fixation distortion (155). In this model the biofilm is grown on glass coverslip sealed with silicone glue to a polycarbonate block with channels that have a design compatible with typical microscope slide mounting apparatus (147, 155). The block is connected with tubing to the source of the medium, pump and bubble traps in one end and to the waste container on the other end. Different staining techniques can be used to visualize biofilms and/or the matrix (EPS) (155), including fluorescent proteins, fluorescent *in situ* hybridization and nucleic acid stains.

1.7 Proteome analysis

The proteome is defined as the entire set of proteins expressed by a genome, cell, tissue, or organism at a certain time. The term was first introduced by Marc Wilkins (156), combining the two words “protein” and “genome”. Compared to the relatively static genome, the proteome is dynamic and complex as protein expression is affected by three main potential modification states (glycosylation, phosphorylation and ubiquitination), and may be followed by additional modification (e.g. another phosphorylation, acetylation, protease cleavage, lipidation, acetylation, etc.). This leads to diverse forms of protein expression, called protein isoforms, and post-translation modifications (157). While genomics and transcriptomics provide basic information on DNA sequences, regulatory elements, and gene expression, proteomics provides quantitative information on the total protein profile of a cell, tissue, or organism at specific time points. It also takes into account the relative abundance, distribution, functions and interactions with other macromolecules (158).

1.6.1 Proteomics of oral bacteria associated with periodontal diseases

Unlike the genome, it is difficult to find the whole proteome expressed by a cell or organism due to the complexity of the proteome, as it changes depending on abundance, post-translation modification, cell location and interaction with other proteins, all of which can change quickly (158).

The characterization of proteins expressed by oral bacteria under a range of *in vitro* growth conditions was started with one species at a time, usually under planktonic growth condition (159), followed later by proteomic analysis for bacteria grown in a biofilm and most recently in multi-species biofilm models (160).

One of the targeted bacteria for proteomic analysis is *P. gingivalis*, due to its strong association with periodontal diseases and the fact that it is the easiest red complex member to grow and manipulate (71). Differential protein expression by *P. gingivalis*

in response to secreted epithelial cell components was studied by Zhang et al. and 1014 proteins (46% of the total theoretical proteome) were identified in four independent analyses (161). Among the proteins up-regulated in the presence of epithelial cell components was a homolog of the internalin proteins of *Listeria monocytogenes* and subunits of the ATP-dependent Clp protease complex (161). Proteomic analysis of *P. gingivalis* grown in an oral microbial community with *F. nucleatum* and *Streptococcus gordonii* showed a decrease in proteins involved in cell shape and the formation of the cell envelope, as well as thiamine, cobalamin, and pyrimidine synthesis and DNA repair (141). An overall increase was seen in proteins involved in protein synthesis and HmuR, a TonB dependent outer membrane receptor, was up-regulated in the community (141). In a polymicrobial biofilm composed of the anaerobic proteolytic species *P. gingivalis*, *T. denticola* and *T. forsythia* which are said to be strongly associated with chronic periodontitis (65), proteomic analysis showed a change of plan in iron acquisition by *P. gingivalis* due to large increases in the abundance of HusA and HusB in the polymicrobial biofilm, while HmuY and other iron/haem transport systems decreased (162). Significant changes in the abundance of peptidases and enzymes involved in glutamate and glycine catabolism suggest syntrophy (162). In a study of the outer membrane vesicles of *P. gingivalis*, it has been shown that they contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors (85). Two recent studies of the extracellular proteome of *P. gingivalis* have now identified the most abundant proteins, major virulence related proteins, outer membrane proteins (138, 163) and citrullinated extracellular proteins (163).

A. actinomycetemcomitans is another putative periodontal pathogen that has received significant attention due to its strong association with severe periodontitis in younger individuals (164). The cell envelope proteome of *A. actinomycetemcomitans* shows a broad range of different proteins, including surface adhesins, porins, lipoproteins, numerous influx and efflux pumps, multiple sugar, amino acid and iron transporters, and components of the type I, II and V secretion systems (165). In another study, the secretome of *A. actinomycetemcomitans* has been shown to contain putative virulence

determinants including DegQ, fHbp, LppC, Macrophage Infectivity Protein (MIP), NlpB, Pcp, PotD, TolB, and TolC (166). The interactions between *A. actinomycetemcomitans* and other bacterial species in an *in vitro* 10-species "subgingival" biofilm model have been studied using proteomic analysis (167), and there were shown to be distinct protein regulation patterns, with the regulated groups of proteins being primarily responsible for changes in the metabolic rate, the ferric iron-binding, and the 5S RNA binding capacities, at the universal biofilm level (167). While the presence of *A. actinomycetemcomitans* did not disturb the numeric composition or absolute protein numbers of the other biofilm species, it triggered qualitative changes in their overall protein expression profile (167).

F. nucleatum, a bacterial species known for its capacity to coaggregate with other species within the oral biofilm, acting as a bridge between early and late colonizers, has also been subjected to multiple proteomic studies (96, 143, 168-170). Zilm et al. have explored how the proteomic profile of *F. nucleatum* is regulated by growth pH. Differentially expressed proteins associated with increased energy (ATP) production via the 2-oxoglutarate and Embden-Meyerhof pathways appeared to be directed towards either cellular biosynthesis or the maintenance of internal homeostasis (168). The ampicillin resistant *F. nucleatum* showed up-regulated expression of these proteins, a class D beta-lactamase, ATP-binding cassette (ABC) transporter ATP-binding protein and enolase (169). In response to oxidative stress, three major protein systems of *F. nucleatum* were altered. Proteins of the alkyl hydroperoxide reductase/thioredoxin reductase system were increased in intracellular concentration, glycolytic enzymes were modified by oxidation and the intracellular concentrations of molecular chaperone proteins and related proteins (i.e. ClpB, DnaK, HtpG, and HrcA) were increased (96). In alkaline-induced *F. nucleatum* biofilms, the intracellular concentration of stress response proteins including heat shock protein GroEL and recombinational protein RecA increased markedly in an alkaline environment (170). There was increased abundance of an adhesin, Fusobacterial outer membrane protein A (FomA), known for its capacity to bind to a vast number of bacterial species and human epithelial cells and its increased abundance has been associated with biofilm

formation (138, 170). The proteomics of *F. nucleatum* in a microbial community model with *S. gordonii* and *P. gingivalis* showed extensive changes in energy metabolism, and all multispecies comparisons showed reductions in amino acid fermentation and a shift toward butanoate as a metabolic byproduct (141), with functional analysis showing reduced translation, lipopolysaccharide, and cell wall biosynthesis, DNA replication and DNA repair in the community (141).

A proteomic overview of regulated pathways of host-biofilm interaction models, provides insights into the early events of periodontal pathogenesis (171). An *in vitro* periodontal organotypic tissue model in a perfusion bioreactor system was used in co-culture with an 11-species subgingival biofilm, and *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* were among the species grown in that biofilm (171). Most secreted bacterial biofilm proteins derived from their cytoplasmic domain and in the presence of the tissue, the levels of *F. nucleatum*, *Actinomyces oris* and *Campylobacter rectus* proteins were significantly regulated, and the functions of the upregulated intracellular (biofilm lysate) proteins were associated with cytokinesis (171).

2. Aim of the study

The main aim of the project was to study in depth and characterize a dual species biofilm composed of *F. nucleatum* and *P. gingivalis* *in vitro* using molecular imaging techniques and proteomics. Furthermore, we explored the extracellular polymeric substances in the biofilm matrix of the dual and mono-species biofilm, followed by protein identification and analysis of their differential expression.

The specific aims:

- To establish and maintain an *in vitro* model for dual species biofilm composed of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.
- To characterize extracellular polymeric substances in the biofilm matrix and to analyze enzymatic effects on early and mature biofilms formed by *F. nucleatum* and *P. gingivalis*.
- To identify and quantify proteins in the EPM of *F. nucleatum* and *P. gingivalis* when grown in mono- or dual-species biofilms.
- To study the functional characterization of the protein profiles of *F. nucleatum* and *P. gingivalis* when grown as mono- or dual-species biofilms or under planktonic growth conditions.

3. Materials and Methods (mainly derived from Paper I - III)

3.1 Paper I

Bacteria and growth medium

Fusobacterium nucleatum subsp. *nucleatum*, type strain ATCC 25586 and *Porphyromonas gingivalis* type strains ATCC53978 (W50), ATCC 33277 and ATCC BAA-1703 (FDC 381) were used in this study.

The bacterial strains were grown on fastidious anaerobic agar (FAA) plates at 37°C in anaerobic condition (5% CO₂, 10% H₂, and 85% N₂) (Anoxomat System) for 48h and then inoculated in liquid medium prepared with the following : tryptone (Oxoid Ltd., London) (15 g/L); NaCl, (5 g/L); KH₂PO₄, 1.5 g/L); Na₂HPO₄.2H₂O, (3.5 g/L) ;NaHCO₃, (0.5 g/L) and yeast extract (Oxoid), (3.0 g/L). Filter sterilized ascorbic acid (1 mg/L), vitamin B12 (0.1 mg/L), glucose (5.5 g/L) and hemin (5 mg/L) were added to the autoclaved part of the medium (172). The bacteria were incubated for 24h at 37°C in anaerobic condition and used as the source of culture inoculum in the dynamic and static biofilm models.

The flow cell biofilm

Biofilms were grown at 37°C in three-channel flow cells with individual channel dimensions of 1 x 4 x 40 mm. The flow system was assembled and prepared as described by Christensen et al (173). A glass cover slip (24 x 50 mm) was used as substratum for biofilm growth. Before each experiment, the flow cell system was autoclaved, and after assembling, the system was sterilized by pumping a 0.5% (wt/vol) hypochlorite solution into the system and leaving it there for 4 h. The system was flushed with 2L of sterile water after which the flow chamber was filled with

media and allowed to sit overnight at 37°C to let the system equilibrate with the medium. Inocula were prepared as follows: bacteria grown for 48h on FAA plates were re-suspended in liquid media and incubated overnight at 37°C. After adjusting the optical density at 550nm to 0.5, aliquots of 250 µl cultures were injected into each channel of the flow cell after stopping the medium flow and clamping off the silicon tubing to prevent back flow into the system. The flow cell was inverted for one hour to allow for adhesion of cells to the glass surface without flow. Then the flow was resumed and the clamps removed. During growth of biofilms the fresh medium was pumped through the flow cells at a constant rate of 3.3 ml/h/channel by using a peristaltic pump (Watson-Marlow, Falmouth, UK) (174).

The biofilm for EPS extraction

Petri dishes with a diameter of 9 cm (Nunc, Rochester, NY, USA) containing 20 ml of liquid medium each were inoculated with 100 µl of bacterial suspension ($OD_{550nm}=1$). The dishes were incubated in anaerobic conditions (without shaking) at 37°C for 5 days. Then the medium was removed and the biofilm samples washed twice with phosphate buffered saline (PBS) before the biofilms were harvested by scraping with cell scraper (Nunc, Rochester, NY, USA). The biofilm samples were suspended in 1ml PBS and stored at -20°C until processing.

Enzymatic treatment of harvested biofilm

The biofilm samples were homogenized with FastPrep FP120 Thermo Savant homogenizer (Qbiogene, Cedex, France) at a speed of 4 m/sec for 20 seconds, then Proteinase K (Sigma-Aldrich, MO, USA) was added to 500 µl of each sample to yield a final concentration of 5 µg/ml as described (18, 29). Samples with added distilled water were used as controls. Enzyme treated samples and controls were incubated at 37°C for 1h. After enzymatic treatment, the biofilm samples and controls were filtered through 0.2 µm pore size acrodisc syringe filters (Pall, BioSciences, Ann Arbor, MI, USA). Aliquots from the eluate were used for quantification of proteins and carbohydrates and extraction of DNA.

Protein concentration assay

For the measurement of the protein concentration the samples and controls were diluted 10 times in distilled water and then 0.5 ml of Lowry reagent was added to 0.5 ml of this sample. After 20 min at room temperature, 0.5 ml of Folin and Ciocalteu's phenol reagent working solution (Sigma-Aldrich, MO, USA) was added to the mixture and left for another 30 min at room temperature (25). The absorbance of the standards and samples were measured at 750 nm and compared to a standard curve obtained by serial dilution of bovine serum albumin.

Carbohydrate assay

The carbohydrate concentration in EPM was measured by the anthrone method with the modifications described by Raunkjær et al (18, 175), using glucose as a reference standard. The samples and controls were prepared by 10 times dilution in distilled water, and then 100 µl of each diluted sample was mixed with 200 µl of anthrone reagent (0.125% anthrone [wt/vol] in 94.5% [vol/vol] H₂SO₄). Samples and controls were placed in a water bath at 100°C for 14 min and then cooled at 4°C for 5 min. The absorbance at 595 nm was measured using microtitre plate reader (Multiskan MS Type 352, Labsystems, Finland).

eDNA extraction and quantification

Extraction of eDNA was performed by using Fast DNA spin kit (MP Biomedicals, Solon, Ohio, USA) according to the manufacturer's instructions. Measurements of DNA concentration in 500 µl from each sample were done by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA).

The eDNA was electrophoresed on a 0.8% agarose gel from SeaKem (FMC BioProducts, Rockland, ME, USA) and stained with GelRed™ (Biotium, Hayward, CA, USA) using 0.5x TBE buffer at 100V for 40 minutes. EZ load 100-bp molecular ruler (Bio-Rad, CA, USA) was used as DNA standard.

Static biofilm microtitre plate assay

Black 96 well clear flat bottom polystyrene untreated microplates (cat. no. 3631, Corning, NY, USA) were used to grow biofilms. The effect of the enzymes was evaluated on biofilm formation and mature biofilm (29). Deoxyribonuclease I (DNase I) (Sigma-Aldrich, MO, USA) from bovine pancreas was prepared in enzyme buffer (0.15 mM NaCl and 5mM MgCl₂) and proteinase K was prepared in distilled water. The two enzymes were used in different concentrations (0.125, 0.25, 0.5, and 1 mg/ml). The enzyme buffer (for DNase I) and distilled water (for proteinase K) were used for the controls. The bacteria were prepared by diluting overnight grown bacterial cultures to prepare suspensions of 1.2×10^7 cfu/ml.

A total of 200 μ l from the bacterial suspension was used in each well of the microplate to grow biofilm, for dual species biofilm equal amounts (100 μ l) from each bacteria were used.

To evaluate the effect of DNase I and proteinase K on biofilm formation the enzymes were added and then the microplates were incubated in anaerobic conditions at 37° C for 48h. To evaluate the effects on mature biofilm a 48 h old biofilm was washed with PBS, and then the enzymes were added in their respective buffers and incubated for 1h at 37° C.

The medium and enzymes were removed and the wells were washed once with distilled water. The biofilm was then stained with 150 μ l of crystal violet (0.5 %) for 15 min, the stain was removed and the biofilm was washed twice with distilled water and left to dry. To solubilize the stain 150 μ l of 95% ethanol was added to each well, the absorbance was read at 570 nm in an automatic ELISA microplate reader (Multiskan MS Type 352, Labsystems, Finland).

CLSM was used to visualize the effect of enzymes on biofilm formation and on mature biofilm. In brief, the biofilm was grown in μ -clear bottom, chimney well, surface treated, sterile 96 wells microtitre plates (cat. no. 635090, Greiner Bio-One, Frickenhausen, Germany) in the same conditions as described above. The

concentrations of DNase I and proteinase K used on the biofilm examined by CLSM were 1mg/ml.

CLSM of biofilms in flow cells and microtitre plates

The biofilms were examined using a Zeiss LSM 510 META equipped with a water-immersion 63x objective (Carl Zeiss, Jena, Germany). The biofilms were stained for 15 min with 100 μ l LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen Corporation, NY, USA), The final concentrations of Syto-9 and propidium iodide (PI) were 0.01 mM and 0.06 mM, respectively. The SYPRO® Ruby biofilm matrix stain (Invitrogen Corporation, NY, USA) was used to stain proteins in the EPM. The green fluorescence and red fluorescence of SYTO 9 and PI were excited using an argon laser beam with excitation lines at 488 nm and a helium/neon at 543nm, respectively. The SYPRO® Ruby stain was excited at 405nm with diode laser. The CLSM image stacks were analysed by the image-processing software COMSTAT (176). The biomass, average thickness and maximum thickness were the parameters used to compare different biofilms.

Statistical analyses

The IBM SPSS 19.0 software package was used for the statistical analyses. The means and standard deviations of carbohydrates and eDNA concentrations in harvested biofilms treated with proteinase K were calculated and Mann–Whitney U-test was used to compare the means. The means and standard deviation of absorbance values representing the effect of DNase I and proteinase K on early biofilm formation or mature biofilm were calculated for each enzyme concentration and each tested biofilm. Multiple comparisons within groups were performed by Kruskal-Wallis test and if significant Mann–Whitney U-test as post hoc testing is used. The significance level was set to $p < 0.05$.

3.2 Paper II & III

Bacteria and growth medium

Fusobacterium nucleatum subsp. *nucleatum* type strain ATCC 25586 and *Porphyromonas gingivalis* type strain ATCC 33277 were used in the current study. The bacterial strains were grown on fastidious anaerobic agar (FAA) plates at 37°C in anaerobic conditions (5% CO₂, 10% H₂, and 85% N₂) (Anoxomat System, MART Microbiology, Lichtenvoorde, Netherlands) for 48h. A few colonies of each species were then used to inoculate Brucella broths (Becton Dickinson, Sparks, MD, USA) supplemented with 5 µg/ml hemin and 0.25 µg/ml vitamin K. The bacteria were grown overnight in the liquid medium under 37°C under anaerobic conditions. The overnight cultures were adjusted to an absorbance of 0.15 at 600 nm (A_{600}), whereof 10 ml was transferred to a separate 25 cm² (area) polystyrene cell culture flask (cat.no 90026, TPP, Trasadingen, Switzerland) to prepare mono species biofilms, and 5 ml from each species was transferred to prepare dual species biofilm. We cultured the biofilms in an *in vitro* static biofilm model (177), the flasks were incubated at 37°C under anaerobic conditions for 4 days without any additional supply of fresh medium. After medium removal, the biofilm samples were washed once with phosphate buffered saline (PBS) to remove free-floating bacteria and the attached biofilm was harvested with a cell scraper (Nunc, Rochester, NY, USA). The collected biofilms were then re-suspended in 500 µl PBS and stored at -20°C until further processing. The planktonic cultures were grown with same medium in 10 ml glass tubes for 4 days, and the bacteria collected by centrifugation.

Biofilm Viability by Colony forming unit (CFU) counting

The viability of the bacterial cells was determined by counting CFU of the initial inoculum and of the mature 4 days-old biofilm. Three independent biological replicates were serially diluted, selected dilutions plated on FAA medium, and incubated anaerobically at 37°C for 4 days. The colonies formed on the plates were counted and used for calculating estimated numbers of viable cells.

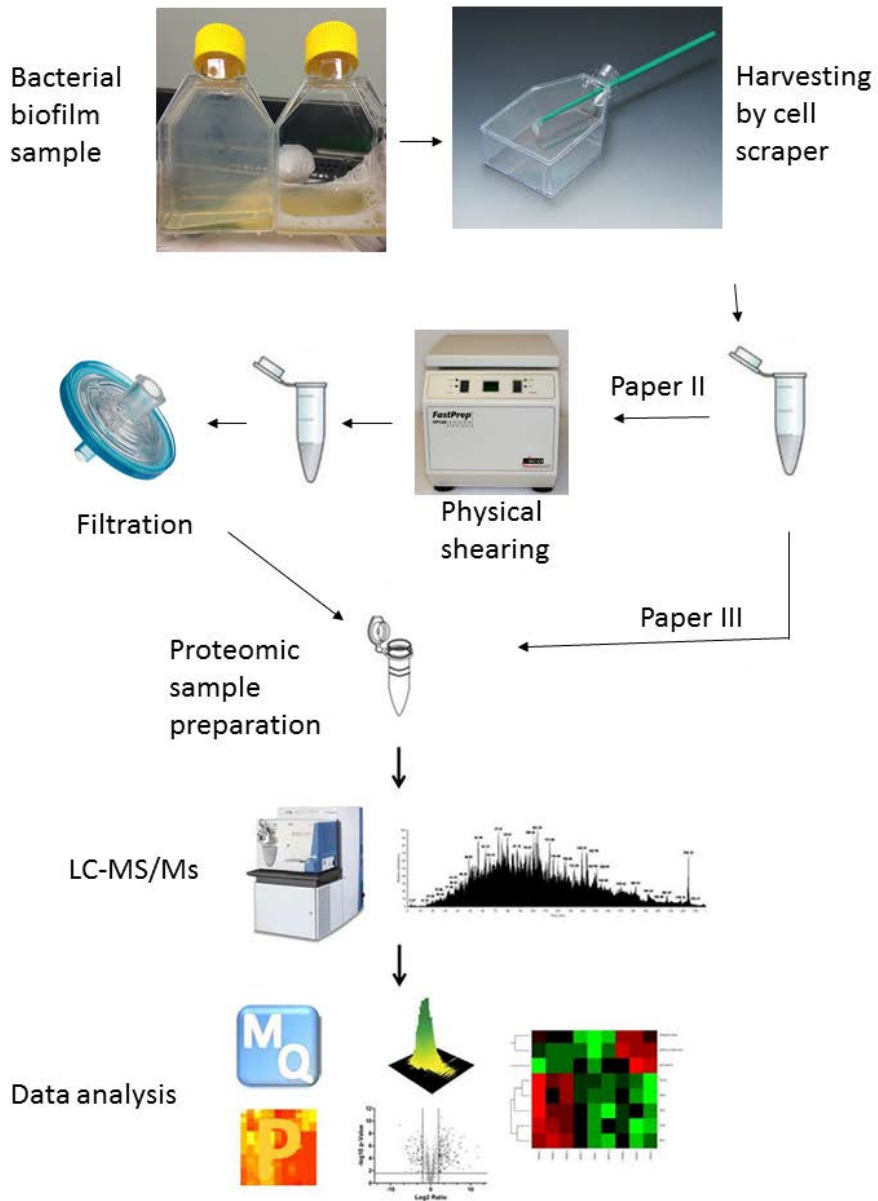


Figure 7. The experimental workflow included the harvesting of biofilm samples, processing of cell extracts, preparation of peptide mixtures for LC-MS/MS, and data analysis by MaxQuant (MQ) and Perseus (P) software.

Extraction of EPM

The biofilm samples were mechanically sheared with FastPrep FP120 Thermo Savant homogenizer (Qbiogene, Cedex, France) at a speed of 4 m/sec for 20 seconds, in Eppendorf tubes, without any cell-disrupting beads, to avoid contamination from cellular proteins. The samples were then filtered through 0.2 μm pore size acrodisc syringe filters (Pall, BioSciences, Ann Arbor, MI, USA) to remove cells and cellular debris (18). Aliquots from each eluate were used in further work. Direct Detect® Spectrometer (Merck Millipore, Darmstadt, Germany) was used for protein concentration measurements. Low concentration samples of *P. gingivalis* EPM were concentrated by using Amicon Ultra-0.5 centrifugal filter devices with 3K Da cutoffs (Merck Millipore, Darmstadt, Germany).

Sample preparation for the proteomic analysis

In order to generate a statistically robust proteomic dataset, samples with EPM extracts of mono- and dual-species biofilms from different culture flasks were prepared in four biological replicates (Paper II) or three biological replicates (Paper III). Filter Aided Sample Preparation (FASP) method developed by Wisniewski and co-workers (178), was used with minor modifications for the samples processing. Briefly, EPM samples were mixed in a solution of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (NH_4HCO_3) [solution to total protein ratio (v/w) 1:10] and incubated for 45 min at 56°C. Microcon device YM-10 filters (Merck Millipore, Darmstadt, Germany) were first conditioned by adding 100 μl of urea buffer (8M urea, 10mM HEPES, pH 8.0) and centrifuged at 14,000xg for 5 min. This and the following steps were carried out at room temperature, unless otherwise stated. Aliquots of EPM samples containing 50 μg of protein were mixed with 200 μl urea buffer in the filter unit and centrifuged at 14,000xg for 15 min and this step was repeated one more time. The filtrate was discarded and 100 μl of 0.05 M iodoacetamide was added to each sample. The samples were mixed at 600 rpm for 1 min in a thermo-mixer and incubated without mixing in the dark for 20 min, followed by centrifugation at 14,000xg for 10 min, three washes with 100 μl urea buffer and another three washes with 100 μl 40 mM NH_4HCO_3 in H_2O . EPM remaining on the filter were digested with trypsin (Thermo Fisher Scientific, IL, USA) in 40 mM NH_4HCO_3 buffer [enzyme to protein ratio 1:50 (w/w)] at 37°C for 16 h. The released peptides were collected by adding 50 μl of mass spectrometry grade water followed by centrifugation at 14,000xg for 15 min. This step was repeated twice.

Samples were concentrated (to 20-40 µl volume) in a vacuum concentrator (Eppendorf, Hamburg, Germany).

Filtration and desalting

StageTips for filtration and desalting were prepared by packing 3M Empore C18 extraction disks (3M, MN, USA) in 200 µl pipet tips by a blunt ended needle and a plunger or metal rod that helped to fit the extracted disks in the pipet tips, according to the protocol developed by Rappsilber and colleagues (179). The disks were wetted by passing 20 µl of methanol, followed by 20 µl of elution buffer [80% acetonitrile (ACN), 0.1% formic acid (FA)]. The disks were then conditioned and equilibrated with 20 µl of 0.1% FA just before the last residue of the previous buffer left the tip to avoid drying of the disks. Samples (volumes 20-40 µl) were loaded on top of the StageTip. The disks with samples were desalted by washing with 20 µl of 0.1% FA and were transferred to new tubes. Peptides were eluted and collected by adding 20 µl elution buffer twice. The collected samples were dried in a vacuum concentrator and stored at -80°C for further analysis. Peptide samples were resuspended by adding 1 µl of 100% FA and 19 µl of 2% ACN prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Data analysis

The acquired MS raw data were processed using the MaxQuant software (180), version 1.5.2.8, with default settings. Label-Free Quantification (LFQ) (181) and match between runs, which is based on retention time alignment between different replicates, were optional software features, which were used in the MS/MS data searches. The MS spectra were searched against protein databases of either *F. nucleatum* type strain ATCC 25586 or *P. gingivalis* type strain ATCC 33277. The respective files were downloaded from the UniProt knowledgebase on the 4th of February 2015. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE (182) partner repository with the dataset identifiers PXD004888 (Paper II) and PXD008288 (Paper III).

Post MaxQuant analysis included filtering of the generated 'proteingroups.txt' table for contaminants, only identified by site and reverse hits by the Perseus software (183). Each protein identified in at least two out of four replicates was considered valid. To discriminate

differential expressions of proteins present both in the mono- and dual-species biofilm, *t*-test with p -value ≤ 0.05 was used.

Functional protein annotation was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (184). Predictions of the identified proteins subcellular localization were performed by web-based application SOSUI-GramN (185). VirulentPred (186) was employed to predict the virulence factors among identified bacterial proteins, and the predictions were derived from the Cascaded SVM (Support Vector Machine) module (187). The protein lists were also searched for beta-barrels integral outer membrane proteins with the BOMP web-based tool (188).

4. Summary of the results

4.1 Paper I

Proteins and carbohydrates were the major components of the biofilm matrix, and extracellular (eDNA) was also present. The average concentration of proteins among the tested samples was 666 µg/ml, for carbohydrates it was an average of 682 µg/ml, for eDNA an average of 25 µg/ml. The matrix component showed variation among the species, with proteins and carbohydrates highest in EPM extracted from *F. nucleatum* and *P. gingivalis* W50 biofilms and lowest in *F. nucleatum* and *P. gingivalis* FDC381 biofilm. Proteinase K treatment had no effect on the concentration of the yielded eDNA or carbohydrate from the treated matrices.

DNase I and proteinase K treatments had no significant effect on biofilm formation or on mature biofilms under the conditions used.

In the flow-cell biofilm model, *F. nucleatum* was able to grow under partially oxygenated conditions while *P. gingivalis* failed to form biofilm alone in similar conditions but it can grow with *F. nucleatum* as dual species biofilm.

4.2 Paper II

We identified 542, 93 and 280 proteins from the matrix of *F. nucleatum*, *P. gingivalis*, and the dual-species biofilm, respectively. Nearly 70% of all EPM proteins in the dual-species biofilm originated from *F. nucleatum*, and a majority of these were cytoplasmic proteins, suggesting an enhanced lysis of *F. nucleatum* cells. The proteomic analysis also indicated an interaction between the two species: 22 *F. nucleatum* proteins showed differential levels between the mono and dual-species EPMS, and 11 proteins (8 and 3 from *F. nucleatum* and *P. gingivalis*, respectively) were exclusively detected in the dual-species EPM. Oxidoreductases and chaperones

were among the most abundant proteins identified in all three EPMs. The biofilm matrices also contained several known and hypothetical virulence proteins, which can mediate adhesion to the host cells and disintegration of the periodontal tissues.

4.3 Paper III

Comparisons between the protein profiles for the two bacterial species showed significant changes under all the conditions tested, i.e. when they were grown in biofilm or planktonic conditions, and when they were grown in mono- or in dual-species biofilm settings. In the *F. nucleatum* biofilm 5 proteins showed changes from the planktonic condition, including increased proteins involved in vitamin B metabolic processes. In the *P. gingivalis* biofilm 40 proteins were changed: 30 increased and 10 decreased. Among the increased proteins, putative cell division trigger factor and riboflavin biosynthesis proteins were the most increased in this biofilm. To describe interactions between the two species at the protein level, we grew the bacteria both individually and together. In the mixed species biofilm culture, 112 proteins showed significant changes, including 72 proteins derived from *F. nucleatum* and 40 proteins from *P. gingivalis*. By comparing dual-species to mono-species in biofilm and under planktonic growth conditions, *P. gingivalis* showed more proteins with a decreased level in the dual-species conditions.

5. Discussion

5.1 Methodological considerations

The present study was done *in vitro* using different laboratory methods, material and equipment. Growing anaerobic bacteria in a biofilm model is technically challenging because it needs to be practically valid, reproducible and representative.

Paper I

A flow cell biofilm model was used to grow the biofilms. The main advantage of this model is the flow conditions can be controlled. This model in combination with confocal microscopy is perfect for the organisms that can be fluorescently tagged, and it gives a good spatiotemporal overview of the bacterial biofilm (155). In a flow cell model *P. gingivalis* was not able to form biofilm in partially oxygenated condition but biofilm is easily formed under strict anaerobic conditions (189). In our study, we showed that *F. nucleatum* was able to grow in partially oxygenated conditions and it can support the growth of *P. gingivalis* in the dual species biofilm (30). A limitation of this model is that our bacteria were not fluorescently tagged and we used live/dead stain to view the biofilm, thus we were unable to do time point analysis for the same biofilm. In addition, it was difficult to harvest enough biofilm to do further analysis of the samples, and contamination with aerobic bacteria was highly prevalent. We therefore changed to other models that allowed us to harvest mature biofilm with dense EPS matrix for further analyses.

To visualize the effect of enzymatic treatment on biofilm shape and structure, we used μ -clear 96 well plates, which have a thin glass bottom that allows visualization under confocal microscopy. Although there was some small effect of DNase enzyme on the

shape of the biofilm under confocal microscopy (Figure 8) this did not affect the main structure of the biofilm.

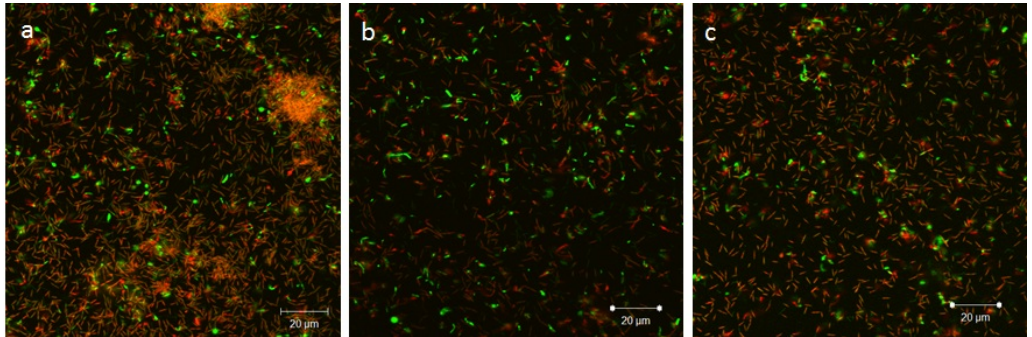


Figure 8: Confocal images of *F.nucleatum* 25586 and *P.gingivalis* 33277 in 48h old biofilms. (a) without enzyme treatment (b) treated with 1mg/ml DNase I (c) treated with 1 mg/ml proteinase K.

Paper II

In order to understand basic principles of biofilm organization at a molecular level, identification of ECM components is essential. Most extraction methods for extracellular polymeric substances (EPS) were adapted from the marine or environmental microbiology fields. The extraction methods can be classified as either physical or chemical (190). Unfortunately, there is no universal standard EPS isolation method, and the extraction procedure has to be modified to the specific type of biofilm under investigation (12). In our study, we elected to physically shear the biofilm using a homogenizer without disrupting beads in order to avoid contamination from cellular proteins. We also avoided the use of any chemicals in the extraction procedure because the presence of certain chemicals in EPS extracts results in severe underestimation of proteins from the samples (190). Physical shearing was followed by filtration to remove cells and any cellular debris.

Paper II and III

Mass spectrometry has emerged as a core tool for large-scale protein analysis. In the past decade there has been rapid improvement in the resolution, mass accuracy, sensitivity and scan rate of mass spectrometers used to analyze proteins. In addition, hybrid mass analyzers have been introduced recently (e.g. Linear Ion Trap-Orbitrap) which have significantly improved proteomic analysis (191). Shotgun proteomics provides an indirect measurement of proteins through peptides derived from proteolytic digestion of intact proteins and is considered as a “bottom-up” protein analysis (191). Relative quantification of proteins, especially label-free quantification in high throughput shotgun proteomics, have also developed rapidly in recent years. It can help to avoid additional sample processing steps, cost of labeling reagents, inefficient labeling, difficulty in analysis of low abundance peptides, and limitation of sample number which are all drawbacks associated with the use of labeling techniques. With the advent of a large number of fast, accurate, and sensitive instruments and software programs for validation, label free quantification is becoming a common substitute for the use of labeling methods (191).

5.2 Discussion of the main findings

There is a shortage of studies on EPM of oral subgingival biofilms. However, exopolysaccharides in the supragingival biofilm have been widely studied and identified for a long time (192-194). In the present study, we have isolated EPM and tested the effects of DNase I and proteinase K on biofilms of anaerobic periodontal disease associated bacteria grown *in vitro* (Paper I). Furthermore, we used high-resolution proteomics to identify and quantify proteins in the EPM of *F. nucleatum* and *P. gingivalis* grown in both mono- and dual-species biofilms (Paper II). Finally, we compared the proteome profiles of *F. nucleatum* and *P. gingivalis* grown in biofilm or planktonic mono- and dual-species growth conditions.

Extracellular polymeric matrix

The EPM of the bacterial biofilms is characterized by the presence of macromolecular complexes of carbohydrates, proteins and nucleic acids. The enzymatic treatment of the harvested biofilms with proteinase K was performed to discover if it would increase the liberation of eDNA or carbohydrates compared with simple vortexing or homogenizing. This treatment did not result in a significant difference in the yield of eDNA or carbohydrates. Wu and Xi found similar results for carbohydrates in *Acinetobacter* sp. strain AC811 biofilm matrix, but the eDNA yield was increased after enzyme treatment (18).

Although proteins were abundant in the biofilm matrix of our *F. nucleatum* and *P. gingivalis* dual species biofilms, treatment with proteinase K was shown to be insufficient to disperse the biofilm matrix. The high carbohydrate concentration in the matrix might be responsible for this ineffectiveness, which has also been proposed for staphylococcal biofilms (195, 196). The eDNA detected in the EPM in our biofilms had a size around 100 bp as demonstrated by agarose gel electrophoresis. This is higher than described for other biofilms, however, the size of the eDNA has been reported to range from less than 100 bp to 10 kb (197). Treating the eDNA with DNase I in the mono or dual species biofilm matrix had no significant effects with respect to preventing biofilm formation or dispersing mature biofilms. This is in contrast to enzymatic treatment of *P. aeruginosa* biofilms (22). One suggested function of eDNA is gene transfer (198, 199). The biofilm may offer an environment conducive to DNA exchange as the cells are in close juxtaposition and DNA can be trapped within the extracellular matrix (46). Genus *Fusobacterium* and other genera of oral bacteria contain conjugative transposons that facilitate DNA transfer between bacteria through conjugation. *P. gingivalis* also shows a large degree of variation between strains, suggesting that this organism has gone through frequent genetic rearrangements (46, 200).

An improved understanding of the EPM of subgingival biofilm and complex multispecies biofilms in general should lead to more effective control strategies. The

management of biofilm growth does not require direct killing of the bacteria in the biofilm, but might be directed to degradation or dispersal of the biofilm matrix to reverse the biofilm mode of growth to a planktonic state which is significantly easier to treat and manage, for example by using antibiotics.

Flow-cell model

We have shown that *F. nucleatum* can grow in a flow-cell biofilm model in a non-strictly anaerobic environment, while this was not true for *P. gingivalis*. There appears to be a synergistic enhancement in biofilm formation when these two species are grown together, even in a partially oxygenated condition suggesting that *F. nucleatum* might have the capacity to protect *P. gingivalis* from oxidative stress. This has also been reported in other studies (98, 99). Nearby *in vivo* association between these two microorganisms might indicate that they support each other, as has been shown in biofilm and mouse models (122, 131). *F. nucleatum* and *P. gingivalis* have been found to co-aggregate *in vitro* and *in vivo*, which may play a role in biofilm formation and pathogenesis as reported in mouse model experiments (130).

Proteins of the biofilm matrix

F. nucleatum and *P. gingivalis* are found in the normal flora of the mouth, they have documented roles in the periodontal disease, and their genomes have been sequenced (76, 201). The bacteria were cultured for four days which gave a mature biofilm with adequate amount of the EPM (30), and also had a minimal effect on the number of viable cells in the biofilms. To identify EPM-associated proteins of *F. nucleatum* and *P. gingivalis* biofilms, the bacteria were grown in cell culture flasks on a plastic surface both individually and together, an approach that allowed for investigation of possible interactions between the two species at the protein level.

The number of identified proteins was similar to previous studies on EPMs of bacterial biofilms, which reported between 150 and 270 EPM proteins (202, 203). Reasons for the high number of protein identifications in the EPM of *F. nucleatum* (542 proteins), when compared to the numbers derived from *P. gingivalis* and dual-

species EPMs (93 and 280 proteins, respectively), are not entirely clear. The high number of *F. nucleatum* proteins in the biofilm matrix could be caused by more intensive cell lysis, when compared to *P. gingivalis*. However, only moderate changes in the number of viable cells were observed in the growth period of four days. A possible mechanism behind cell lysis might be programmed cell death (PCD). *F. nucleatum* has Cid/Lrg homologues of so-called holins, small membrane proteins responsible for PCD in the bacteria, whose role is mainly associated with permeabilisation of the cytoplasmic membrane and with concomitant protein export (204, 205). For example, in *S. aureus* CidA contributes to biofilm adherence both *in vitro* and *in vivo* by affecting cell lysis and the release of genomic DNA (206, 207). The murein hydrolase exporter (FN0467) and murein hydrolase export regulator (FN1531), which are the holins of *F. nucleatum*, are both found in the proteome of *F. nucleatum* when grown in biofilm or under planktonic conditions (205). Moreover, a high number of nucleotide-binding proteins in the EPMs of *F. nucleatum* and the dual species biofilms agree with our previous results showing DNA as a major component in the biofilm matrix (30), and further support the occurrence of cell lysis during biofilm formation.

P. gingivalis is an asaccharolytic microorganism (*i.e.* unable to metabolize carbohydrates) while *F. nucleatum* is able to utilize amino acids, peptides and sugars (208). Accordingly, we noticed that most of the detected metabolic pathways in *P. gingivalis* EPM had a role in amino acid metabolism (143). This finding and a generally high percentage of other metabolic enzymes detected in the EPMs of both *F. nucleatum* and the dual species biofilms are supportive of the role of the matrix as an external source of nutrition and energy production as previously suggested in other bacterial biofilms (12, 209).

Studies on these two organisms found evidence of physiological support between the species (99, 131). *F. nucleatum* is a moderate anaerobe, however, its ability to adapt to and reduce an oxygenated environment is extremely high (99). On the other hand, *P. gingivalis* cannot survive in an aerated environment above 6% O₂ when grown as a monoculture, but when grown as a co-culture with *F. nucleatum*, *P.*

gingivalis can survive O₂ levels of up to 20% (99). Proteins associated with oxidative stress were abundant in the matrix of the studied biofilms, similar to findings described in a study of the *P. aeruginosa* EPM (202). Oxidative stress response proteins were also previously shown to be up-regulated in the biofilms of *T. forsythia* (210) and *Campylobacter jejuni* (211), when compared to planktonic growth. It has been suggested that mixed species biofilms enhance the production of oxidative stress proteins because the more strict anaerobes are dependent on oxygen tolerant bacteria (212).

The two-species biofilm model used in this study represents a limited model since periodontal diseases develop in a polymicrobial environment. Although biofilm models with multiple bacterial species could represent *in vivo* condition more closely (213), such models are difficult to control and manipulate. Biofilm models with only two bacteria, such as the *F. nucleatum* - *P. gingivalis* model, are more straightforward for interpreting possible interactions between the two species. The proteomic analysis showed differential production of 22 *F. nucleatum* proteins between the mono and dual-species EPMs and 11 proteins were detected only in the dual-species EPM. These results indicate that the two species specifically influence each other at the protein level, further supporting synergistic action between these two oral pathogens (99, 131).

Among the most abundant proteins identified in the EPM were molecular chaperons. Previously, typical cytosolic proteins GroEL and DnaK were described as being associated with membranes and extracellular fractions of *F. nucleatum* (214). Targeting GroEL could represent an antimicrobial strategy with broad-spectrum application, and recently a high-throughput screening effort to discover chemically and structurally diverse inhibitors of GroEL/GroES has been undertaken (215). Moreover, *P. gingivalis* GroEL immunization was reported to significantly reduce the levels of alveolar bone loss induced by multiple periodontopathic bacteria in an animal model (216). Finally, the presence of oxidoreductases and various chaperone proteins in the EPM of oral bacteria biofilms is not only of interest regarding

periodontal diseases, but also for possible associations of bacterial biofilms with systemic inflammatory and autoimmune diseases (217-219).

Twenty-five of the proteins detected in the EPM of *P.gingivalis* biofilm were previously identified as outer membrane vesicles' (OMV) proteins (85). *P. gingivalis* is able to specifically concentrate and release a large number of its virulence factors into the environment in the form of OMV, and these vesicles have been linked to biofilm formation for example in *Helicobacter pylori* (220). Our results show that *P.gingivalis* OMV proteins represent a significant portion of the EMP proteome and OMV are therefore likely contributors to the biofilm development. An example of *P. gingivalis* OMV protein identified both in mono- and dual-species EPMs was hemagglutinin HagA, a surface protein that can function as an adhesin attaching bacteria to the host cells (82). We identified several other *P. gingivalis* virulence proteins in the biofilm matrix, such as fimbriae that are key factors in adhesion of the bacterial cells to the host tissue, its colonization and invasion of host cell membranes (221). Another virulence protein that contributes to the destruction of periodontal tissues is Lys-gingipain (kgp), and in our dataset, it was one of the most abundant *P. gingivalis* EPM proteins. Gingipains degrade collagen and fibronectin and inhibit interactions between host cells and the extracellular matrix. In addition, they degrade various cytokines, resulting in a disruption of the host cytokine network (221). Another *P. gingivalis* protein identified both in mono- and dual-species EPM was PGN_0898, a bacterial peptidylarginine deiminase (PAD) (Additional file 2: Table S1). It gives *P. gingivalis* a unique ability to citrullinate proteins (222). Citrullinated bacterial and host peptides may cause an autoimmune response in rheumatoid arthritis (222, 223).

Sequence-based prediction of the proteins subcellular localization is an important part of the identified proteome description and an essential step in the search for novel vaccine or drug targets (224, 225). Our data provided evidence that the matrix proteome consists of secreted proteins, proteins from cell debris, and OMPs. The prediction of OMPs in this study was of particular importance due to their involvement in adhesive properties and coaggregation of *F. nucleatum* and *P.*

gingivalis with other bacteria, as well as attachment to host cells. The latter interaction has significance both in the pathogenesis of infection and in the immune response of the host (225). The SOSUI-Gram and BOMP tools identified 40 and 42 OMPs, respectively, and there was variation in the prediction outcomes of the two bioinformatics methods. This observation illustrates that the use of several bioinformatics methods is both beneficial and necessary, and cross-referencing with available literature should complement the importance of the predictions. The BOMP tool specifically predicts membrane proteins containing β -barrel integral domains, which can have many different functions including enzymatic, transport and structural support (226). An example of such OMP is FomA (FN1859) which was identified in both mono- and dual-species EPM (Table 4). It is a nonspecific porin which acts as a virulence factor, and a major antigen of *F. nucleatum* (227, 228) that plays a role in binding to *P. gingivalis* (229). FomA of *F. nucleatum* represents a potential target protein for the prevention of bacterial co-aggregation by vaccination (229, 230). Other detected putative *F. nucleatum* virulence factors were auto-transporter fusobacterium outer membrane protein (FN1526) and serine protease (FN1426). Both proteins are involved in protein secretion pathways (205), and the latter has peptidase and hydrolase activity, which allows degradation of fibronectins, fibrinogens and collagens. The capacity of *F. nucleatum* to degrade proteins of the extracellular matrix of host connective tissues has been described as a significant contributor to invasion of the gingival tissue and subsequent damage of periodontal tissues (205, 231).

Proteomic comparisons between different growth conditions

The bacterial proteins show fluctuations under different conditions of growth, i.e. in biofilm, planktonic, mono-species and dual-species settings, and the regulated proteins have various and complex functional classifications.

The most abundant proteins have functions as oxidoreductases, acyltransferases, outer membrane proteins and proteases among others. Levels of a number of virulence factors were among the most abundant proteins under both biofilm and

planktonic growth conditions. Some of these proteins were shown to be abundant in the extracellular polymeric matrix of the biofilms (204). Proteins involved in vitamin B1 (Thiamine) and vitamin B2 (Riboflavin) metabolic processes were among those significantly increased in *F. nucleatum* biofilms. It was not previously known if vitamin B1 or B2 were aiding in biofilm formation, but *Thermotoga maritima* biofilm cells exhibited increased transcription of genes involved in biosynthesis of thiamine (232). As in *F. nucleatum* biofilm, riboflavin biosynthesis protein (RibBA or PGN_0643) was also increased in the *P. gingivalis* biofilm. This agrees with a transcriptomic study that showed this protein to be upregulated in a *P. gingivalis* biofilm (233).

Interestingly, the FadA adhesion protein (FN0249) displayed an almost 8-fold reduction in the dual-species biofilm compared to the planktonic state, but showed no change in the mono-species cultures. This adhesion protein helps *F. nucleatum* to adhere and invade host epithelial and endothelial cells (234) and promotes colorectal carcinogenesis by modulating E-cadherin/b-catenin signaling (113). In a recent study, *P. gingivalis* suppressed the invasion of *F. nucleatum* in gingival epithelial cells (235) and this was attributed to the degradation of E-cadherin by *P. gingivalis* gingipains, which has been previously reported (236). This work also suggests that *P. gingivalis* effects the FadA protein in addition to the E-cadherin.

When comparing mono-species to dual-species protein levels, results indicate minimal changes in the *F. nucleatum* protein levels in biofilm and in planktonic growth conditions. The majority of *P. gingivalis* proteins that show changes in the dual species growth condition showed reduction under both biofilm and planktonic settings, that confirmed the finding that shows how community provides favorable environment to *P. gingivalis* and reduces its stress (141)

6. Conclusion and future perspectives

In conclusion, in this study we demonstrated that proteins and carbohydrates are the major components in the EPM of *in vitro* grown biofilms of *F. nucleatum* and *P. gingivalis*; however, eDNA might also play a role in the structure of the biofilm. More structural and functional studies of EPM of subgingival biofilms are still needed to identify new targets to control biofilm growth. Improved models with more complex systems that involve *in vivo* studies are clear objectives for further work.

Proteins in the extracellular matrix of biofilms formed by the oral bacteria *F. nucleatum* and *P. gingivalis* have different functional classifications. Potential virulence proteins, outer membrane proteins and various binding proteins (DNA-binding, ATP-binding, and metal ion binding) were among the abundant proteins identified in the biofilm EPM. These proteins represent potential candidates that might be targeted for the inhibition of biofilm development. Furthermore, identification and quantification of these proteins will provide a molecular basis for their role in the formation of EPM. This might contribute to an understanding of the role played by *F. nucleatum* and *P. gingivalis* in the development of periodontal and systemic diseases, and lead to improved treatment options for these diseases.

The dynamics of bacterial proteins' representation in biofilm, planktonic, mono-species and dual-species settings is influenced by the different conditions of bacterial growth. Different bacterial functions show changes in protein levels as conditions change, for instance increased vitamin B synthesis in *F. nucleatum* and *P. gingivalis* biofilms, and increased translation and binding proteins in the dual species biofilms. In general, *P. gingivalis* showed greater protein changes compared to *F. nucleatum* in both settings (biofilm vs planktonic and mono-species vs dual-species setting). When dual-species were compared between biofilm and planktonic growth conditions, *P. gingivalis* had fewer changes, suggesting their dependency on *F. nucleatum*, which showed greater proteomic changes.

Based on these results, we conclude that adding another bacteria to the environment can trigger changes in the protein levels; this can be seen when bacteria grow in biofilm or in the planktonic state. Thus proteomic studies of multispecies biofilms are an important area for future investigation in bacterial proteomics.

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Characterization of extracellular polymeric matrix, and treatment of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNase I and proteinase K

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Background: Biofilms are organized communities of microorganisms embedded in a self-produced extracellular polymeric matrix (EPM), often with great phylogenetic variety. Bacteria in the subgingival biofilm are key factors that cause periodontal diseases; among these are the Gram-negative bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. The objectives of this study were to characterize the major components of the EPM and to test the effect of deoxyribonuclease I (DNase I) and proteinase K.

Methods: *F. nucleatum* and *P. gingivalis* bacterial cells were grown in dynamic and static biofilm models. The effects of DNase I and proteinase K enzymes on the major components of the EPM were tested during biofilm formation and on mature biofilm. Confocal laser scanning microscopy was used in observing biofilm structure.

Results: Proteins and carbohydrates were the major components of the biofilm matrix, and extracellular DNA (eDNA) was also present. DNase I and proteinase K enzymes had little effect on biofilms in the conditions used. In the flow cell, *F. nucleatum* was able to grow in partially oxygenated conditions while *P. gingivalis* failed to form biofilm alone in similar conditions. *F. nucleatum* supported the growth of *P. gingivalis* when they were grown together as dual species biofilm.

Conclusion: DNase I and proteinase K had little effect on the biofilm matrix in the conditions used. *F. nucleatum* formed biofilm easily and supported the growth of *P. gingivalis*, which preferred anaerobic conditions.

Keywords: *Subgingival biofilm; extracellular polymeric matrix; Fusobacterium nucleatum; Porphyromonas gingivalis; static and dynamic biofilm models; confocal laser scanning microscopy*

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A biofilm has been defined as a structured community of bacterial cells enclosed in a self-produced extracellular polymeric matrix (EPM) and adherent to an inert or living surface (1). All biofilms share several common features – these include the production of extracellular polymeric substances (EPS), which are hydrated biopolymers secreted by bacteria. The biopolymers surround and immobilize microbial aggregates, make the macroscopic appearance of biofilms, and are frequently referred to as ‘slime’ (2).

In general, it is estimated that the microorganisms account for less than 10% of the dry weight of the biofilms, whereas the matrix can account for more than 90% (2). The EPM increases resistance to host defences and antimicrobial agents, compared with more vulnerable free-floating (planktonic) cells, and it forms a hydrated barrier between cells and their external environment. The function of the matrix includes adhesion, aggregation of microbial cells, cohesion of biofilm, retention of water, sorption of organic and inorganic material, enzymatic

activity, nutrient source, exchange of genetic information, and export of cell components (2).

The EPM is chemically complex, varying with respect to bacterial species/strains and culture conditions (2, 3). Extracellular polysaccharides and proteins have been shown to be the key components of the matrix (2). Recent studies also indicate that extracellular DNA (eDNA) plays an important role in the establishment of biofilm structure (3–6). Some studies showed that removing eDNA reduces initial adhesion and aggregation of bacteria to surfaces (7, 8), and others have shown that eDNA is a major matrix component in some species biofilm (9), including *Pseudomonas aeruginosa* biofilm (10), where eDNA seems to induce antibiotic resistance (11). In *Staphylococcus aureus* biofilm, it was shown that cell lysis and the presence of eDNA were critical for attachment of biofilm during the initial stages of development and during biofilm maturation (12). Characterization of EPM components is mandatory in understanding biofilm structure and function. However, efficient EPM isolation is demanding because the isolation procedures might damage the cells causing contamination. Enzymatic treatment of biofilm was found to be helpful in the extraction of biofilm matrix, with no noticeable cell lysis (13, 14). Proteinase K is one of the enzymes that is used or included in the enzymatic extraction methods to degrade proteins in the matrix to increase nucleic acid release (14, 15).

Dispersal of biofilms by enzymes has been used in recalcitrant biofilms (e.g. using DNase I) on *P. aeruginosa* biofilms in cystic fibrosis patients (16). Treatment of biofilms with DNase I has also been shown to enhance the effect of antibiotics (17).

Oral bacterial biofilms are the key factors in the etiology of dental caries and periodontal diseases. The diversity, complexity, and multispecies community of the oral biofilm have been extensively reviewed (18, 19), but are still not fully clarified.

Fusobacterium nucleatum and *Porphyromonas gingivalis* are among the important species in the oral biofilm involved in the pathogenesis of periodontitis (20). *F. nucleatum* is commonly cultivated from the subgingival plaque from periodontitis patients, and because of its ability to aggregate with many oral bacteria, it works as a bridge between early and late colonizers in the dental biofilm (21). *P. gingivalis* is a member of the Socransky's red complex (bacteria strongly associated with periodontal disease) and has many virulent factors such as fimbriae, lipopolysaccharides, cysteine proteinases, and end products of metabolism (22).

The aim of this study was to characterize EPM main components and to analyse the effects of DNase I and proteinase K on early and mature biofilms formed by *F. nucleatum* and *P. gingivalis*. Confocal laser scanning

microscopy (CLSM) was used in structural studies applying dynamic and static biofilm models.

Materials and methods

Bacteria and growth medium

Fusobacterium nucleatum subsp. *nucleatum*, strain ATCC 25586 and *P. gingivalis* type strains ATCC 53978 (W50), ATCC 33277, and ATCC BAA-1703 (FDC 381) were used in the current study.

The bacteria were grown on fastidious anaerobic agar (FAA) plates at 37°C in anaerobic condition (5% CO₂, 10% H₂, and 85% N₂) (Anoxomat System, MART Microbiology, Lichtenvoorde, The Netherlands) for 48 h and then inoculated in liquid medium prepared with the following: tryptone (Oxoid Ltd., London) (15 g/L); NaCl (5 g/L); KH₂PO₄ (1.5 g/L); Na₂HPO₄·2H₂O (3.5 g/L); NaHCO₃ (0.5 g/L); and yeast extract (Oxoid) (3.0 g/L). Filter sterilized ascorbic acid (1 mg/L), vitamin B12 (0.1 mg/L), glucose (5.5 g/L), and hemin (5 mg/L) were added to the autoclaved part of the medium (23). The bacteria were incubated for 24 h at 37°C in anaerobic conditions and were used as the source of culture inoculum in the dynamic and static biofilm models (see beneath).

The flow cell biofilm

Biofilms were grown at 37°C in three-channel flow cells with individual channel dimensions of 1 × 4 × 40 mm. The flow system was assembled and prepared as described by Christensen et al. (24). A glass cover slip (24 × 50 mm) (product # 1014; Assistant, Sondheim/Rhön, Germany) was used as substratum for biofilm growth. Before each experiment was carried out, the flow cell system was autoclaved, and after assembling, the system was sterilized by pumping a 0.5% (wt/vol) hypochlorite solution into the system and leaving it there for 4 h. The system was flushed with 2 L of sterile water. The flow chamber was then filled overnight with media at 37°C to let the system equilibrate with the medium. Inocula were prepared as follows: bacteria grown for 48 h on FAA plates were re-suspended in liquid media and incubated overnight at 37°C. After adjusting the optical density at 550 nm to 0.5, aliquots of 250 µl cultures were injected into each channel of the flow cell after stopping the medium flow and clamping off the silicon tubing to prevent back flow into the system. The flow cell was inverted for 1 h to allow for adhesion of cells to the glass surface without flow. The flow was then resumed and the clamps were removed. During the growth of biofilms, the fresh medium was pumped through the flow cells at a constant rate of 3.3 ml/h/channel by using a peristaltic pump (model 205S; Watson-Marlow, Falmouth, UK) (25).

The biofilm for EPS extraction

Petri dishes with 9 cm diameter (Nunc, Rochester, NY, USA) each containing 20 ml of liquid medium were inoculated with 100 μ l of bacterial suspension ($OD_{550nm}=1$). The dishes were incubated in anaerobic conditions (without shaking) at 37°C for 5 days. The medium was then removed and the biofilm samples were washed twice with phosphate-buffered saline (PBS) before being harvested by scraping with a cell scraper (Nunc, Rochester, NY, USA). The biofilm samples were suspended in 1 ml PBS and stored at -20°C until processing.

Enzymatic treatment of harvested biofilm

The biofilm samples were homogenized with FastPrep FP120 Thermo Savant homogenizer (Qbiogene, Cedex, France) at a speed of 4 m/sec for 20 sec, then Proteinase K was added to 500 μ l of each sample to a final concentration of 5 μ g/ml as described (14, 26). Samples with added distilled water were used as controls. Enzyme treated samples and controls were incubated at 37°C for 1 h. After enzymatic treatment, the biofilm samples and controls were filtered through 0.2 μ m pore size acrodisc syringe filters (Pall, BioSciences, Ann Arbor, MI, USA). Aliquots from the eluate were used for quantification of proteins and carbohydrates and extraction of DNA.

Protein concentration assay

For measurement of the protein concentration, the samples and controls were diluted 10 times in distilled water, and then 0.5 ml of Lowry reagent was added to 0.5 ml of sample dilution. After 20 min at room temperature, 0.5 ml of Folin and Ciocalteu's phenol reagent working solution (Sigma-Aldrich, MO, USA) were added to the mixture and left for another 30 min at room temperature (25). The absorbance of the standards and samples were measured at 750 nm and compared to a standard curve obtained by serial dilution of bovine serum albumin.

Carbohydrate assay

The carbohydrate concentration in EPM was measured by the anthrone method with the modifications described by Raunkjær et al. (14, 27), using glucose as a reference standard. The samples and controls were prepared by 10 times dilution in distilled water, and then 100 μ l of each dilution was mixed with 200 μ l of anthrone reagent (0.125% anthrone [wt/vol] in 94.5% [vol/vol] H₂SO₄). Samples and controls were placed in a water bath at 100°C for 14 min and then cooled at 4°C for 5 min. The absorbance at 595 nm was measured using microtitre plate reader (Multiskan MS Type 352, Labsystems, Finland).

eDNA extraction and quantification

Extraction of eDNA was performed using Fast DNA spin kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. Measurements of DNA concentration in 500 μ l from each sample were carried out using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA).

The eDNA was electrophoresed on an 0.8% agarose gel from SeaKem (FMC BioProducts, Rockland, ME, USA) and stained with GelRed™ (Biotium, Hayward, CA, USA) using 0.5 \times TBE buffer at 100 V for 40 min. EZ load 100-bp molecular ruler (Bio-Rad, CA, USA) was used as DNA standard.

Static biofilm microtitre plate assay

Ninety-six Well Black with Clear Flat Bottom Polystyrene Not Treated Microplates (cat. no. 3631, Corning, NY, USA) were used to grow biofilms. The effect of the enzymes was evaluated on biofilm formation and mature biofilm (26). Deoxyribonuclease I (DNase I) (Sigma-Aldrich, MO, USA) from bovine pancreas was prepared in an enzyme buffer (0.15 mM NaCl and 5 mM MgCl₂), and proteinase K (Sigma-Aldrich, MO, USA) was prepared in distilled water. The two enzymes were used in different concentrations (0.125, 0.25, 0.5, and 1 mg/ml). The enzyme buffer (for DNase I) and distilled water (for proteinase K) were used for the controls. The bacteria were prepared by diluting overnight grown bacterial cultures to prepare suspensions of 1.2×10^7 cfu/ml.

A total of 200 μ l from the bacterial suspension was used in each well of the microplates to grow biofilm. For dual species biofilm, equal amounts (100 μ l) from each bacterium were used.

To evaluate the effect of DNase I and proteinase K on biofilm formation, the enzymes were added and then the microplates were incubated in anaerobic conditions at 37°C for 48 h. To evaluate the effects on mature biofilm, a 48-h-old biofilm was washed with PBS, and then the enzymes were added in their respective buffers and incubated for 1 h at 37°C.

The medium and enzymes were removed, and the wells were washed once with distilled water. The biofilm was then stained with 150 μ l of crystal violet (0.5%) for 15 min, the stain was removed, and the biofilm was washed twice with distilled water and left to dry. To solubilize the stain, 150 μ l of 95% ethanol was added to each well, and the absorbance was read at 570 nm in an automatic ELISA microplate reader (Multiskan MS Type 352, Labsystems, Finland).

CLSM was used to visualize the effect of enzymes on biofilm formation and on mature biofilm. In brief, the biofilm was grown in μ -clear bottom, chimney well, surface treated, sterile 96 Well Microtitre plates (cat. no. 635090, Greiner Bio-One, Frickenhausen, Germany)

under the same conditions as described above. The concentrations of DNase I and proteinase K used on the biofilm examined by CLSM were 1 mg/ml.

CLSM of biofilms in flow cells and microtitre plates

The biofilms were examined by Zeiss LSM 510 META equipped with a water-immersion of 63 × objective (Carl Zeiss, Jena, Germany). The biofilms were stained for 15 min with 100 µl LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen Corporation, NY, USA). The final concentrations of Syto-9 and propidium iodide (PI) were 0.01 mM and 0.06 mM, respectively. The SYPRO® Ruby biofilm matrix stain (Invitrogen Corporation, NY, USA) was used to stain proteins in the EPM. The green fluorescence and red fluorescence of SYTO 9 and PI were excited using an argon laser beam, with excitation lines

at 488 nm and a helium/neon at 543 nm, respectively. The SYPRO® Ruby stain was excited at 405 nm with diode laser. The CLSM image stacks were analysed by the image-processing software COMSTAT (28). The biomass, average thickness, and maximum thickness were the parameters used to compare different biofilms.

Statistical analyses

The software package IBM SPSS 19.0 was used for the statistical analyses. The means and standard deviations of carbohydrates and eDNA concentrations in harvested biofilms that had been treated with proteinase K were calculated, and Mann–Whitney U-test was used to compare the means. The means and standard deviation of absorbance values representing the effect of DNase I and proteinase K on early biofilm formation or mature

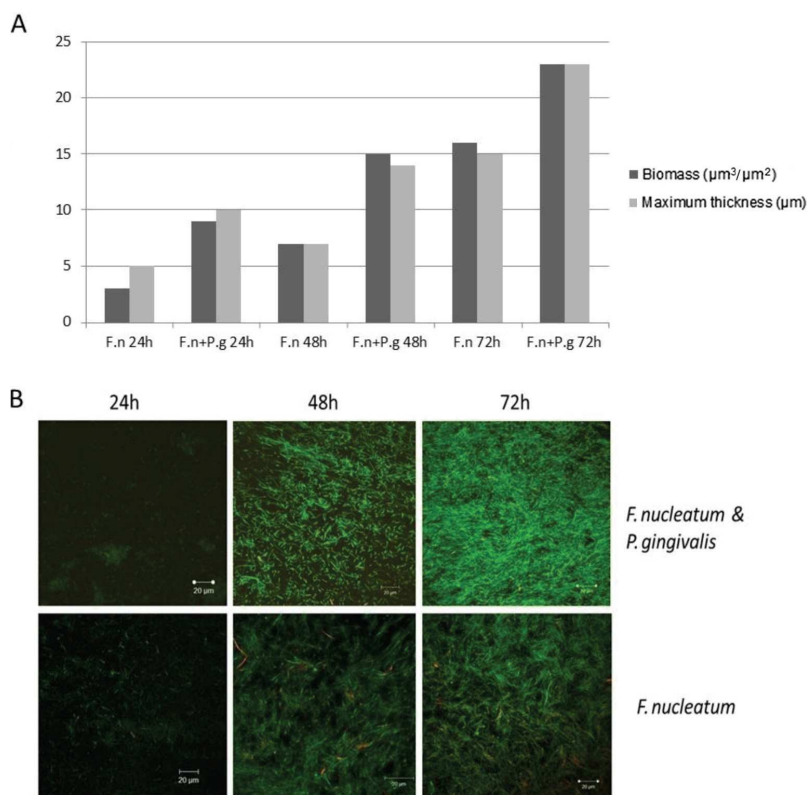


Fig. 1. (A) Biomass and maximum thickness of *Fusobacterium nucleatum* ATCC 25586 (F.n) when grown alone or together with *Porphyromonas gingivalis* W50 (P.g) biofilm and calculated from one point z-stack confocal microscopy images taken from the middle of the flow cell and analysed by COMSTAT software program. (B) Representative CLSM images of 24-h- (left), 48-h- (middle), and 72-h- (right) old biofilms showing mutualistic growth of *F. nucleatum* ATCC 25586 and *P. gingivalis* W50 (upper) compared to mono-species *F. nucleatum* ATCC 25586 (lower). The biofilms were grown in flow cells and were stained with Cyto9 (green) and propidium iodide (red).

biofilm were calculated for each enzyme concentration and each tested biofilm. Multiple comparisons within groups were performed by the Kruskal–Wallis test, and, if significant, the Mann–Whitney U-test was used as a post hoc test. The significance level was set to $p < 0.05$.

Results

Biofilm growth in flow cell

F. nucleatum was able to form biofilm in partially oxygenated conditions where the biofilm developed shortly (2–4 h) after inoculation. *P. gingivalis* failed to form biofilm alone in similar conditions. However, the growth of *P. gingivalis* was initiated when grown together with *F. nucleatum*. Biofilm formation and maturation were enhanced by co-culture of the two species (Fig. 1A and B).

The CLSM images revealed irregular topography of the biofilm, without clear mushroom-shaped structure (Figs. 1B and 2). The biofilm thickness after 3 days of cultivation ranged from 20–30 μm in our experimental setting.

The EPM major components

The EPM components were extracted from the static biofilm. Proteins and carbohydrates were major components of the biofilm matrix, and the protein concentration in the samples of the extracellular biofilm ranged from 374 to 982 $\mu\text{g}/\text{ml}$, with an average of 666 $\mu\text{g}/\text{ml}$, and for carbohydrate, the concentration ranged from 348 $\mu\text{g}/\text{ml}$ to 990 $\mu\text{g}/\text{ml}$, with an average of 682 $\mu\text{g}/\text{ml}$. For DNA, the concentration ranged from 17 to 46 $\mu\text{g}/\text{ml}$, with an average of 25 $\mu\text{g}/\text{ml}$.

Chemical analysis of EPM showed that the contents of proteins and carbohydrates were highest in EPM extracted from *F. nucleatum* and *P. gingivalis* W50 biofilm and lowest in *F. nucleatum* and *P. gingivalis* FDC381 biofilm (Fig. 3).

Proteins in the EPM of the dual species *F. nucleatum* and *P. gingivalis* biofilm grown in flow cells were visualized by CLSM after staining with Live/Dead and SYPRO® Ruby biofilm matrix stain, and it showed abundant amounts of proteins distributed within the biofilm matrix (Fig. 2).

No statistical significant differences were found in the concentrations of carbohydrates and eDNA between Proteinase K-treated and non-treated harvested biofilms (Fig. 3). The extracted DNA was analysed by agarose gel electrophoresis and the size of eDNA was found to be around 100 bp as shown (Fig. 4).

Enzyme effect on biofilm formation and mature biofilm

To test the effect of DNase I and proteinase K on biofilm formation, the enzymes were added at time zero, and the biofilm was analysed after 48 h. To test the effect on

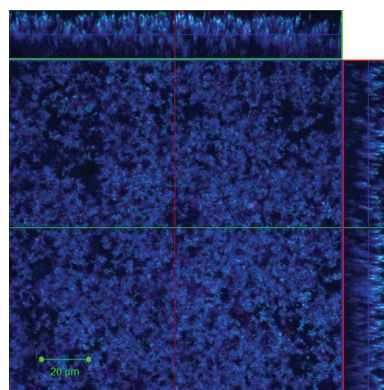


Fig. 2. Representative CLSM image shows proteins in EPM of 24-h dual species biofilm (*Fusobacterium nucleatum* ATCC 25586 and *Porphyromonas gingivalis* W50) grown in flow cells. The proteins were stained with SYPRO® Ruby stain (blue). Sideviews, XZ (top) and YZ (right) are sagittal sections of the biofilm. Scale Bar=20 μm .

mature biofilm, the enzymes were added at 48 h, and the effect was analysed after 1 h of incubation. *F. nucleatum* type strain ATCC 25586 and *P. gingivalis* type strain ATCC 33277 were tested when they were grown as a monoculture or as a dual species culture. In the static biofilm model (microtitre plates), the effects of these enzymes were not statistically significant (Fig. 5).

These findings were confirmed by CLSM image analyses, where bacterial biomass, maximum thickness, and average thickness of the biofilm were measured (Fig. 6). These parameters have little or no variation after enzymatic treatment, and the biofilm shape and structure remained unchanged.

Discussion

There is a lack of studies on EPM of subgingival biofilm. However, exo-polysaccharides in the supragingival biofilm have been extensively studied and known for a long time (29–31). In the present study, we have isolated EPM and tested effects of DNase I and proteinase K on biofilms from periodontal-disease-associated bacteria grown *in vitro*.

The EPM of the bacterial biofilms in our study are characterized by the presence of macromolecular complexes of carbohydrates, proteins, and nucleic acids. The enzymatic treatment of the harvested biofilms with proteinase K was performed to find out if it would increase the liberation of eDNA or carbohydrates, compared with only vortexing or homogenizing. This treatment did not result in noticeable difference in the yielded eDNA or carbohydrates (Fig. 3). Wu and Xi found similar results for carbohydrates in *Acinetobacter* sp. strain AC811 biofilm

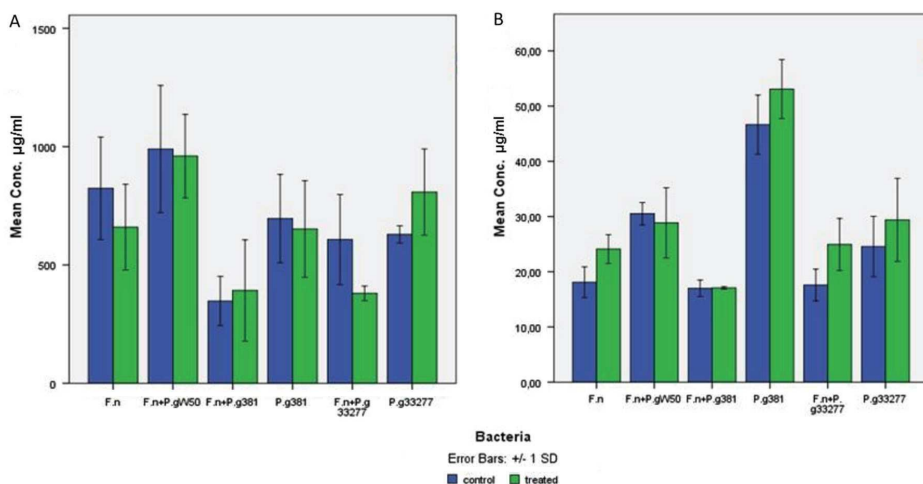


Fig. 3. Comparison of (A) carbohydrate and (B) eDNA yields from the biofilm matrix samples treated with proteinase K enzyme and non-treated samples. The matrix of 5-day-old biofilm was treated with 5 µg/ml proteinase K at 37°C for 1 h. F.n, *Fusobacterium nucleatum* ATCC 2558; P.g W50, *Porphyromonas gingivalis* W50; P.g 381, *P. gingivalis* FDC381; P.g 33277, *P. gingivalis* ATCC 33277. The bars represent the means with standard deviations from five samples (carbohydrates) and three replicates (eDNA).

matrix, but the eDNA yield was increased after enzyme treatment (14).

Even though proteins were abundant in the biofilm matrix of our *F. nucleatum* and *P. gingivalis* dual species

biofilm as shown in (Fig. 2), treatment with proteinase K was shown to be insufficient to disperse the biofilm matrix. A carbohydrate-rich matrix might be the reason for this ineffectiveness, which has also been suggested for

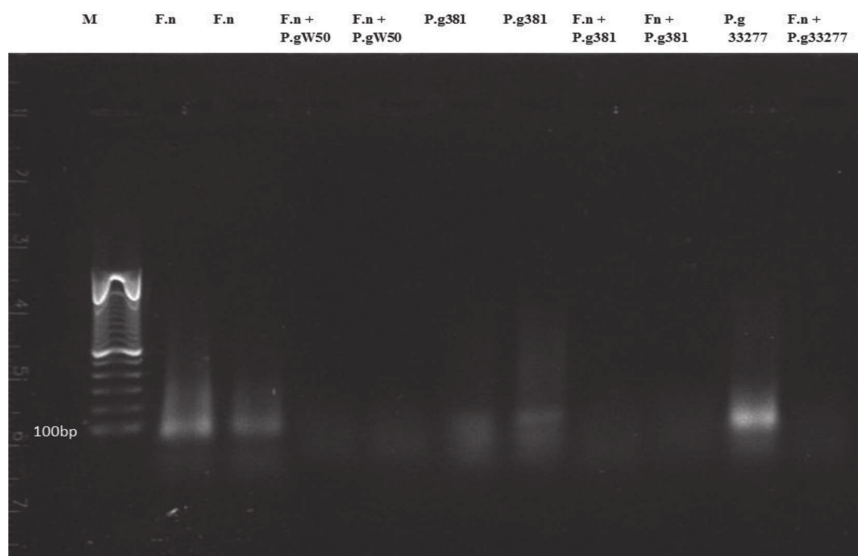


Fig. 4. Gel electrophoresis (0.8% agarose gel) of the extracellular DNA. The eDNA was extracted from the matrix of 5-day-old biofilm of these species. F.n, *Fusobacterium nucleatum* ATCC 25586; P.g W50, *Porphyromonas gingivalis* W50; P.g 381, *P. gingivalis* FDC381; P.g 33277, *P. gingivalis* ATCC 33277. Lane M, EZ load 100-bp molecular ruler (Bio-Rad).

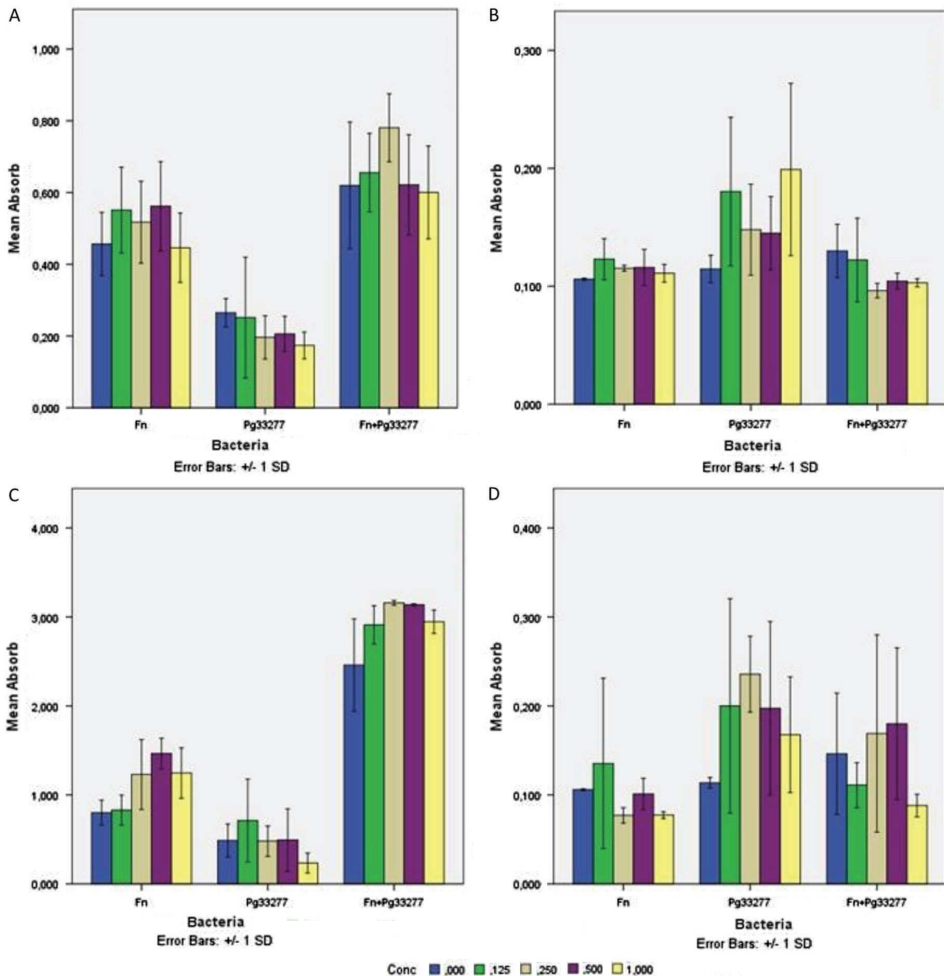


Fig. 5. Effect of DNase I and proteinase K on the biofilm formation (time zero) and on 48-h-old biofilm. *Fusobacterium nucleatum* type strain ATCC 25586 (F.n) and *Porphyromonas gingivalis* type strain ATCC 33277 (Pg 33277) bacterial species were tested when they were grown as monoculture or as dual species culture. (A) DNase I effect on biofilm formation, (B) DNase I effect on 48-h biofilm, (C) Proteinase K effect on biofilm formation, (D) Proteinase K effect on 48-h biofilm. The colored columns refer to the enzyme concentrations (0.125, 0.25, 0.5, and 1 mg/ml). The y-axis represents absorbance at 570 nm. The bars represent the means with standard deviations for 3–5 samples.

staphylococcal biofilms (32, 33). The eDNA detected in the EPM in our biofilms had a size around 100 bp, as demonstrated with agarose gel electrophoresis (Fig. 4). This size is usually higher than described for other biofilms; however, the size of the eDNA has been reported to range from less than 100 bp to 10 kb (34). Targeting the eDNA with DNase I in the mono or dual species biofilm matrix in our study gave no obvious effects with respect to prevention of biofilm formation or

dispersion of mature biofilm. This is in contrast to enzymatic treatment of *P. aeruginosa* biofilm (4). One suggested function of eDNA is in gene transfer (35, 36). The biofilm may offer an excellent milieu for DNA exchange, as the cells are in close juxtaposition and DNA can be trapped within the extracellular matrix (37). Genus *Fusobacterium* and other genera of oral bacteria contain conjugative transposons that facilitate the DNA transfer between bacteria through conjugation.

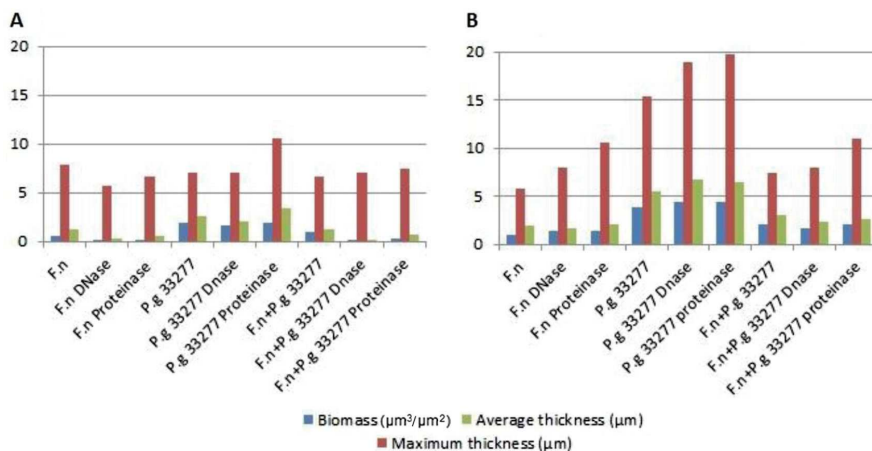


Fig. 6. Effect of DNase I and proteinase K enzymes at a concentration of 1 mg/ml on biofilm formation, (A) time zero and (B) 48-h-old biofilm. The graphs show the biomass ($\mu\text{m}^3/\mu\text{m}^2$), maximum thickness (μm), and average thickness (μm) of one point z-stack confocal images analysed by COMSTAT software program. F.n, *Fusobacterium nucleatum* ATCC 25586; P.g 33277, *Porphyromonas gingivalis* ATCC 33277.

P. gingivalis also shows a large degree of variation between strains, proposing that this organism has gone through frequent genetic rearrangements (37, 38).

In this study, we have shown that *F. nucleatum* can grow in a flow cell biofilm model in a non-strictly anaerobic environment, while this was not true for *P. gingivalis*. It seems to be a synergistic enhancement in the biofilm formation when these two species are grown together even in a partially oxygenated condition, which indicates that *F. nucleatum* might have the capacity to protect *P. gingivalis* from oxidative stress. This has also been reported in other studies (39, 40). Nearby *in vivo* association between these two microorganisms might indicate that they support each other, as shown in biofilm and mouse models (41, 42). *F. nucleatum* and *P. gingivalis* have been found to co-aggregate *in vitro* and *in vivo*, which could play a role in biofilm formation and pathogenesis, as also reported from mouse model experiments (43).

In general, a better understanding of the EPM of subgingival biofilm and complex multispecies biofilms should lead to more efficient control strategies. The management of biofilm growth does not necessarily require direct killing of the bacteria in the biofilm, but it might be directed toward degradation or dispersal of the biofilm matrix to reverse the biofilm mode of growth to a planktonic state, which is significantly easier to treat and manage, for example, by antibiotics.

In conclusion, in this study, we demonstrate that proteins and carbohydrates are major components in the EPM biofilms of *F. nucleatum* and *P. gingivalis* grown *in vitro*; however, eDNA might also play a role in the

structuring of the biofilm. More detailed structural and functional studies of EPM of subgingival biofilms are needed to identify and to attack new targets to control biofilm growth. Improved models and more complex systems and *in vivo* studies are clear objectives for further work.

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Conflict of interest and funding

The authors declare that they have no conflict of interest.

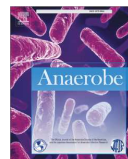
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Pathogenesis and toxins

Quantitative proteomic analysis of extracellular matrix extracted from mono- and dual-species biofilms of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*



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ABSTRACT

The Gram-negative bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are members of a complex dental biofilm associated with periodontal disease. In this study, we cultured *F. nucleatum* and *P. gingivalis* as mono- and dual-species biofilms, and analyzed the protein composition of the biofilms extracellular polymeric matrix (EPM) by high-resolution liquid chromatography-tandem mass spectrometry. Label-free quantitative proteomic analysis was used for identification of proteins and sequence-based functional characterization for their classification and prediction of possible roles in EPM. We identified 542, 93 and 280 proteins in the matrix of *F. nucleatum*, *P. gingivalis*, and the dual-species biofilm, respectively. Nearly 70% of all EPM proteins in the dual-species biofilm originated from *F. nucleatum*, and a majority of these were cytoplasmic proteins, suggesting an enhanced lysis of *F. nucleatum* cells. The proteomic analysis also indicated an interaction between the two species: 22 *F. nucleatum* proteins showed differential levels between the mono and dual-species EPMS, and 11 proteins (8 and 3 from *F. nucleatum* and *P. gingivalis*, respectively) were exclusively detected in the dual-species EPM. Oxidoreductases and chaperones were among the most abundant proteins identified in all three EPMS. The biofilm matrices in addition contained several known and hypothetical virulence proteins, which can mediate adhesion to the host cells and disintegration of the periodontal tissues. This study demonstrated that the biofilm matrix of two important periodontal pathogens consists of a multitude of proteins whose amounts and functionalities vary largely. Relatively high levels of several of the detected proteins might facilitate their potential use as targets for the inhibition of biofilm development.

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1. Background

Most bacterial species can adopt either a single-cell planktonic lifestyle or a multicellular state, *i.e.* community lifestyle with

Abbreviations: EPM, Extracellular Polymeric Matrix; LFQ, Label-Free Quantification; FAA, Fastidious Anaerobic Agar; PBS, Phosphate Buffered Saline; CFU, Colony Forming Unit; FASP, Filter Aided Sample Preparation; LC-MS/MS, Liquid Chromatography-tandem mass spectrometry; LTQ, Linear Trap Quadrupole; DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes; OMP, Outer Membrane Protein OMVs; Outer Membrane Vesicles; PCD, Programmed Cell Death.

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bacterial cells anchored to one another and to surfaces in an ordered structure known as a biofilm [1,2]. However, much of what is currently known about bacteria has been obtained from free-floating bacteria that grow in suspension [1], which can have considerably different gene expression pattern from sessile bacteria [3]. Bacterial biofilms play significant roles in human infections and diseases, and it is estimated that 65–80% of the microbial infections are caused by bacteria adhered to surfaces [4]. These biofilms also have an important role in increasing bacterial resistance to antibiotics [4]. In biofilms, poor antibiotic penetration, nutrient limitation, slow growth, adaptive stress responses, and formation of persister cells are assumed to establish a multi-layered defense [5]. An extracellular matrix provides the biofilms with their macroscopic appearance and can account for more than 90% of the

whole biofilm dry weight [6]. It is usually composed of polysaccharides, proteins and extracellular DNA and lipids, which altogether are called extracellular polymeric matrix (EPM) [7].

Periodontitis, which is defined as inflammation of the gingiva extending into the whole periodontium, is a classic example of biofilm mediated diseases [8]. It starts in a mild form called gingivitis, which is highly prevalent, and can affect up to 90% of the worldwide population. However, gingivitis does not affect the underlying supporting structures of the teeth and is reversible. On the other hand, more severe periodontitis can result in loss of connective tissue and bone support and is a main cause of tooth loss in adults [9]. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are among the important species in the subgingival biofilm that are involved in the pathogenesis of periodontitis [10]. *F. nucleatum* is frequently cultivated from the subgingival biofilm, and because of its ability to aggregate with many oral bacteria, it works as a bridge between early and late colonizers in the development of dental biofilm [11]. *P. gingivalis* is a member of the Socransky's red complex (*i.e.* bacteria strongly associated with periodontal disease) and has many virulence factors such as fimbriae, lipopolysaccharides, cysteine proteinases, and end-products of metabolism [12].

We have previously shown that EPM of a biofilm composed of *F. nucleatum* and *P. gingivalis* is prosperous with proteins [13]. In general the proteins in EPM are lectins, sugar binding proteins which enable cell-to-cell or cell-to-matrix interactions [14], and auto-transporters. The latter group is a family of outer membrane and secreted proteins of Gram-negative bacteria that possess unique structural properties facilitating their independent transport across the bacterial membrane to the cell surface, and may work in adhesion, aggregation, invasion, biofilm formation and toxicity [15,16]. In addition, pili and fimbriae are proteinaceous appendages that can contribute to the structure of the biofilm matrix, and were previously shown to be up-regulated in different biofilms compared to planktonic cultures [15,17].

In this study, we used a label-free quantitative proteomic approach for identification and relative quantification of proteins in the EPM of *F. nucleatum* and *P. gingivalis* when grown in mono- or dual-species biofilms. We report changes in the abundance of EPM proteins that depend on whether the bacteria have been grown in mono- or dual species biofilm.

2. Methods

2.1. Bacteria and growth medium

Fusobacterium nucleatum subsp. *nucleatum* type strain ATCC 25586 and *Porphyromonas gingivalis* type strain ATCC 33277 were used in the current study. The bacterial strains were grown on fastidious anaerobic agar (FAA) plates at 37 °C in anaerobic conditions (5% CO₂, 10% H₂, and 85% N₂) (Anoxomat System, MART Microbiology, Lichtenvoorde, Netherlands) for 48 h. A few colonies of each species were then used to inoculate Brucella broths (Becton Dickinson, Sparks, MD, USA) supplemented with 5 µg/ml hemin and 0.25 µg/ml vitamin K. The bacteria were grown overnight in the liquid medium at 37 °C in anaerobic conditions. The overnight cultures were adjusted to an absorbance of 0.15 at 600 nm (A₆₀₀), whereof 10 ml was transferred to a separate 25 cm² (area) polystyrene cell culture flask (cat.no 90026, TPP, Trasadingen, Switzerland) to prepare mono species biofilms, and 5 ml from each species was transferred to prepare dual species biofilm. We cultured the biofilms in an *in vitro* static biofilm model [18], the flasks were incubated at 37 °C in anaerobic conditions for 4 days without any additional supply of fresh medium. After medium removal, the biofilm samples were washed once with phosphate buffered saline (PBS) to remove free-floating bacteria and the

attached biofilm was harvested with a cell scraper (Nunc, Rochester, NY, USA). The collected biofilms were then resuspended in 500 µl PBS and stored at –20 °C until further processing.

2.2. Biofilm viability by colony forming unit (CFU) counting

The viability of the bacterial cells was determined by CFU counting of the initial inoculum and of the mature 4 days-old biofilm. Three independent biological replicates were serially diluted, selected dilutions plated on FAA medium, and incubated anaerobically at 37 °C for 4 days. The colonies formed on the plates were counted and used for calculating estimated numbers of viable cells.

2.3. Extraction of EPM

The biofilm samples were mechanically sheared with FastPrep FP120 Thermo Savant homogenizer (Qbiogene, Cedex, France) at a speed of 4 m/sec for 20 s, in Eppendorf tubes, without any cell-disrupting beads, to avoid contamination from cellular proteins. The samples were then filtered through 0.2 µm pore size acrodisc syringe filters (Pall, BioSciences, Ann Arbor, MI, USA) to remove cells and cellular debris [19]. Aliquots from each eluate were used in further work. Direct Detect[®] Spectrometer (Merck Millipore, Darmstadt, Germany) was used for protein concentration measurements. Low concentration samples of *P. gingivalis* EPM were concentrated by using Amicon Ultra-0.5 centrifugal filter devices with 3 K Da cutoffs (Merck Millipore, Darmstadt, Germany).

2.4. Sample preparation for the proteomic analysis

In order to generate a statistically robust proteomic dataset, samples with EPM extracts of mono- and dual-species biofilms from different culture flasks were prepared in four biological replicates. Filter Aided Sample Preparation (FASP) method developed by Wisniewski and co-workers [20], was used with minor modifications for the samples processing. Briefly, EPM samples were mixed in a solution of 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (NH₄HCO₃) [solution to total protein ratio (v/w) 1:10] and incubated for 45 min at 56 °C. Microcon device YM-10 filters (Merck Millipore, Darmstadt, Germany) were first conditioned by adding 100 µl of urea buffer (8M urea, 10 mM HEPES, pH 8.0) and centrifuged at 14,000xg for 5 min. This and the following steps were carried out at room temperature, unless otherwise stated. Aliquots of EPM samples containing 50 µg of protein were mixed with 200 µl urea buffer in the filter unit and centrifuged at 14,000xg for 15 min and this step was repeated one more time. The filtrate was discarded and 100 µl of 0.05 M iodoacetamide was added to each sample. The samples were mixed at 600 rpm for 1 min in a thermo-mixer and incubated without mixing in the dark for 20 min, followed by centrifugation at 14,000xg for 10 min, three washes with 100 µl urea buffer and another three washes with 100 µl 40 mM NH₄HCO₃ in H₂O. EPM remaining on the filter were digested with trypsin (Thermo Fisher Scientific, IL, USA) in 40 mM NH₄HCO₃ buffer [enzyme to protein ratio 1:50 (w/w)] at 37 °C for 16 h. The released peptides were collected by adding 50 µl of mass spectrometry grade water followed by centrifugation at 14,000xg for 15 min. This step was repeated twice. Samples were concentrated (to 20–40 µl volume) in a vacuum concentrator (Eppendorf, Hamburg, Germany).

2.5. Filtration and desalting

StageTips for filtration and desalting were prepared by packing 3M Empore C18 extraction disks (3M, MN, USA) in 200 µl pipet tips

by a blunt ended needle and a plunger or metal rod that helped to fit the extracted disks in the pipet tips, according to the protocol developed by Rappsilber and colleagues [21]. The disks were wetted by passing 20 μ l of methanol, followed by 20 μ l of elution buffer [80% acetonitrile (ACN), 0.1% formic acid (FA)]. The disks were then conditioned and equilibrated with 20 μ l of 0.1% FA just before the last residue of the previous buffer left the tip to avoid drying of the disks. Samples (volumes 20–40 μ l) were loaded on top of the Stage Tip. The disks with samples were desalted by washing with 20 μ l of 0.1% FA and were transferred to new tubes. Peptides were eluted and collected by adding 20 μ l elution buffer two times. The collected samples were dried in the vacuum concentrator and stored at -80 °C until further analyses. Peptide samples were resuspended by adding 1 μ l of 100% FA and 19 μ l of 2% ACN prior to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

2.6. LC-MS/MS

The MS/MS analysis was carried out at the Proteomics Unit, University of Bergen (PROBE) on an Ultimate 3000 RSLC system (Thermo Scientific, Waltham, MA, USA) connected to a linear quadrupole ion trap–Orbitrap (LTQ–Orbitrap) mass spectrometer (Thermo Scientific) equipped with a nano-electrospray ion source. Briefly, 0.5–1 μ g protein was loaded onto a pre-concentration column (Acclaim PepMap 100, 2 cm \times 75 μ m i. d. nanoViper column, packed with 3 μ m C18 beads) at a flow rate of 5 μ l/min for 5 min using an isocratic flow of 0.1% trifluoroacetic acid, vol/vol (TFA). Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 270 nl/min) on the analytical column (Acclaim PepMap 100, 50 cm \times 75 μ m i. d. nanoViper column, packed with 3 μ m C18 beads). Solvent A and B was 0.1% FA (vol/vol) in water or ACN (vol/vol), respectively. Separated peptides were sprayed directly into the MS instrument during a 195 min LC run with the following gradient composition: 0–5 min 5% B, 5–6 min 8% B, 6–135 min 7–32% B, 135–145 min 33–40% B, and 145–150 min 40–90% B. Elution of very hydrophobic peptides and conditioning of the column was performed by isocratic elution with 90% B (150–170 min) and 5% B (175–195 min), respectively. Desolvation and charge production were accomplished by a nanospray Flex ion source.

The mass spectrometer was operated in the data-dependent-acquisition mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey of full-scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution of R = 240,000 at m/z 400 (after accumulation to a target of 1,000,000 charges in the LTQ). The method used allowed sequential isolation of the most intense ions (up to 10, depending on signal intensity) for fragmentation on the linear ion trap using collision-induced dissociation at a target value of 10,000 charges. Target ions already selected for MS/MS were dynamically excluded for 18s. General mass spectrometry conditions were as follows: electrospray voltage, 1.8 kV; no sheath; and auxiliary gas flow. Ion selection threshold was 1000 counts for MS/MS, and an activation Q-value of 0.25 and activation time of 10 ms was also applied for MS/MS.

Table 1

Number of colony forming units (CFU) in the mono- and dual-species biofilms of *F. nucleatum* and *Pgingivalis*.

Bacterial species	Initial inoculum ^a (cfu/ml)	4 days mono-species biofilm (cfu/ml)	4 days dual-species biofilm (cfu/ml)
<i>F. nucleatum</i>	2–3 \times 10 ⁷	1–3 \times 10 ⁶	1–2 \times 10 ⁶
<i>P.gingivalis</i>	1–2 \times 10 ⁸	1–4 \times 10 ¹⁰	1–2 \times 10 ⁸

The numbers are an average of three biological replicates analysis.

^a Absorbance of 0.15 at 600 nm.

2.7. Data analysis

The acquired MS raw data were processed by using the MaxQuant software [22], version 1.5.2.8, with default settings. Label-Free Quantification (LFQ) [23] and match between runs, which is based on retention time alignment between different replicates, were optional software features, which were used in the MS/MS data searches. The MS spectra were searched against protein databases of either *F. nucleatum* type strain ATCC 25586 or *P. gingivalis* type strain ATCC 33277. The respective files were downloaded from the UniProt knowledgebase on the 4th of February 2015. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE [24] partner repository with the dataset identifier PXD004888.

Post MaxQuant analysis included filtering of the generated ‘proteingroups.txt’ table for contaminants, only identified by site and reverse hits by the Perseus software [25]. Each protein identified in at least two out of four replicates was considered valid. To discriminate differential expressions of proteins present both in the mono- and dual-species biofilm, *t*-test with *p*-value \leq 0.05 was used.

Functional protein annotation was performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [26]. Predictions of the identified proteins subcellular localization were performed by web-based application SOSUI-GramN [27]. VirulentPred [28] was employed to predict the virulence factors among identified bacterial proteins, and the predictions were derived from the Cascaded SVM (Support Vector Machine) module [29]. The protein lists were also searched for beta-barrels integral outer membrane proteins with the BOMP web-based tool [30].

3. Results

3.1. Biofilm viability

Characterization of the biofilms with respect to the amount of viable cells showed a slight reduction in the numbers of cells in *F. nucleatum* biofilms, both when grown alone and with *P. gingivalis* (Table 1) compared to the initial inoculum. In the *P. gingivalis* biofilm the number of cells was slightly increased after 4 days of growth, however, in the shared biofilm with *F. nucleatum* viable *P. gingivalis* cells were at equivalent levels as the initial inoculum (Table 1).

3.2. Biofilm matrix of *F. nucleatum* is rich in proteins when compared to *P. gingivalis* EPM

Trypsin-digested EPM samples from four biological replicates of the mono- and dual-species biofilms were analyzed by LC-MS/MS. Searching the acquired MS/MS raw data against either *F. nucleatum* or *P. gingivalis* protein databases resulted in identification of 542 and 93 proteins in the matrix of *F. nucleatum* and of *P. gingivalis*, respectively. In the dual-species biofilm matrix we identified 280 proteins in total, with 198 (70.7%) derived from *F. nucleatum* and 82 (29.3%) from *P. gingivalis* (Fig. 1 and Additional file 2: Table S1). The

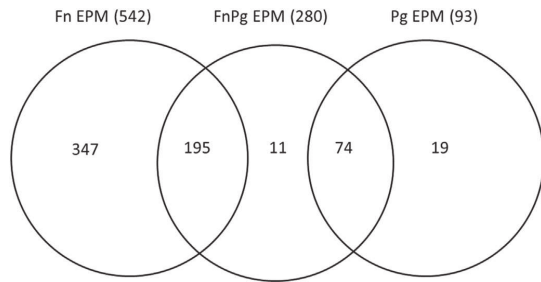


Fig. 1. Summary of proteins identifications in the EPMs of two oral bacteria. The Venn diagram summarizes the number of proteins identified in EPM of *F. nucleatum* (Fn) and *P. gingivalis* (Pg) grown as mono- and dual-species biofilms. The eleven exclusive proteins in the dual-species EPM included 8 proteins derived from *P. gingivalis* and 3 proteins from *F. nucleatum* (additional file 1: Table S4).

correlation between different biological replicates was highest in the samples from *F. nucleatum* EPM (Pearson correlation coefficient R between 0.89 and 0.99), while somewhat lower for the samples of *P. gingivalis* and the dual-species EPM (R between 0.65 and 0.95 and 0.63–0.89 for the EPMs of *P. gingivalis* and dual-species, respectively) (Additional file 1, Fig. S1A).

3.3. Functional analysis of EPM-associated proteins

We next characterized the identified proteins with respect to

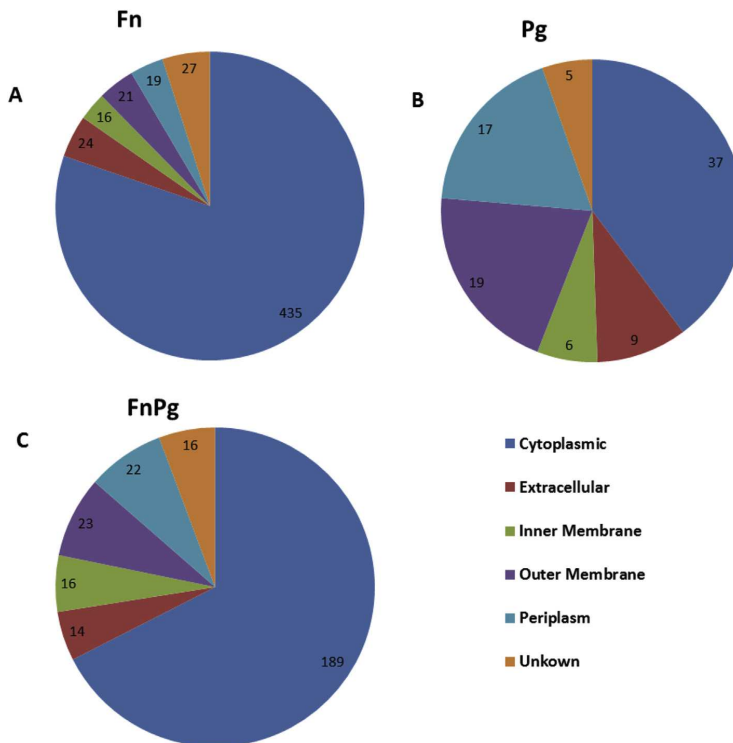


Fig. 2. Predicted subcellular localization of the EPM proteins. Distribution of the identified proteins in the respective EPMs according to their predicted subcellular localization by SOSUI-GramN: A) *F. nucleatum* (Fn) EPM; B) *P. gingivalis* (Pg) EPM and C) Dual-species (FnPg) EPM.

their cellular localization by using a prediction system SOSUI-GramN. A majority of the proteins originated from the cytoplasm, with 80% in *F. nucleatum* EPM and 40% in *P. gingivalis* EPM (Fig. 2). For comparison, the percentage of cytoplasmic proteins in the predicted whole proteomes of *F. nucleatum* and *P. gingivalis* is 65% and 55%, respectively, as determined by using the same software. The number of proteins with cytoplasmic origin was 68% in the dual-species EPM. These results suggest an enhanced cell lysis in the *F. nucleatum* biofilm, and further support the importance of dead cell components in the formation of EPMs.

Functional annotation of the identified proteins, which was extracted from the DAVID database, revealed that *F. nucleatum* EPM proteins participated in translation, oxidation/reduction, proteolysis and various metabolic processes (Additional file 3: Table S2). The latter mentioned metabolic proteins could be divided into different pathways classified by Kyoto Encyclopedia of Genes and Genomes (KEGG), with the most abundant pathways related to amino acid, carbohydrate and nucleotide/nucleic acid metabolism. The annotated proteins from *P. gingivalis* EPM were linked to proteolysis, cellular homeostasis of ions, and metabolism of amino acids and nucleobases (Additional file 3: Table S2). Four *P. gingivalis* proteins were associated with pathogenesis: major fimbrial subunit protein FimA, arginine-specific cysteine proteinase RgpA, hemagglutinin protein HagA and Lys-gingipain [31]. The main biological processes of proteins detected in the dual-species EPM closely resembled those described for mono-species EPM proteins (Additional file 3: Table S2).

Oxidoreductases and chaperones were among the most

abundant proteins identified. The results showed that 41 proteins (7.6%) from the *F. nucleatum* EPM, 6 proteins (6.5%) in *P. gingivalis* EPM and 27 proteins (9.6%) in the dual species EPM were involved in oxidation/reduction (Additional file 3: Table S2). Six different chaperone proteins were identified in the EPM of *F. nucleatum* including: GroEL (60 kDa chaperonin), DnaK, GroES (10 kDa chaperonin), ClpB, HtpG and F1610 (33 kDa chaperonin). DnaK and GroEL were also found in the dual species biofilm matrix (Additional file 2: Table S1).

3.4. Relative abundances of multiple proteins differ in the mono- and dual-species EPMs

As the next step we performed quantitative analysis of the identified proteins based on their LFQ intensity scores. For 87%, 42% and 41% of all proteins described in the *F. nucleatum*, *P. gingivalis* and the dual-species EPMs, respectively, we determined the relative abundance (Additional file 4: Table S3). Correlations of LFQ intensities between the different biological replicas, represented as R, varied between 0.91 and 0.99, 0.73–0.96 and 0.83–0.95 for *F. nucleatum*, *P. gingivalis* and the dual-species EPMs, respectively (Additional file 1, Fig. S1B). The quantitative levels of proteins covered a dynamic range of approximately $10 \log_2$ (Fig. 3). While the distribution of proteins abundances were similar in the *F. nucleatum* and dual-species EPMs (median of \log_2 LFQ equal to 25.0 and 24.6, respectively), amounts of proteins identified in *P. gingivalis* EPM were significantly lower (median of \log_2 LFQ = 22.1). Top 20 abundant proteins according to the averaged \log_2 LFQ are listed in Table 2. Acetyl-CoA acetyltransferase, alkyl hydroperoxide reductase C22 protein, neutrophil-activating protein A, tryptophanase and glutamate dehydrogenase were the top five proteins in the matrix of *F. nucleatum* biofilm. The oxidoreductase NAD-specific glutamate dehydrogenase and the proteolytic and adhesive protein Lys-gingipain were most abundant in *P. gingivalis* EPM. In the dual species EPM, *F. nucleatum* contributed to four out of five proteins, and the most abundant was *F. nucleatum* glutamate dehydrogenase, while NAD-specific dehydrogenase was among the most abundant proteins from *P. gingivalis*. Functional annotation of top abundant proteins revealed their involvement in oxidation/reduction, hydrolase activity, proteolysis and binding (Table 2).

In order to discriminate differential expression of proteins present both in the mono- and dual-species biofilm, we performed two-sample *t*-test on the corresponding LFQ intensities, with *p*-value ≤ 0.05 . The LFQ intensities of 22 proteins in *F. nucleatum* EPM showed significant changes: three increased and 19 decreased in the dual-species EPM compared to the mono-species EPM (Fig. 4). Ethanolamine utilization protein (FN0083), uncharacterized protein (FN1302) and aspartate aminotransferase (FN1152) were among the increased proteins. On the other hand, different binding proteins (FN1423, FN0820, FN0472, FN0652, FN1170, FN0512, FN0278, FN1812), monosaccharide metabolism proteins (FN0262, FN0652) and oxidoreductases (FN0512, FN0652, FN0820, FN1170, FN1423, FN1983) were significantly decreased in dual-species EPM. In *P. gingivalis* EPM biofilms no proteins showed statistically significant difference compared to the dual-species EPM in terms of the protein LFQ intensities.

3.5. Prediction of EPM-associated proteins with virulence properties

To further characterize the EPM-derived protein datasets, we performed prediction of virulence factors by cascaded SVM module of the VirulentPred tool. Proteins with high score in virulence potential (Table 3) were mainly uncharacterized proteins and hypothetical cytosolic proteins that need more elaboration on their

function.

Among the *P. gingivalis* virulence proteins were several fimbriae: the subunit protein of long FimA fimbria was found in the EPM of both mono- and dual-species biofilm and the accessory fimbriae FimCDE and FimDE were found in mono- and dual-species biofilm, respectively. Lys-gingipain (kgp), hemagglutinin HagA and peptidylarginine deiminase (PGN_0898) are *P. gingivalis* virulence factors that were found in the EPM of mono and dual-species biofilms (Additional file 2: Table S1).

Searching for outer membrane proteins (OMPs) in our dataset was of particular interest because OMPs are often involved in adhesive properties and binding of *F. nucleatum* and *P. gingivalis*. Forty proteins were predicted by SOSUI-GramN tool as OMPs (21 and 19 from *F. nucleatum* and *P. gingivalis*, respectively) (Additional file 2: Table S1). In addition, we used the BOMP web-based tool for the prediction of OMPs containing integral β -barrel domains. Fourteen proteins in the dual-species biofilm matrix, 18 in the *F. nucleatum* biofilm matrix and 10 in the *P. gingivalis* biofilm matrix were predicted as integral β -barrel outer membrane proteins (Table 4). The OMPs predictions by the SOSUI-GramN and BOMP tools overlapped for 14 proteins. However, in 11 and 3 cases the SOSUI-GramN suggested extracellular and cytoplasmic localization, respectively, while the BOMP indicated that the respective proteins contain integral β -barrel domain (Table 4).

4. Discussion

In this study we used high-resolution proteomics to identify and quantify proteins in the EPM of *F. nucleatum* and *P. gingivalis* when grown in mono- or dual-species biofilms. The two selected strains are found in the normal flora of the mouth, they have documented role in the periodontal disease, and their genomes have been sequenced [32,33]. The bacteria were cultured for four days which gave a mature biofilm with adequate amount of the EPM [13], and also had a minimal effect on the number of viable cells in the biofilms (Table 1). To identify EPM associated proteins of *F. nucleatum* and *P. gingivalis* biofilms, the bacteria were grown in cell culture flasks on a plastic surface both individually and together, an approach that allowed for investigation of possible interactions between the two species at the protein level. Initially, extracellular matrix was isolated from each biofilm by mechanical shearing and filtration through membranes that removed cellular debris and whole cell contaminants. We avoided sonication and chemical treatment to reduce contamination with cellular proteins. This protocol has been previously validated and confirmed that it does not provoke cellular lysis [19], and a similar procedure with minor modifications has been used to study the matrix proteins in *Pseudomonas aeruginosa* biofilm [34].

The number of identified proteins was similar to other studies on EPMs of bacterial biofilms, which reported between 150 and 270 EPM proteins [34,35]. Reasons for the high number of protein identifications in the EPM of *F. nucleatum* (542 proteins), when compared to the numbers derived from *P. gingivalis* and dual-species EPMs (93 and 280 proteins, respectively), are not entirely clear. The high number of *F. nucleatum* proteins in the biofilm matrix could be caused by more intensive cell lysis, when compared to *P. gingivalis*. However, only moderate changes in the number of viable cells were observed in the growth period of four days (Table 1). A possible mechanism behind cell lysis might be programmed cell death (PCD). *F. nucleatum* has Cid/Lrg homologues of so-called holins, small membrane proteins responsible for PCD in the bacteria, whose role is mainly associated with permeabilisation of the cytoplasmic membrane and with concomitant protein export [36,37]. For example, in *Staphylococcus aureus* CidA contributes to biofilm adherence both *in vitro* and *in vivo* by affecting cell lysis and

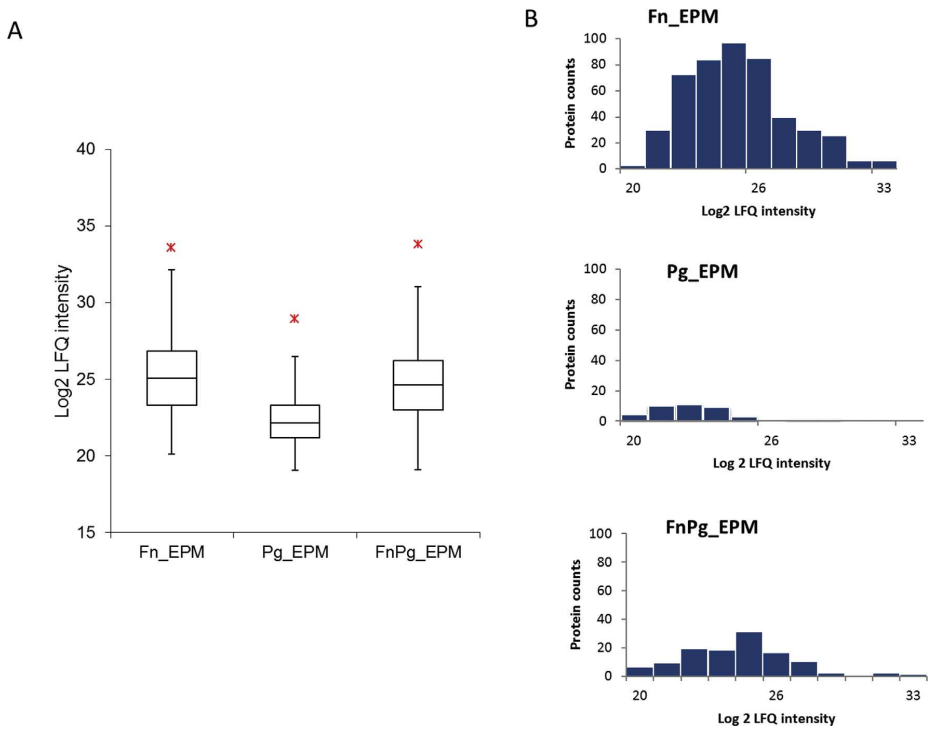


Fig. 3. Quantitative analysis of the identified EPM proteins. A) Boxplots showing distribution of \log_2 transformed LFQ intensities among 471 *F. nucleatum* (Fn), 39 *P. gingivalis* (Pg) and 114 (FnPg) proteins in the mono- and dual-species EPMs, red marks indicate the maximum outliers. B) Histograms showing the distribution of averaged \log_2 LFQ intensities across the samples, the vertical axis shows the counts of identified proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the release of genomic DNA [38,39]. The murein hydrolase exporter (FN0467) and murein hydrolase export regulator (FN1531), which are the holins of *F. nucleatum*, are both found in the proteome of

F. nucleatum when grown in biofilm or under planktonic conditions [37].

Moreover, a high number of nucleotide-binding proteins in the

Table 2

Top abundant proteins identified in the mono- and dual-species EPM of *F. nucleatum* and *P. gingivalis*.

Accession	Gene Name	Protein Description	Major Function	\log_2 LFQ
Top 10 in <i>F. nucleatum</i> EPM				
Q8RG24	FN0495	Acetyl-CoA acetyltransferase	acyltransferase	33.57
Q8RGD3	FN1983	Alkyl hydroperoxide reductase C22 protein	oxidoreductase	33.45
Q8REM0	FN1079	Neutrophil-activating protein A	virulence factor	33.42
Q8RHQ6	FN1943	Tryptophanase	lyase	33.27
Q8RG30	FN0488	Glutamate dehydrogenase	oxidoreductase	33.16
Q8RDT4	FN1419	Methionine gamma-lyase	lyase	32.67
Q8RES5	FN1019	3-hydroxybutyryl-CoA dehydrogenase	oxidoreductase	32.36
Q8RI55	Eno	Enolase	phosphopyruvate hydratase	32.34
Q8RC46	FN0472	Flavodoxin	electron-transfer protein	31.70
Q8RHM6	Tpl	Tyrosine phenol-lyase	lyase	31.57
Top 5 in <i>P. gingivalis</i> EPM				
B2RKJ1	gdh	NAD-specific glutamate dehydrogenase	oxidoreductase	28.90
B2RLK2	kgp	Lys-gingipain	protease/virulence factor	28.06
B2RJ88	PGN_0914	Peptidase M24 family	hydrolase	25.11
B2RIQ1	PGN_0727	4-hydroxybutyryl-CoA dehydratase	oxidoreductase	24.9
B2RKQ8	PGN_1434	Aminoacyl-histidine dipeptidase	proteolysis	24.91
Top 5 in dual-species EPM				
Q8RG30	FN0488	Glutamate dehydrogenase	oxidoreductase	33.80
Q8RG24	FN0495	Acetyl-CoA acetyltransferase	transferase	32.36
Q8RE27	FN1302	Uncharacterized protein	unknown	32.31
B2RKJ1	gdh	NAD-specific glutamate dehydrogenase	oxidoreductase	29.78
Q8REM0	FN1079	Neutrophil-activating protein A	virulence factor	29.40

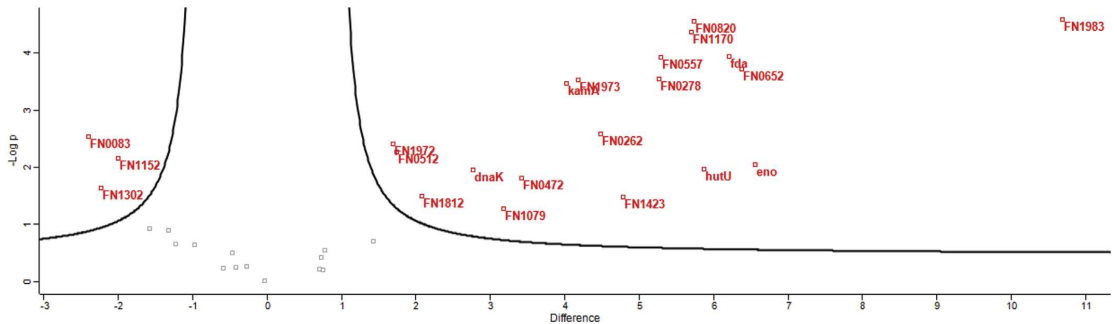


Fig. 4. Differentially expressed *F. nucleatum* proteins in mono- and dual-species EPMS. Volcano plot shows results of two-sample *t*-test with *p*-value ≤ 0.05 , which was performed on \log_2 LFQ intensities of 36 proteins common for the EPMS of mono- and dual-species biofilms. Changes in relative abundances were detected for 22 *F. nucleatum* proteins (shown with the gene name).

EPMS of *F. nucleatum* and the dual species biofilms coincide with our previous results that showed DNA as a major component in the biofilm matrix [13], and further support occurrence of cell lysis during biofilm formation.

P. gingivalis is an asaccharolytic microorganism (*i.e.* unable to metabolize carbohydrates) while *F. nucleatum* is able to utilize amino acids, peptides and sugars [40]. Accordingly, we noticed that most of the detected metabolic pathways in *P. gingivalis* EPM had a role in amino acid metabolism [41]. This finding and generally high percentage of other metabolic enzymes detected in the EPMS of both *F. nucleatum* and the dual species biofilms, are indications of the putative role of the matrix as an external source of nutrition and energy production as previously predicted in other bacterial biofilms [6,42].

Studies on these two organisms found evidence of physiological support between the species [43,44]. *F. nucleatum* is a moderate anaerobe, however, its ability to adapt to and reduce an oxygenated environment is extremely high [43]. On the other hand, *P. gingivalis* cannot survive in an aerated environment above 6% O_2 when grown as a monoculture, but when grown as a co-culture with

F. nucleatum, *P. gingivalis* can survive O_2 levels of up to 20% [43]. Proteins associated with oxidative stress were abundant in the matrix of the studied biofilms, similar to findings described in a study of the *P. aeruginosa* EPM [34]. Oxidative stress response proteins were also previously shown to be up-regulated in the biofilms of *Tannerella forsythia* [45] and *Campylobacter jejuni* [46], when compared to planktonic growth. It has been suggested that mixed species biofilms enhance the production of oxidative stress proteins because the more strict anaerobes are dependent on oxygen tolerant bacteria [47].

The two-species biofilm model used in this study represents a limited example since periodontal diseases develop in a polymicrobial environment. Although biofilm models with multiple bacterial species could represent more closely *in vivo* condition [48], such models are difficult to control and manipulate. Biofilm model with only two bacteria, such as the *F. nucleatum* - *P. gingivalis* model, is more straightforward for interpreting possible interactions between the two species. The proteomic analysis showed differential production of 22 *F. nucleatum* proteins between the mono and dual-species EPMS (Fig. 4) and 11 proteins were detected

Table 3

Top scored virulence-related proteins identified in the mono- and dual-species EPM of *F. nucleatum* and *P. gingivalis*, as predicted by VirulentPred web-based tool.

Accession	Gene Name	Protein Description	Score ^a
Top 10 in <i>F. nucleatum</i> EPM			
Q8RH18	FN2034	Protein YicC	1.15
Q8RGA1	FN0407	Uncharacterized protein	1.14
Q8RGH5	FN0320	Hypothetical cytosolic protein	1.14
Q8RFA8	kdsB	3-deoxy-manno-octulosonate cytidyltransferase	1.14
Q8RH51	FN0062	Hypothetical cytosolic protein	1.13
Q8RI03	FN1835	Uncharacterized protein	1.13
Q8RG53	FN0465	Uncharacterized protein	1.13
Q8RDV3	glsA	Glutaminase	1.13
Q8RH86	FN0024	Hypothetical exported 24-amino acid repeat protein	1.13
Q8RHV9	FN1884	Uncharacterized protein	1.12
Top 5 in <i>P. gingivalis</i> EPM			
B2RGY4	PGN_0110	Putative uncharacterized protein	1.15
B2RLK7	hagA	Hemagglutinin protein HagA	1.11
B2RL17	PGN_0693	Putative uncharacterized protein	1.05
B2RH58	fimD	Minor component FimD	1.04
B2RK12	PGN_1188	Putative uncharacterized protein	1.03
Top 5 in dual species EPM			
B2RGY4	PGN_0110	Putative uncharacterized protein	1.15
Q8RG53	FN0465	Uncharacterized protein	1.13
B2RLK7	hagA	Hemagglutinin protein HagA	1.11
Q8R614	FN1387	Metal dependent hydrolase	1.11
Q8RFM6	FN0666	Uncharacterized protein	1.11

^a Support Vector Machine (SVM) score. The protein is virulent if the score ≥ 0 .

Table 4
 β -barrel integral outer membrane proteins identified in the mono- and dual-species EPM of *F. nucleatum* and *P. gingivalis*, as predicted by BOMP web-based tool.

Accession	Gene Name	Description	BOMP Category ^a	Known as OMP	Prediction by SOSUI-Gram ^b
<i>F. nucleatum</i> EPM					
Q8REA9	FN1200	Uncharacterized protein	5		OM
Q8RIP5	FN1526	Fusobacterium outer membrane protein family	5	[32]	EC
Q8RHY1	FN1859	Major outer membrane protein FomA	4	[32,69]	OM
Q8R608	FN1426	Serine protease	4		OM
Q8RFV3	FN0579	Hypothetical cytosolic protein	3		OM
Q8RI47	FN1787	Tetratricopeptide repeat family protein	3		C
Q8RF62	glgB	1,4- α -glucan branching enzyme GlgB	3		C
Q8RF57	FN0610	Uncharacterized protein	3		EC
Q8RGD6	FN1950	Serine protease	2		OM
Q8RE26	ssb	Single-stranded DNA-binding protein	2		EC
Q8RH76	FN0034	Uncharacterized protein	1		EC
Q8RFG5	FN0735	Cell surface protein	1		OM
Q8RHC7	FN2110	Hypothetical exported 24-amino acid repeat protein	1		EC
Q8RHM8	FN1986	Uncharacterized protein	1		EC
Q8RHV1	FN1893	Fusobacterium outer membrane protein family	1	[32]	EC
Q8RHH1	FN2058	Fusobacterium outer membrane protein family	1	[32]	EC
Q8RE33	FN1296	Uncharacterized protein	1		EC
Q8RCN9	FN0247	Hypothetical cytosolic protein	1		C
<i>P. gingivalis</i> EPM					
B2RL18	PGN_1744	Putative uncharacterized protein	4		OM
B2RLU2	PGN_1818	Putative uncharacterized protein	3		OM
B2RLK7	hagA	Hemagglutinin protein HagA	3		EC
B2RLK2	kgp	Lys-gingipain	3	[70]	OM
B2RHG7	ragA	Receptor antigen A	3	[70]	OM
B2RGP7	porV	Por secretion system protein porV (Pg27, lptO)	1		OM
B2RKPO	PGN_1416	Probable lysyl endopeptidase	1		OM
B2RM93	rgpA	Arginine-specific cysteine proteinase RgpA	1	[70]	OM
B2RIW9	PGN_0795	Putative uncharacterized protein	1		OM
B2RH26	PGN_0152	Immunoreactive 61 kDa antigen	1		EC
Dual species EPM					
Derived from <i>F. nucleatum</i>					
Q8REA9	FN1200	Uncharacterized protein	5		OM
Q8RIP5	FN1526	Fusobacterium outer membrane protein family	5	[32]	EC
Q8R608	FN1426	Serine protease	4		OM
Q8RHY1	FN1859	Major outer membrane protein FomA	4	[32,69]	OM
Q8RFV3	FN0579	Hypothetical cytosolic protein	3		OM
Q8RE33	FN1296	Uncharacterized protein	1		EC
Q8RHH1	FN2058	Fusobacterium outer membrane protein	1	[32]	EC
Derived from <i>P. gingivalis</i>					
B2RL18	PGN_1744	Putative uncharacterized protein	4		OM
B2RHG7	ragA	Receptor antigen A	3	[70]	OM
B2RLK2	kgp	Lys-gingipain	3	[70]	OM
B2RLK7	hagA	Hemagglutinin protein HagA	3		EC
B2RIW9	PGN_0795	Putative uncharacterized protein	1		OM
B2RKPO	PGN_1416	Probable lysyl endopeptidase	1		OM
B2RM93	rgpA	Arginine-specific cysteine proteinase RgpA	1	[70]	OM

^a BOMP Category 1 is the least reliable prediction while category 5 is the most reliable prediction of integral β -barrel domains.

^b OM: outer membrane, EC: extracellular, C: cytoplasmic.

only in the dual-species EPM (additional file 1, Table S4). These results indicate that the two species specifically influence each other at the protein level, further supporting synergistic action between these two oral pathogens [43,44].

Among the most abundant proteins identified in the EPM were molecular chaperons. Previously, typical cytosolic proteins GroEL and DnaK were described to be associated with membranes and extracellular fractions of *F. nucleatum* [49]. Targeting GroEL could represent an antimicrobial strategy with broad-spectrum application, and recently a high-throughput screening effort to discover chemically and structurally diverse inhibitors of GroEL/GroES has been undertaken [50]. Moreover, *P. gingivalis* GroEL immunization was reported to significantly reduce the levels of alveolar bone loss induced by multiple periodontopathic bacteria in an animal model [51]. Finally, the presence of oxidoreductases and various chaperone proteins in the EPM of oral bacteria biofilms is not only of interest regarding periodontal diseases, but also for possible associations of bacterial biofilms with systemic inflammatory and autoimmune diseases [52–54].

Twenty-five of the proteins detected in the EPM of *P. gingivalis* biofilm were previously identified as outer membrane vesicles (OMVs) proteins [55] (Additional file 2: Table S1). *P. gingivalis* is able to specifically concentrate and release a large number of its virulence factors into the environment in the form of OMVs, and these vesicles have been linked to biofilm formation for example in *Helicobacter pylori* [56]. Our results show that *P. gingivalis* OMVs proteins represent a significant portion of the EMP proteome and OMVs are therefore likely contributors to the biofilm development. An example of *P. gingivalis* OMVs protein identified both in mono- and dual-species EPMS was hemagglutinin HagA, a surface protein that can function as an adhesin attaching bacteria to the host cells [57]. We identified several other *P. gingivalis* virulence proteins in the biofilm matrix, such as fimbriae that are key factors in adhesion of the bacterial cells to the host tissue, its colonization and invasion of host cell membranes [58]. Another virulence protein that contributes to the destruction of periodontal tissues is Lys-gingipain (kgp), and in our dataset it was one of the most abundant *P. gingivalis* EPM proteins (Table 2 and Additional file 4: Table S3).

Gingipains degrade collagen and fibronectin and inhibit interactions between host cells and the extracellular matrix. In addition, they degrade various cytokines, resulting in a disruption of the host cytokine network [58]. Another *P. gingivalis* protein identified both in mono- and dual-species EPM was PGN_O898, a bacterial peptidylarginine deiminase (PAD) (Additional file 2: Table S1). It gives *P. gingivalis* a unique ability to citrullinate proteins [59]. Citrullinated bacterial and host peptides may cause an autoimmune response in rheumatoid arthritis [59,60].

Sequence-based prediction of the proteins subcellular localization is an important part of the identified proteome description and an essential step in the search for novel vaccine or drug targets [61,62]. Our data provided evidence that the matrix proteome consists of secreted proteins, proteins from cell debris, and OMPs. The prediction of OMPs in this study was of particular importance due to their involvement in adhesive properties and coaggregation of *F. nucleatum* and *P. gingivalis* with other bacteria, as well as attachment to host cells. The latter interaction has significance both in the pathogenesis of infection and in the immune response of the host [62]. The SOSUI-Gram and BOMP tools identified 40 and 42 OMPs, respectively, and there was variation in the prediction outcomes of the two bioinformatics methods. This observation illustrates that the use of several bioinformatics methods is both beneficial and necessary, and cross-referencing with available literature should complement the importance of the predictions. The BOMP tool specifically predict membrane proteins containing β -barrel integral domains, which can have many different functions including enzymatic, transport and structural support [63]. An example of such OMP is FomA (FN1859) that was identified in both mono- and dual-species EPM (Table 4). It is a nonspecific porin which acts as a virulence factor, and a major antigen of *F. nucleatum* [64,65] that plays a role in binding to *P. gingivalis* [66]. FomA of *F. nucleatum* represents a potential target protein for the prevention of bacterial co-aggregation by vaccination [66,67]. Other detected putative *F. nucleatum* virulence factors were auto-transporter fusobacterium outer membrane protein (FN1526) and serine protease (FN1426). Both proteins are involved in protein secretion pathways [37], and the latter has peptidase and hydrolase activity, which allows degradation of fibronectins, fibrinogens and collagens. The capacity of *F. nucleatum* to degrade proteins of the extracellular matrix of host connective tissues has been described as a significant contributor to invasion of the gingival tissue and subsequent damage of periodontal tissues [37,68].

5. Conclusion

To our knowledge this is the first study to explore proteins in the extracellular matrix of biofilms formed by the oral bacteria *F. nucleatum* and *P. gingivalis*. Potential virulence proteins, outer membrane proteins and various binding proteins (DNA-binding, ATP-binding, and metal ion-binding) were among the abundant proteins identified in the biofilm EPM. These proteins represent potential candidates to be targeted for the inhibition of biofilm development. Furthermore, identification and quantification of the proteins provides a molecular basis for further revealing their role in the formation of EPM and might contribute to an understanding of *F. nucleatum* and *P. gingivalis* role in the development of periodontal and systemic diseases.

6. Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE [24] partner repository with the dataset identifier PXD004888.

Competing interests

The authors have declared that no competing of interests exists.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anaerobe.2017.03.002>.

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