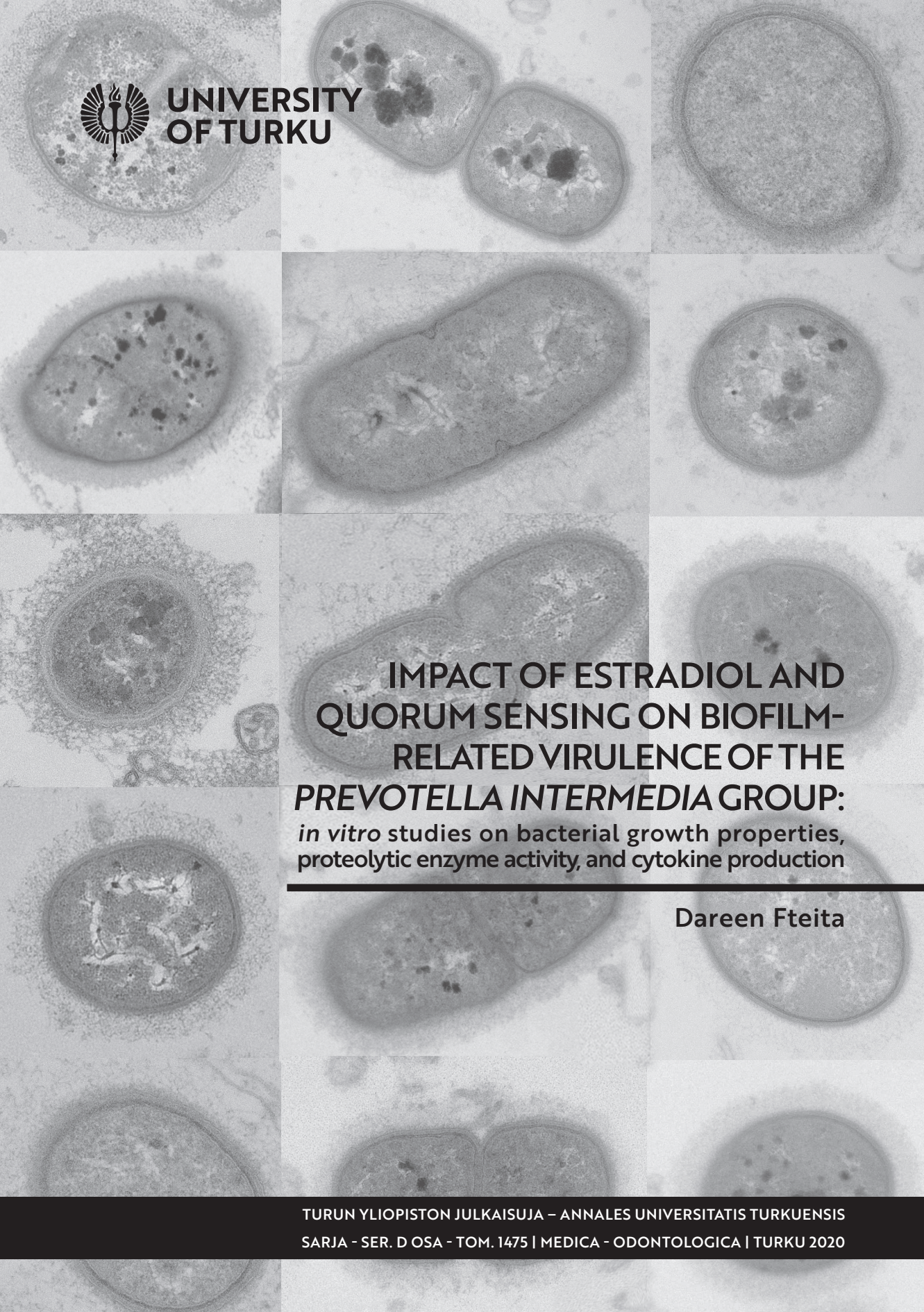


UNIVERSITY OF TURKU

IMPACT OF ESTRADIOL AND QUORUM SENSING ON BIOFILM-RELATED VIRULENCE OF THE *PREVOTELLA INTERMEDIA* GROUP:

in vitro studies on bacterial growth properties, proteolytic enzyme activity, and cytokine production

Dareen Fteita

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1475 | MEDICA - ODONTOLOGICA | TURKU 2020

UNIVERSITY OF TURKU

IMPACT OF ESTRADIOL AND QUORUM SENSING ON BIOFILM-RELATED VIRULENCE OF THE *PREVOTELLA INTERMEDIA* GROUP:

in vitro studies on bacterial growth properties, proteolytic enzyme activity, and cytokine production

Dareen Fteita

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA – TOM. 1475 | MEDICA – ODONTOLOGICA | TURKU 2020

University of Turku

Faculty of Medicine
Institute of Dentistry
Department of Periodontology
Finnish Doctoral Program in Oral Sciences (FINDOS-Turku)

Supervised by

Professor Eija Könönen, DDS, PhD
Department of Periodontology
Institute of Dentistry
University of Turku, Finland

Associate Professor Ulvi Gürsoy, DDS, PhD
Department of Periodontology
Institute of Dentistry
University of Turku, Finland

Reviewed by

Professor Gilad Bachrach, BS, PhD
Faculty of Dental Medicine
Hadassah School of Dental Medicine
The Hebrew University, Israel

Associate Professor Tülay Yucel-Lindberg, MSc, PhD
Department of Dental Medicine
Karolinska Institutet, Sweden

Opponent

Associate Professor Rory M. Watt
Applied Oral Sciences &
Community Dental Care
Faculty of Dentistry
University of Hong Kong, China

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

ISBN 978-951-29-8009-3 (PRINT)
ISBN 978-951-29-8010-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy, Turku, Finland 2020

“The more I study science, the more I believe in God.”
— Albert Einstein

To Bakkuri, our little angel in heaven
To mom, my first and last teacher
To Ahmed, my soul mate and rightest choice ever
To Salem, Rahaf, and Sujud, the true meaning of the word ‘‘happiness’’

University of Turku
Faculty of Medicine
Institute of Dentistry
Department of Periodontology
Finnish Doctoral Program in Oral Sciences (FINDOS-Turku)
DAREEN FTEITA: Impact of estradiol and quorum sensing on biofilm-related virulence of the *Prevotella intermedia* group: – *in vitro* studies on bacterial growth properties, proteolytic enzyme activity and cytokine production.
Doctoral Dissertation, 124 pp.
April 2020

ABSTRACT

The *Prevotella intermedia* group organisms (*P. intermedia*, *Prevotella nigrescens*, *Prevotella pallens*, and *Prevotella aurantiaca*) are Gram-negative anaerobes predominantly existing in the human oral cavity. *P. intermedia* and *P. nigrescens* are characterized by their ability to consume maternal steroids; estrogen and progesterone. During pregnancy, microbial shifts in subgingival microbiota favoring *P. intermedia,* the tendency for gingival bleeding and elevated serum levels of maternal steroids occur. *P. intermedia* sensu lato (*P. intermedia* and *P. nigrescens*) is able to utilize estrogen and progesterone as a nutritional source, thus, it is here hypothesized that estradiol, the most potent estrogen during pregnancy, has an impact on the growth properties and pathogenicity of the *P. intermedia* group organisms. The aim of this study series was to examine the effect of estradiol on the growth behavior, biofilm formation and proteolytic enzyme activity of the *P. intermedia* group bacteria. Secondly, the impact of estradiol and QS on gingival keratinocyte response against the cell extracts of *P. intermedia* group bacteria, was determined based on the cytokine response. Thirdly, the role of quorum sensing (QS) molecules as enzyme and biofilm inhibitors was evaluated. The nine strains of *P. intermedia* group bacteria used in the study were: *P. intermedia* ATCC 25611^T and AHN 8290, *P. nigrescens* ATCC 33563^T and AHN 8293, *P. pallens* NCTC 13042^T and AHN 9283, and *P. aurantiaca* AHN 37505, AHN 37552, and CCUG 57723. In addition, *Fusobacterium nucleatum* ATCC 25586^T was used in the coaggregation assays. Colony forming unit, spectrophotometric, Bradford and phenol-sulfuric acid methods were used to examine the planktonic growth, coaggregation, biofilm formation, and protein and polysaccharide levels, respectively. Enzyme activities of the *P. intermedia* group bacteria were determined with fluorometric methods and zymography, while cytokine responses were analyzed by Luminex multiplex immunoassay. The study indicated that estradiol has a significant regulatory effect on the biofilm-related virulence of *P. intermedia* group organisms in a dose- and strain-dependent manner. QS molecules may be considered a promising therapeutic target in the treatment of oral chronic conditions related to bacterial biofilms, including pregnancy-associated gingivitis.

KEYWORDS: sex hormone, *Prevotella*, pregnancy, enzyme activity, biofilm, virulence

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Hammaslääketieteen laitos
Parodontologia
Kansallinen suun terveystieteiden tohtoriohjelma (FINDOS-Turku)
DAREEN FTEITA: Estradiolin ja quorum sensing –molekyylien vaikutus *Prevotella intermedia* -ryhmän bakteerien biofilmiin liittyvään virulenssiin: *in vitro* -tutkimuksia bakteerien kasvuominaisuuksista, proteolyyttisen entsyymiaktiivisuuksista ja epiteelin puolustusvasteesta.
Väitöskirja, 124 s.
Huhtikuu 2020

TIIVISTELMÄ

Prevotella intermedia -ryhmään kuuluvat organismit (*P. intermedia*, *Prevotella nigrescens*, *Prevotella pallens* ja *Prevotella aurantiaca*) ovat Gram-negatiivisia anaerobisia bakteereita, joita esiintyy ihmisillä pääasiassa suuontelossa. *P. intermedia* –ryhmän lajeille on ominaista niiden kyky hyödyntää kasvussaan naissukupuolihormoneja, estrogeenia ja progesteronia. Raskauden aikana subgingivaalisessa mikrobistossa tapahtuu muutoksia, jotka suosivat *P. intermedia* sensu lato (*P. intermedia* ja *P. nigrescens*) kasvua ja lisäävät taipumusta ienverenvuodolle. Tämän työn hypoteesinä oli, että estradioli vaikuttaa *P. intermedia* -ryhmän bakteerien kasvuun ja patogeenisuuteen. Tutkimuksen tavoitteena oli selvittää estradiolin vaikutusta *P. intermedia* -ryhmän bakteerien kasvuun, biofilmin muodostumiseen ja proteolyyttiseen entsyymiaktiivisuuteen. Lisäksi estradiolin vaikutusta puolustusvasteeseen *P. intermedia* -ryhmän bakteereja vastaan tutkittiin mittaamalla ikenen keratinosyyttisolulinjan sytokiinivastetta. Lisäksi arvioitiin quorum sensing (QS) -molekyylien merkitystä entsyymi- ja biofilmi-inhibiittorina. Tutkimuksessa käytettiin yhdeksää *P. intermedia* -ryhmän bakteerikantaa: *P. intermedia* ATCC 25611^T ja AHN 8290, *P. nigrescens* ATCC 33563^T ja AHN 8293, *P. pallens* NCTC 13042^T ja AHN 9283 sekä *P. aurantiaca* AHN 37505, AHN 37552 ja CCUG 57723. Lisäksi koaggregaatiomäärityksissä käytettiin *Fusobacterium nucleatum* ATCC 25586^T. Bakteerien kasvua, koaggregoitumista, biofilmin muodostumista sekä proteiini- ja polysakkaridimääriä mitattiin pesäkelaskennalla, spektrofotometrisillä sekä Bradford- ja fenoli-rikkihappomenetelmillä. *P. intermedia* -ryhmän bakteerien entsyymiaktiivisuuksia määritettiin fluorometrisillä menetelmillä ja zymografialla, kun taas sytokiinivasteet analysoitiin Luminex-multiplex-immunomäärityksellä. Tutkimus osoitti, että estradiolilla on merkittävä vaikutus *P. intermedia* –ryhmän lajien biofilmiin liittyvään virulenssiin annoksesta ja lajista ja kannasta riippuen. QS-molekyylejä voidaan pitää lupaavana terapeuttisena kohteena suun kroonisten sairauksien, kuten raskauteen liittyvän ientulehduksen, hoidossa.

AVAINSANAT: naishormoni, *Prevotella*, raskaus, entsyymiaktiivisuus, biofilmi, virulenssi

Table of Contents

Abbreviations

AI	autoinducer
AHL	acyl homoserine lactone
AHN	anaerobe Helsinki negative
ANOVA	analysis of variance
AP-PCR	arbitrarily primed-polymerase chain reaction
NUG	necrotizing ulcerative gingivitis
ATCC	American type culture collection
BHI	brain heart infusion
BSA	bovine serum albumin
CCL20	chemokine (C-C motif) ligand 20
CCUG	culture collection University of Gothenburg
CD26	cluster of differentiation 26
CFU	colony forming unit
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPD	dihydroxy-2,3-pentanedione
DPPIV	dipeptidyl peptidase IV
EG	Eggerth–Gagnon
ELISA	enzyme-linked immunosorbent assay
FAA	fastidious anaerobe agar
GCF	gingival crevicular fluid
HGF	human gingival fibroblast
HMK	human gingival keratinocyte
H-Ala-Pro-AFC	H-Ala-Pro-7-amido-4- trifluromethylcoumarin
hsp60	heat shock protein 60
Ig	immunoglobulin
IL	interleukin
InpA	interpain A
ISH	in situ hybridization

JCM	Japan collection of microorganisms
LPS	lipopolysaccharide
MALDI- TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MMP	matrix metalloproteinase
NCTC	national collection of type cultures
NF-$_K$B	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
QS	quorum sensing
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
pH	power of Hydrogen
PMNL	polymorphonuclear leukocyte
RAPD	random amplification of polymorphic DNA
REA	restriction endonuclease analysis
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS_PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
sp. nov.	species nova
spp.	species
subsp.	subspecies
TEM	transmission electron microscopy
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
T-RFLP	terminal restriction fragment length polymorphism
UV	ultraviolet
WCE	whole cell extract
VEGF	vascular endothelial growth factor
VPI	*Vibrio* pathogenicity island

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-IV):

I **Fteita D**, Könönen E, Söderling E, Gürsoy UK. Effect of estradiol on planktonic growth, coaggregation, and biofilm formation of the *Prevotella intermedia* group bacteria. Anaerobe 2014; 27:7-13.

II **Fteita D**, Könönen E, Gürsoy M, Söderling E, Gürsoy UK. Does estradiol have an impact on the dipeptidyl peptidase IV enzyme activity of the *Prevotella intermedia* group bacteria? Anaerobe 2015; 18:14-18.

III **Fteita D**, Musrati AA, Könönen E, Ma X, Gürsoy M, Peurla M, Söderling E, Sintim HO, Gürsoy UK. Dipeptidyl peptidase IV and quorum sensing signaling in biofilm-related virulence of *Prevotella aurantiaca*. Anaerobe 2017; 48:152-159.

IV **Fteita D**, Könönen E, Gürsoy M, Ma X, Sintim HO, Gürsoy UK. Quorum sensing molecules regulate epithelial cytokine response and biofilm-related virulence of three *Prevotella* species. Anaerobe 2018; 3:128-135.

Additionally, some unpublished data are presented.

1 Introduction

The *Prevotella intermedia* group organisms are pigmented Gram-negative anaerobes, which include four human species: *P. intermedia*, *Prevotella nigrescens*, *Prevotella pallens,* and *Prevotella aurantiaca,* while the fifth species, *Prevotella falsenii*, is isolated from monkeys. They are detected as members of the oral microbiome but also in mixed anaerobic infections in the human oral cavity, which is the primary and predominant site of isolation. *P. intermedia* is predominant in subgingival biofilms of diseased periodontal tissues (Socransky et al., 1998), *P. nigrescens* has been isolated from periodontic and endodontic infections, and from subgingival biofilms of the healthy periodontium as well (Shah and Gharbia, 1992; Gharbia et al., 1994). Whereas, *P. pallens* has been mainly associated with periodontal health (Könönen et al., 1998a; 1998b). The description of the newest member of the group, *P. aurantiaca*, was based on an isolate from a deep periodontal pocket of a periodontitis patient (Sakamoto et al., 2010).

Related to the periodontal health, pregnancy is a period of major physiological and hormonal changes, which are most marked in gingival tissues (Laine, 2002). Pregnancy-associated gingivitis has long been classified under the dental plaque-induced gingival diseases, which are modified by systemic conditions (Armitage, 1999). Due to lack of correlation between the amount of dental plaque at the gingival margin and the severity of inflammation, pregnancy-associated gingivitis has been recently reclassified under local conditions affected by systemic risks or modifying immune responses, which exacerbate gingival inflammation (Chapple et al., 2018). Among these systemic risk factors, elevations in sex steroid hormones lead to complex biological reactions within the gingival tissues, which exaggerate the inflammatory response to relatively insignificant amounts of dental plaque.

Female sex hormones and the main maternal steroids, estrogen and progesterone, are responsible for both sexual development and reproduction. Estradiol is one of the three active estrogen metabolites, and it is the most predominant and potent estrogen in healthy women of reproductive age (Mariotti, 1994; Mariotti and Mawhinney, 2013).

Some human hormones, e.g., stress hormones, regulate the growth behavior of anaerobic periodontal bacteria (Jentsch et al., 2013). From the same hormonal

family, maternal steroids can be utilized by *P. intermedia* as an essential nutritional source when cultured in estrogen- and progesterone-containing media (Kornman and Loesche, 1982). Clinical studies have demonstrated a correlation between elevated female sex hormones and increased numbers of *P. intermedia* and *P. nigrescens* in subgingival biofilms (Mariotti, 1994; Gürsoy et al., 2009; Carrillo-de-Albornoz et al., 2010).

In pregnancy-associated gingivitis, the increased tendency for gingival bleeding and inflammation during the second and third trimesters seems to be related to elevations in maternal steroids, which in turn lead to microbial shifts in subgingival plaque favoring the growth of *P. intermedia* sensu lato (*P. intermedia* and *P. nigrescens*) (Gürsoy et al., 2009; 2013).

The present thesis work provides novel data on: 1) the biofilm-related pathogenicity of the *P. intermedia* group bacteria under the effect of estradiol, 2) the regulatory role of quorum sensing (QS) molecules as a potential anti-biofilm agent and an enzyme inhibitor, and 3) the growth properties of the *P. intermedia* group organisms and the enzymatic activities by which the strains exhibit their virulence aspects.

2 Review of the Literature

2.1 The indigenous oral microbiota in health and disease

2.1.1 The oral microbiota

The human oral microbiota comprise a complex and diverse microbial community of at least 700 predominant taxa of which less than one-third as yet being uncultured *in vitro* (Huttenhower et al., 2012; Baker et al., 2017). The term "microbiome" describes the ecological community and collective genomes of the commensal, symbiotic, and pathogenic diverse microbial taxa occupying the human body and significantly determine its health and disease condition. The major representative bacterial phyla of the oral microbiome are *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Chlamydiae*, *Chloroflexi*, *Euryarchaeota*, *Spirochaetes*, *Synergistetes*, *Fusobacteria* and *Tenericutes* (Dewhirst et al., 2010; Wade, 2013). In addition, approximately, the same or more of other species remain as yet uncultured phyla and divisions (Siqueira and Rôças, 2013).

The diverse ecological niches tend to colonize the oral cavity in a surface-preference manner, and these surfaces are: teeth, gingival crevice, tongue, cheek mucosa, and hard and soft palate. Connected with the oral cavity, tonsils, pharynx, trachea, distal esophagus, inner ear, nasal vestibules, and sinuses are considered contiguous extensions of the oral microbial surfaces. The indigenous microbiota colonized on these anatomical sites include bacteria, viruses, protozoa, fungi, and archaea (Marcotte and Lavoie, 1998). Despite their commensalism, the diverse oral microbial communities, including the resident (more prevalent) and the transient microbiota (less prevalent), are often affected by host genetics and environmental changes. Thus, they are responsible for two important oral diseases: dental caries and periodontal disease (Dewhirst et al., 2010; Takahashi and Nyvad, 2011; Huttenhower et al., 2012; Baker et al., 2017).

Bacterial symbiosis is a spatiotemporal and interactive relationship where different bacterial species cohabit in a mutualism, commensalism, or parasitism (Chow et al., 2010). Oral commensal microbiota play a key role in maintaining intrinsic symbiosis and resisting the colonization of pathogens by self-colonization

to oral cavity habitats limiting the available binding sites on different oral surfaces and simulating the host immune system at the same time (Vollaard and Clasener, 1994; Chow et al., 2010). However, if the stability of the habitat is disturbed, indigenous bacteria may cause local infections that harm the host (Marsh et al., 2003; Abusleme et al., 2013; Olsen et al., 2018).

It was long believed that the incidence of oral anaerobic bacteria which colonize the oral cavity, is chronologically associated with tooth eruption, where the availability of optimum oxygen-depleted environment obtained from the gingival crevice (Socransky and Manganiello, 1971; Bimstein, 1991). Nevertheless, several anaerobic species seem to colonize the oral cavity and gradually establish their community there at an earlier onset. For instance, *Veillonella* spp. followed by *Prevotella melaninogenica* were the most frequently isolated anaerobic species from saliva samples of two-month-old infants, up to the first year of age when the *Fusobacterium nucleatum* and *Capnocytophaga* spp. start to proliferate with a remarkably increased frequency (Könönen et al., 1992; 1999). *P. intermedia* group organisms were late colonizers in the dental plaque formation process.

2.1.2 Dental plaque as an oral biofilm

Biofilm is a complex and diverse microbial community, in which bacterial cells adhere to each other and attach to a surface within a self-produced extracellular polymeric matrix consisting of exopolysaccharides, globular glycoproteins, polypeptides, extracellular enzymes and nucleic acids (Bowen et al., 2018). In the oral cavity, the internal environment is humid and nutrition-rich, which provides optimal conditions for many intrinsic microorganisms to colonize and build biofilms.

Dental biofilms benefit the teeth primarily to prevent colonization by exogenous species. However, some dominant species may play a critical role in the aetiology of dental caries and periodontal disease, depending on host immunity and microbial diversity (Rosan and Lamont, 2000; Dewhirst et al., 2010; Takahashi and Nyvad, 2011).

Dental plaque formation is a dynamic process starting with the attachment of a protein film called “acquired pellicle” on tooth surfaces a few minutes after performing oral hygiene measures. Acquired pellicles are originated from host-derived molecules including high molecular weight salivary proteins, e.g. mucins, agglutinin, and amylases, which are recognized by the early colonizers that attach to these proteins in a pattern predominated by Gram-positive species (Nyvad and Kilian 1987; Kolenbrander et al., 2002). For instance, during the spatiotemporal stage of dental plaque formation, the *Streptococcus* spp. proportionally increases with time at the expense of the *Actinomyces* species (Socransky et al., 1977, Kolenbrander et al., 1990; 2010). As an active player during dental plaque maturation, the oral

commensal *Fusobacterium nucleatum* acts as a "bridge" species to facilitate the coaggregation and binding between early and late colonizers, and with the synergy of the early colonizers. It provides adhesion receptors for the attachment of Gram-negative species favoring *Porphyromonas* spp. (mainly *P. gingivalis*), *Treponema* spp., and *Prevotella* spp. (Kolenbrander et al., 2002), the species with a key role in the initiation and progression of periodontal disease (Socransky et al., 1998).

Plaque early colonizers are Gram-positive aerobes and facultative anaerobes, while late colonizers are mainly Gram-negative obligate anaerobes (Nyvad and Kilian 1987; Kolenbrander et al., 2002). In a study conducted by Ammann et al. to investigate the impact of early colonizing species on the structure of a 10-subgingival species biofilm model, the elimination of streptococci from the model system has enhanced *P. intermedia* to become dominant and form filamented chains that resemble the absent species (Ammann et al., 2013).

2.1.3 Pigmented Gram-negative anaerobes with potential pathogenicity

Due to the heterogeneity and diversity of the group, the taxonomy of pigmented Gram-negative anaerobic bacteria has undergone several changes in the nomenclature since *Bacterium melaninogenicum* (later *Bacteroides melaninogenicus*) was described (Oliver and Wherry, 1921), and later, oral *Bacteroides* species were separated into pigmented *B. melaninogenicus* and non-pigmented *Bacteroides oralis* (Holbrook and Duerden, 1974; Holdeman and Johnson, 1982). The pigmentation of colonies is strongly affected by the contents of the inoculation medium, thus, the design of the subspecies divisions from *B. melaninogenicus* considered the difference in glucose fermentation as a criterion for identification (Holbrook and Duerden, 1974).

The lack of particular dehydrogenase enzymes made the three subspecies of *B. melaninogenicus* to be reclassified as saccharolytic subsp. *melaninogenicus* and moderately saccharolytic subsp. *intermedius,* and asaccharolytic subsp., *asaccharolyticus* (Holdeman and Moore, 1970) as presented in Figure 1.

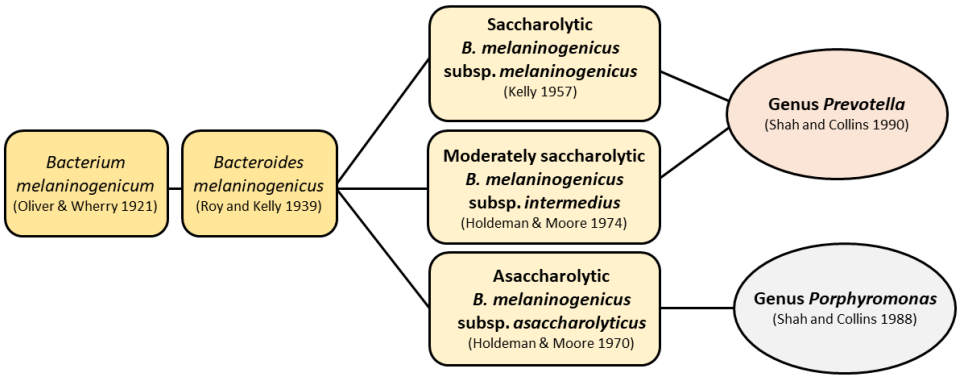


Figure 1. Early taxonomical changes of pigmented Gram-negative anaerobes.

Clinical identification of novel *Prevotella* species relies on varieties of simple and reproducible methods for differentiation between distinctive taxa, e.g., the polyphasic approaches using multilocus enzyme and SDS-PAGE electrophoreses (Frandsen et al., 1995; van Steenbergen et al., 1997), the restriction endonuclease analysis (REA) and ribotyping (Teanpaisan et al., 1996; Pearce et al., 1996; Mättö et al., 1996a), the terminal restriction fragment length polymorphism (T-RFLP) (Milsom et al., 1996), the commercial phenotypic systems (Rodríguez-Cavallini et al., 2011), and the 16S rRNA sequencing or bacterial fingerprint detection by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) in which the identification relies on the protein mass spectra analysis of the isolated bacterial profiles (Nagy et al., 2012; Gürsoy et al., 2017; Rams et al., 2018).

Within these two pigmented Gram-negative anaerobic genera, *Prevotella* and *Porphyromonas*, some new species have been described and their potential pathogenicity has been widely discussed. For instance, *Porphyromonas gingivalis* is the most intensively studied periodontopathogen through almost the last three decades to be continuously documented and confirmed as a keystone pathogen not only in adult periodontitis but also in periodontitis-related systemic disorders (Tanner et al, 1979; Slots et al, 1986; Hajishengallis et al., 2012; Gölz et al., 2016).

The genus *Prevotella* includes a large number of species, which represent a major portion of the oral microbiota and some are among the first colonizers of the oral cavity in infants (Könönen et al., 1992; 1999). *Prevotella* species are prevalent commensals of the oral cavity, but also predominant in the respiratory mucosal surfaces and one of major bacterial enterotypes of the gut microbiome ecosystem (Hilty et al., 2010; Arumugam et al., 2011; Charlson et al., 2011). The initial association between *Prevotella* species and chronic inflammatory disease of the periodontium was described by Burdon in 1928 when he reported the presence of black colonies of Gram-negative anaerobes associated with periodontal disease and

tooth loss (Dahlén, 1993a). Significantly, oral *Prevotella* spp. are able to modify oral symbiosis under certain conditions (i.e. increased bacterial load, suppression of beneficial bacteria, and/or reduction in host immune response) (Kolenbrander 2000; Quince et al., 2009; Zaura et al., 2009). To a lesser extent, they also contribute to non-oral infections such as recurrent tonsillitis, infected human bites, and bacterial vaginosis (Mättö et al., 1997; Talan et al., 2003; Jensen et al., 2013; Datcu et al., 2014).

2.2 *P. intermedia* group organisms

2.2.1 General features

P. intermedia group organisms (namely *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella pallens*, and *Prevotella aurantiaca*) are Gram-negative strict anaerobes with shared biochemical and phylogenetical aspects, saccharolytic fermentation properties, and pigmentation characteristics (Van Winkelhoff et al., 1988a, 1988b; Könönen et al., 1998a, 1998b; Sakamoto and Ohkuma, 2010; Sakamoto et al., 2010) . All species belonging to the group are non-sporing, non-motile, rods or coccobacilli, and favor growth on supplemented Brucella sheep blood agar plates where they form smooth, raised, circular, shiny and convex colonies with interspecies variation in pigmentation intensity. Despite their existence in extraoral infections (Mättö et al., 1997; Bik et al., 2006), the human oral cavity remains the preliminary and predominant site of isolation for the group (Van Winkelhoff et al., 1988a, 1988b; Könönen et al., 1998a; 1998b; Sakamoto and Ohkuma, 2010; Sakamoto et al., 2010).

2.2.2 Taxonomy and phenotypic characteristics

Due to the significant influence of the environment on bacterial phenotypes, phylogenetic analysis became an essential tool to identify bacterial species at a molecular level. In order to construct a phylogenetic tree, genetic distance between base pairs of a certain organism is calculated and a matrix of similarities is created accordingly to be further analysed by molecular characterization methods (Olsen et al., 1994).

Phylogenetic analyses of the *P. intermedia* group have shown that the variations in the 16S rRNA gene sequencing were relatively low compared with the clear species clustering, which was more than 7% in case of *P. intermedia* and *P. nigrescens* (Kuhnert et al., 2002) (Figure 2).

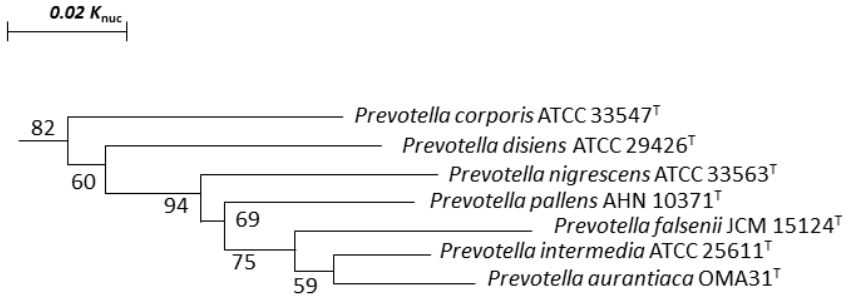


Figure 2. Phylogenetic tree demonstrating the relationship between different species of the *P. intermedia* group and closely related species (modified from Sakamoto et al., 2010).

P. intermedia sensu lato

In the 1990s, *Bacteroides melaninogenicus* subspecies *intermedius* was reclassified into a new genus, *Prevotella* as *P. intermedia* (sensu lato). Based on DNA-DNA hybridization and multilocus enzyme electrophoresis, Shah and Gharbia (1992) proposed the division of the phenotypically identical *P. intermedia* and *P. nigrescens* into two genetically distinct species (Shah and Gharbia, 1992). The two homology groups of *P. intermedia* sensu lato (Johnson and Holdeman 1983) included *P. intermedia* sensu stricto, in correspondence to the homology group VPI 4197, and *P. nigrescens*, in correspondence to the homology group VPI 8944. The difference in their pathogenic potential was recognized e.g., *P. intermedia* sensu stricto was considered to be associated with periodontal infections, while *P. nigrescens* has been detected in both healthy and diseased periodontal sites (Shah and Gharbia, 1992; Gharbia et al., 1994; Mättö et al., 1996a). Although culture media dependent, their dark-pigmented colony morphology they tend to build on Brucella agar, is a typical phenotypic character in preliminary identification of the species (Tanner, 1992).

P. intermedia and *P. nigrescens* are of high haemolytic and hemagglutinating activities, which are crucial for their growth, and for supporting the virulence of other pigmented anaerobes, such as *P. gingivalis*, in subgingival plaque (Okamoto et al., 1999). The red auto-fluorescence they exhibit under a long-wave length (365 nm) of UV light (Slots 1986; Shah and Gharbia 1992), and the lactose fermentation negative reaction of the fluorescence test were well established phenotypic methods for their identification (Alcoforado et al., 1987; Gürsoy et al., 2017). For instance, the detection of *P. intermedia* in mature dental plaque was based on its red fluorescence (Nomura et al., 2017). Table (1) lists some phenotypic features of the *P. intermedia* group species (Conrads et al., 2019).

P. pallens

In 1998, a new indole-positive species, consistently distinguished from *P. intermedia* and *P. nigrescens*, was proposed and named as *P. pallens* (Könönen et al., 1998b). The name was taken from the descriptive term "pale", referring to the faint color of the weakly pigmented (cream) colonies inoculated on blood agar. In addition to its weak pigmentation, *P. pallens* differed from *P. intermedia* and *P. nigrescens* based on its negative lipase reaction.

Table 1. Phenotypic characteristics that differentiate *P. intermedia* group species (adapted from Könönen et al., 1998b; Jousimies-Somer and Summanen, 2002; Sakamoto and Ohkuma, 2010; Sakamoto et al., 2010).

Prevotella spp.	Production of:					Fermentation of:				Hydrolysis of:	
	Pig	Ind	Lip	α-Fuc	β-NAG	Glu	Lact	Suc	Arab	Gel	Esc
Prevotella intermedia	++	+	+	+	-	+	±	+	-	+	-
Prevotella nigrescens	++	+	+	+	-	+	±	+	-	+	-
Prevotella pallens	+	+	-	+	-	+	±	+	-	-	-
Prevotella aurantiaca	+	+	-	+	-	+	-	+	-	+	-

Pig: pigmentation, Ind: indole, Lip: lipase, α-Fuc: fucosidase, β-NAG: N-acetyl-β-glucosidase, Glu: glucose, Lact: lactose, Suc: sucrose, Arab: arabinose, Gel: gelatin, Esc: esculin, spp.: species, ++: black pigment, ±: occasional lactose fermentation.

P. aurantiaca

The newest *P. intermedia*-like organism was isolated from a periodontal pocket of a patient with periodontitis, and these *Prevotella*-like strains were phenotypically closely related to *P. intermedia* JCM 12248^T and *P. nigrescens* JCM 12250^T (Sakamoto et al., 2009). The partial hsp60 and 16S rRNA gene sequence analyses revealed the taxonomic status of the new isolates being closely related to *P. intermedia* ATCC 25611^T with 96.4 % gene sequence similarity, *P. pallens* AHN 10371^T (96.1 %), and *Prevotella falsenii* JCM 15124^T (95.3 %) (Sakamoto et al., 2010). Based on the analyses, the strain OMA31^T was suggested as the type strain of *P. aurantiaca*, a novel species of the *P. intermedia* group (Sakamoto et al., 2010).

P. falsenii

P. falsenii is a non-human species of the *P. intermedia* group, isolated from the oral cavity of a monkey (Sakamoto et al, 2009). Based on mannose fermentation and α-fucosidase activity, eight anaerobic strains were characterized phenotypically to

represent a novel species, *P. falsenii* sp. nov. These strains were most closely related to *P. intermedia* ATCC 25611^T with 95.0 %, *P. pallens* 92.7%, and *P. nigrescens* 92.1 % gene sequence similarity.

2.2.3 Cultivation

Among several scenarios of why some *Prevotella* strains resist conventional *in vitro* cultivation, the primary reason might be related to these strains dependency on the synergism of the naturally occurring co-cultures and/or on the shared molecular signaling "cross talk" within mixed communities (Vartoukian et al., 2010). However, except for a few difficult-to-culture strains, most of the *Prevotella* species are possible to culture *in vitro* in laboratories (Vartoukian et al., 2016).

Different compositions for optimum growth media for *Prevotella* have been suggested, including some essential components shared between almost all culture media used for Gram-negative anaerobes. For example, non-selective enriched Brucella blood agar plates are commonly used for primary isolation supplemented with hemin, bacto-agar, defibrinated blood (sheep or horse), and vitamin K_1 or menadione (from which *P. intermedia* and *P. nigrescens* colonies gain cell-surface accumulation of haem). Selective kanamycin vancomycin laked blood agar, rabbit laked blood agar, Fastidious Anaerobe Agar (FAA), and egg yolk agar are commonly used for cultivating pigmented *Prevotella* species (Jousimies-Somer et al., 2002; Gürsoy et al., 2009). Due to their strictly anaerobic nature, anaerobic incubation is obligatory at 37ºC for 3-7 days (strain-dependent) within automatic jars of evacuation-replacement system or anaerobic workstations or cabinets containing 7-10% H_2, 5-8% CO_2, and 85% N_2 (Brazier and Smith 1989; Haraldsson et al., 2005). However, for *P. aurantiaca*, the cultivation of the strains on Eggerth–Gagnon (EG) agar supplemented with horse blood for 2 days only and at 37ºC in an atmosphere containing 100% pure CO_2 have provided satisfying growth results (Sakamoto et al., 2010).

Regarding their metabolism, oral *Prevotella* anaerobes are well-known for their haem requirement for growth, persistence, and pathogenicity (Smalley et al., 2003). In order to obtain the required haem (iron protoporphyrin IX) from haemoglobin, it expresses a proteolytic enzyme of cysteine protease activity called interpain A (InpA), which has the capability to metabolize and degrade haemoglobin (Byrne et al., 2009). Moreover, in inflamed gingival crevices and periodontal pockets, a significant contribution to haem acquisition by *P. intermedia* (from haemoglobin, haemalbumin and haem-haemopexin) has been suggested under limited conditions of oxygen and higher pH of gingival crevicular fluid (GCF) (Hanioka et al., 2005; Byrne et al., 2015).

2.2.4 Molecular detection methods of the *P. intermedia* group

In order to obtain sufficiently conserved DNA sequence from an organism, molecules such as ribosomal RNA (rRNA) genes, RNA polymerase, and elongation factor G were successfully used to deliver phylogenetic characteristics and complete gene sequence analyses (Tanner et al., 1994). While conventional methods failed to recognize nearly 50% of uncultivable phylotypes of the oral microbiota, designed PCR primers have made it possible to characterize them (Wade 1999; 2002; Harper-Owen et al., 1999). For the genus *Prevotella*, hsp60 gene sequences are considered precise classifiers of almost all *Prevotella* species (Sakamoto et al., 2010; Sakamoto and Ohkuma, 2010; 2012). However, their failure to distinguish between *P. multiformis* and *P. denticola* isolates at intra- and interspecies levels has given a superior advantage to gyrB1 and rpoB housekeeping genes when the aim is to perform comparative studies (Chen et al., 2015).

In 1995, Slots and colleagues demonstrated the sensitivity and reproducibility of the PCR technique by detecting many putative periodontal pathogens in subgingival plaque samples (Slots et al., 1995). However, the selected primers for *P. intermedia* had cross-reactions with some closely related species and thus failed to reveal accurate amplification with distant but related species. The same research group has successfully detected *P. intermedia* and *P. nigrescens* in subgingival plaque of gingivitis and advanced periodontitis patients by using the restriction enzyme digestion method to confirm the specificity of the amplifications. The results of the study showed no cross-reactivity with related species (Ashimoto et al., 1996). Later, Mättö and co-authors confirmed the simplicity and applicability of the arbitrarily primed PCR (AP-PCR) in the differentiation and clonal analysis of *P. intermedia* and *P. nigrescens* (Mättö et al., 1996a). Further improvement in the identification strategies of oral *Prevotella* species appeared when the first-generation PCR-DNA probe assays specific for *P. intermedia* and *P. nigrescens* were constructed based on combining the nucleic acid probes with PCR and amplifying the random amplification of polymorphic DNA (RAPD) fingerprinting, which has the ability to generate species-specific markers and, thus, enhance the sensitivity (Guillot and Mouton, 1997).

Simultaneous detection of *P. intermedia* and *P. nigrescens* species in oral samples by reverse primer combination of multiplex PCR was carried out through direct targeting of specific 16S rRNA gene similarities and subsequent microarray hybridization (García et al., 1998; Conrads et al., 1999; Stubbs et al., 1999; Yoshida et al., 2005; Könönen et al., 2007; Cao et al., 2012). Additional methods, such as the closed-tube PCR and a quantitative real-time PCR for enumerating target bacteria in largescale analyses, have provided a sensitive and reliable approach to quantitatively enumerate specific periodontopathogens in clinical samples including *P. intermedia*

(Doung-udomdacha et al., 2000; Nonnenmacher et al., 2004; Boutaga et al., 2005; Hyvärinen et al., 2009). Species-specific PCR primers, such as Pig27 DNA probe in case of *P. intermedia* and Pn23 DNA probe in case of *P. nigrescens*, have been recommended as useful identifiers for the detection of *Prevotella* and related species (Nagashima et al., 2005; Kim et al, 2011a; 2011b). Although literature reports on *P. pallens* and *P. aurantiaca* are not yet as enormous as the available data on *P. intermedia* and *P. nigrescens*, some reports have described methods to detect these two species by established biochemical methods and molecular approaches such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), RAPD-PCR and AP-PCR analyses, DNA hybridization, and FISH assays (Könönen et al., 1999; 2000; Mättö et al., 1999; Robertson et al., 2000; Riggio et al., 2008; Sakamoto et al., 2010; Gürsoy et al., 2017).

Along with the traditional PCR techniques, deeper insights into the phylogenetic aspects of bacterial communities have led to the development of next generation sequencing methods, such as pyrosequencing and Illumina high-throughput sequencing, which show a high specificity in identifying a wide range of oral microbial communities including many *Prevotella* species, e.g., *P. intermedia* and *P. nigrescens* (Keijser et al., 2008; Lazarevic et al., 2009; 2010; Huang et al., 2014; Shin et al., 2018).

2.2.5 Activation of immune responses by the *P. intermedia* group

Many oral and non-oral isolates of *P. intermedia* have the ability to inhibit lymphocyte functions (B- and T-cell proliferation) by altering endogenous host enzymes such as mitogen-induced DNA, RNA, and protein kinases and syntheses (Shenker and Slots 1989; Shenker et al., 1991). Moreover, lipopolysaccharides (LPS) from *P. intermedia* is capable of stimulating the expression of interleukin (IL)-8 in human gingival fibroblasts and invading the human oral epithelial cells derived from the HeLa cell line, which is known as KB cells (Keratin-forming tumor cell line HeLa) (Nagaoka et al., 1996; Dorn et al, 1998). When human dental stem cells were treated with LPS from *P. intermedia*, a decreased chemotaxis, phagocytosis, and impairment of anti-inflammatory activity have been observed (Hieke et al., 2016). When incubated under oxygen-tension conditions, the oral epithelial cells revealed an ascending pro-inflammatory responses to some periodontal bacteria, including *P. intermedia*, which was detected by elevated levels of epithelial inflammation markers e.g. nuclear factor kappa-activated B cells (NF-$_{K}$B), IL-8 and tumor necrosis factor-α (TNF-α) (Graves, 2008; Grant et al., 2010).

In the same regard, *P. nigrescens* has been shown to promote the T helper 17 (Th17) expression through the production of IL-1β by dendritic cells (DC), which

have been activated by the Toll-like receptor 2 (TLR2) (Figure 3). Neutrophil recruitment dysfunction is an obvious characteristic of chronic periodontal diseases. Some studies suggest that *Prevotella* organisms are capable of inducing periodontal inflammation by guiding neutrophil recruitment through the Th17 immune pathway. Such a mechanism is thought to mediate alveolar bone loss and tissue destruction due to the inability of the recruited neutrophils to maintain bacterial clearance and resolution (Matsui et al., 2014; Uriarte et al., 2016; Schincaglia et al., 2017).

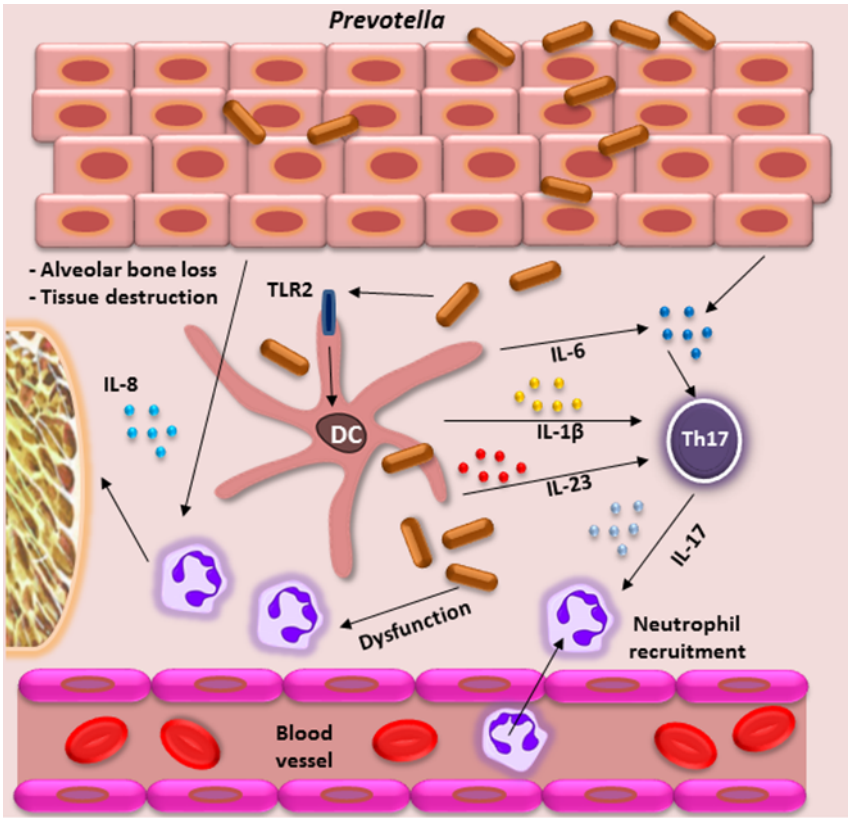


Figure 3. Illustration of 1) the dysbiotic *Prevotella*-mediated host response where interleukin (IL)-1β, IL-6 and IL-23 released by dendritic cells (DC) through the activation of Toll-like receptor 2 (TLR2), 2) the stimulation of epithelial cells by *Prevotella* burden results in the production of IL-8, IL-6 and Chemokine (C-C motif) ligand 20 (CCL20), which induces dysfunction in recruited neutrophils. Modified from Larsen, 2017.

2.3 Biofilm-related virulence of *P. intermedia*

2.3.1 Planktonic growth versus biofilm formation

Planktonic bacteria have markedly different growth behavior to their biofilm-grown counterparts. Bacteria at planktonic phase are mainly responsible for acute infections and are easier to be eradicated by antimicrobials than the bacteria which succeeded to attach to a surface (Sun and Song 2011).

Since the survival rate of planktonic bacteria is short, they rather adhere to a surface, coaggregate, and form biofilms (Jakubovics and Kolenbrander, 2010). Proceeding from the planktonic phase of growth, cell attachment, cell assembly into micro-colonies, and cell differentiation into a mature biofilm are the main spatiotemporal stages to build a 3-D architecture of a biofilm (Kolenbrander, 1995; Kolenbrander et al., 2002; Jakubovics and Kolenbrander., 2010; Bowen et al., 2018). Bacterial biofilms are able to shield the colonizing bacterial cells against the recognition of the immune cells by impairing phagocytosis and complement system (Domenech et al., 2013). Studies using a two-stage chemostat vessel system (aerobic and anaerobic) revealed that cultivable Gram-negative anaerobes, including some *Prevotella* species, synergistically succeed to tolerate and survive oxygen stress at both planktonic and biofilm phases of growth (Marsh et al., 1983; Bradshaw et al., 1996; 1997). However, *Prevotella* species have shown a stronger resistance to antimicrobial agents at the biofilm phase due to antibiotic restoring property of their outer membranes when grown in biofilms (Takahashi et al., 2006; Yu et al., 2007).

To form a stable oral biofilm resisting disruption by external forces like salivary flow or antimicrobials, oral anaerobes use certain surface molecules and cellular components that establish a steady environment for their growth and proliferation. Among these components used by *Prevotella* are LPS, fimbriae, proteases, and outer membrane vesicles or proteins (Botta et al., 1994; Byrne, 2009). During the maturation stage of oral biofilm in a periodontal pocket, *P. intermedia* adjusts the environmental acidity to facilitate the duty of *F. nucleatum* to modify the pH of GCF from 5.0 to 7.0 and to provide ammonia for *P. gingivalis*, which favor a neutral growth medium (Takahashi, 2003).

2.3.2 Coaggregation

Oral microbes tend to colonize, adhere to a surface, and auto- or coaggregate with each other in order to build biofilms capable of resisting host defense mechanisms and anti-microbial agents. *F. nucleatum* is a Gram-negative anaerobe with the capability of withstanding oxidative stress, it also proliferates in intra- and extra-oral diseased sites with superior adhesive properties, colonizes gingival epithelial cells,

and stimulates periodontal tissue destruction (Bakken et al., 1990; Socransky et al., 1988; Xie et al., 1991; da Silva et al., 2015; Gürsoy et al., 2008; Castellarin et al., 2012). Physical interactions, by which *F. nucleatum* connects the early and late colonizers in dental plaque, allow it to serve as a key bacterium of bridging properties and coaggregation capabilities (Socransky et al., 1998; Xie et al., 1991).

The spatiotemporal process of dental plaque formation starts with early colonizers adhering the acquired pellicles on tooth and epithelial surfaces, while late colonizers, e.g., *F. nucleatum*, *P. gingivalis*, *T. forsythia*, and *P. intermedia*, have a higher tendency to adhere to predecessor microbes (Kolenbrander and London 1993; Kolenbrander, 2000). The more pathogenic Gram-negative anaerobes are involved in dysbiotic dental biofilms, the higher the incidence of periodontal disease initiation and progression, and the more chronic oral infections exist (Costerton et al., 1999). The synergistic potency of coaggregation between the genetically distinct colonizers in multispecies biofilms is a prerequisite for chronic oral infections (Kolenbrander and London, 1993).

2.3.3 Dipeptidyl peptidase IV (DPPIV) enzyme activity

The subgingival environment demonstrates bacterial communities with great capability to induce inflammatory responses and oral purulent infections, e.g., periodontal tissue destruction by *P. intermedia* and *P. nigrescens* proteolytic enzymes such as cysteine and serine proteinases and elastolytic serine proteases (Shibata et al., 1993; Yanagisawa et al., 2006). Dipeptidyl peptidase IV (DPPIV) is a serine protease, which hydrolyses the penultimate X-Pro or X-ala dipeptides from the N-terminal amino acid chains of chemokines, neuropeptides, and other synthetic oligopeptides (Augustyns et al., 1999). *In vivo*, host CD26 is transmembrane glycoprotein with known DPPIV activity expressed on the surface of most cell types including human gingival fibroblasts (Abbott et al., 1994; Nemoto et al., 1999). The enzymatically active form of CD26/DPPIV is widely involved in autoimmune diseases, human malignancies including oral squamous cell carcinoma, as well as, its key physiological function as a maintainer of chemokine regulation and glucose homeostasis (Uematsu et al., 2004; Metzemaekers et al., 2016; Deacon, 2019).

As a protease of bacterial origin, the catalytic action of DPPIV by periodontal bacteria has a destructive effect on periodontal tissues through the degradation of collagenous matrix (Kumagai et al., 2005; Yost and Duran-pinedo, 2018). Thus, it has been frequently detected in saliva of patients with periodontitis, and often considered as a diagnostic marker for periodontal disease (Banbula et al., 1999; Elgün et al., 2000; Aemaimanan et al., 2009). *Prevotella* species, including *P. intermedia* and *P. nigrescens*, have shown to express DPP-like activities in chronic oral infections (Yanagisawa et al., 2006). Among periodontopathogens, well-known

DPPIV producers like *Capnocytophaga spp.*, *P. gingivalis*, *Prevotella spp.*, and *Treponema denticola* contribute significantly to the activation of host defense pathways through the inhibition of the human complement, like degrading the complement factor C3 (Gazi et al., 1997; Banbula et al., 2000; Potempa et al., 2000; Yost and Duran-pinedo, 2018).

2.3.4 Quorum sensing (QS)

QS in bacteria is a cell-to-cell communication through diffusible signal molecules, which facilitate the cross-talk between bacterial phenotypes favoring several physiological and pathological activities, e.g., adhesion, dysbiosis, expression of virulence, gene transformation, motility, as well as, biofilm formation (Miller and Bassler, 2001). Besides, the QS phenomenon regulates the expression of endotoxins (capsular polysaccharides synthesis), and bacterial proteolytic and hemolytic activities such as elastase, protease, and hemolysin production (George et al., 2005; Lee et al., 2013; Guo et al., 2018).

At a genetic level, QS in bacteria is defined as the transcriptional regulation of gene expression in response to the cell-population variabilities occurring in bacterial-cell density up to a least stimulatory threshold or "quorum" (Miller and Bassler, 2001). Such fluctuations are exchanged among genetically identical species when typified by autoinducer-1 (AI-1), and among genetically distinct cells when mediated by autoinducer-2 (AI-2) (Bassler et al., 1997; Miller and Bassler, 2001). In Gram-negative bacteria, the amphiphilic N-acyl homoserine lactones AHLs, which represent the QS molecules, are encoded by the gene *luxI* homolog (operon), which transcripts the AHL synthetase enzyme, while the gene *luxR* homolog (regulon) encodes the signal-receptor protein or regulator (Pearson et al., 1999; Miller and Bassler, 2001). AI-1 is *N*-(3-hydroxybutanoyl)-HSL identified in the marine bacterium *Vibrio fischeri* and can efficiently couple gene expression using a signaling cascade of *LuxI*/*LuxR* circuits for the biosynthesis and regulation of HSL molecules. Unlike AI-1, the AI-2 is a universal QS molecule derived from S-4, 5-dihydroxy-2,3-pentanedione (DPD), was first described in the marine bacterium *Vibrio harveyi*, and it does not belong to the HSLs but to the furanosyl borate diester or tetrahydroxy furan family (based on the producing species) (Miller and Bassler, 2001). In case of AI-2, the *LuxS*/*LuxR* circuts are activated in a manner where the *LuxS* gene converts key enzymes responsible for the biosynthesis of DPD, while the *LuxR* biosensor is a cell-density dependent transcription regulator for the activation/repression of target genes (Zhu and Winans, 2001; Waters and Bassler, 2005).

The basic mechanism of action in QS starts with the extracellular production of AIs by bacteria, and these molecules remain undetectable until they accumulate to

reach a certain threshold of a relatively high local concentration, enabling the initiation of response by the provoke of the DNA binding domain of the responsible gene. Then, AI receptors conjugate the molecules with their compatible genes to tandemly coordinate gene transcription and repression (Novick et al., 1995) (Figure 4).

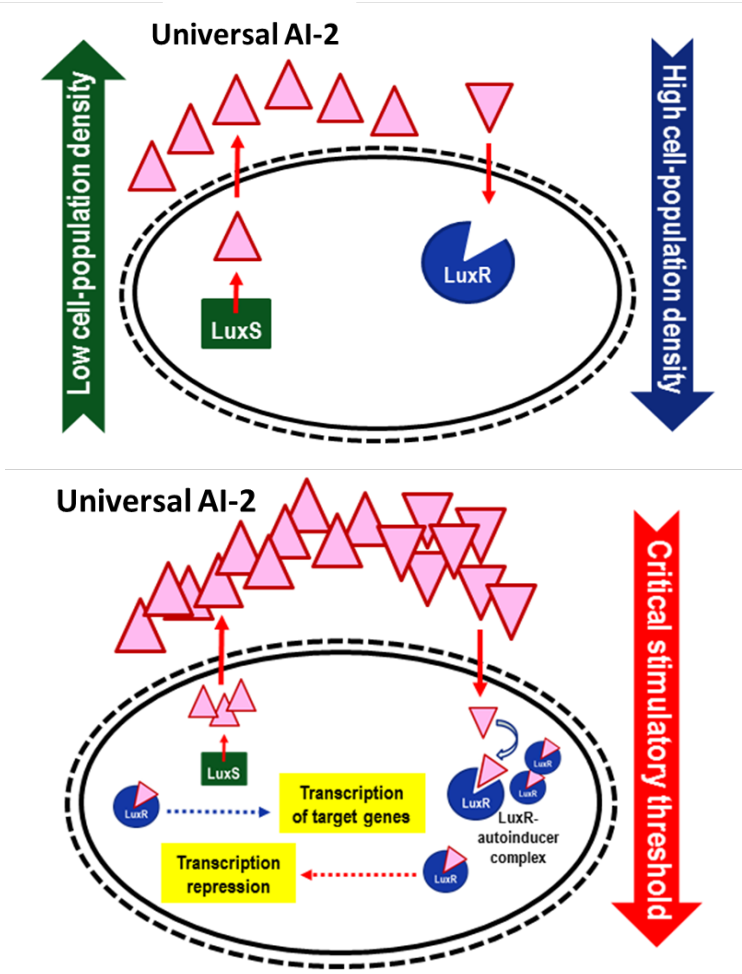


Figure 4. Mechanism of quorum sensing signalling-sequence from autoinducer molecule production to regulation of target gene transcription. *LuxS*: autoinducer synthase, *LuxR*: autoinducer receptor and DNA-binding transcriptional activator. Modified from Miller and Bassler, 2001.

Some periodontal bacteria, i.e. *P. intermedia*, *F. nucleatum*, and *P. gingivalis* significantly produce AI-2 under the experimental atmosphere tested (Frias et al., 2001). AI-2 of *F. nucleatum* has been found to enhance the expression of adhesion

molecules and to play a role in the inhibition of pathogenic dental biofilm formation (Jang et al., 2013). On the other hand, some prominent periodontopathogens, such as *P. gingivalis* and *T. denticola*, have demonstrated an interference with QS-dependent virulence properties of *S. mutans* by exhibiting an antagonizing action, weaken their antimicrobial resistance and virulence of the cariogenic species (Wang et al., 2011).

2.3.5 Antimicrobial resistance (AMR)

Resistance to antimicrobial agents is a common behavior of mature biofilms, where the microbe gains the ability to resist the medication effect that earlier happened to successfully treat a disease or condition caused by that microbe.

AMR is usually a result of one of three common mechanisms: natural resistance, genetic mutation, or acquired resistance from other species. Gram-negative bacteria are able to overcome the antimicrobial effect of the β-lactam antibiotics by a variety of mechanisms: production of β-lactamase enzymes that hydrolyze the β-lactam rings, alterations in the active site of penicillin-binding proteins (transpeptidase and D-alanyl carboxypeptidase), disruption of outer membrane proteins to lower the permeability of β-lactams passing through porin-channels, and upregulated efflux pumps, which export the diffused drug out of the bacterial cell wall (Drawz and Bonomo, 2010). Several antibiotics, such as metronidazole, azithromycin, β-lactam antibiotics combined with β-lactamase inhibitors like amoxicillin-clavulanate, are generally valid to be used against pigmented *Prevotella* species. However, due to their active production of β-lactamase enzymes, resistance of pigmented *Prevotella* species to penicillin is increasing (Walker et al., 1996; Walker and Bueno 1997; Mättö et al., 1999; Takahashi et al., 2006; Moon et al., 2013). *P. intermedia* demonstrates higher prevalence of β-lactamase-producing strains in orofacial odontogenic infections than elsewhere in the human body (Fosse et al., 1999; Kuriyama et al., 2001).

Among all Gram-negative anaerobes, *P. intermedia* sensu lato is a prevalent species in β-lactamase production, with efficient β-lactam resistance through the inhibition of transpeptidase enzymes, which are responsible for cross-linking the peptidoglycans in bacterial cell wall (Bernal et al., 1998; Van Winkelhoff et al., 2000). *P. intermedia* sensu lato exhibits resistance against many antimicrobials, including penicillin, cephalosporin, doxycycline and tetracycline (Andrés et al., 1998; Fosse et al., 2002).

Remarkably, the resistant *P. intermedia* strains have the capability to act as a reservoir for antimicrobial resistance genes that could be exchanged between related and unrelated genera through horizontal gene transfer, i.e., plasmid conjugation (Yu et al., 2007; Fernández-Canigia et al., 2015). For instance, the *tet*(Q) gene, which

encodes for tetracycline resistance in *P. intermedia*, has been linked to the property of the same species to induce conjugal transfer of the β-lactamase production genes to other *Prevotella* and *Bacteroides* species (Walker and Bueno, 1997).

2.3.6 Morphological changes

Gram-negative bacteria may express virulence and self-defense mechanisms via morphological modifications and morphogenetic changes. These include filament formation, fimbriae pattern, membrane thickness, intracellular inclusions, and LPS (Nakao et al., 1986; Buijs et al., 2008; Signoretto et al., 2011). For instance, when *P. intermedia* was treated with a sub-inhibitory and an inhibitory concentration of mushroom or chicory extracts, clear cell elongation was observed with several filaments and interrupted septa (Signoretto et al., 2011).

Exopolysaccharide-producing *P. intermedia* strains demonstrate higher virulence against the host innate immune response in comparison to non-exopolysaccharide-producing strains (Yamanaka et al., 2011). Regarding the role of fimbriae in the virulence of *P. intermedia*, C type fimbriae and specific cytoskeletal rearrangement are found to be prerequisites to *P. intermedia* to invade the oral epithelial cell lines (Dorn et al., 1998).

2.4 *P. intermedia* group in human infections

2.4.1 Role in periodontal diseases and other oral infections

Periodontal diseases are generally classified into gingivitis and periodontitis. In plaque-induced gingivitis, dental plaque accumulation on tooth surfaces at the gingival margin induces a localized or generalized inflammatory process by the synergistic effect of multi-species bacterial communities. Despite the wide heterogeneity of the oral microbiota, a narrow spectrum includes major periodontopathogens contributing to the active process of periodontal inflammation and tissue destruction (Slots et al, 1986; Dzink et al., 1988; Dahlén, 1993b; Harvey, 2017). Several studies have described *P. intermedia* as a potential pathogen due to its involvement in periodontal pathogenesis, for example, in necrotizing ulcerative gingivitis (NUG), periodontitis, and pregnancy-related gingivitis (Kornman and Loesche 1980; Loesche et al., 1982; Moore et al., 1985; Slots et al., 1986). Based on their colonization pattern and unique surface characteristics, *P. intermedia* and *P. nigrescens* may have different roles in periodontal pathogenesis; *P. intermedia* associates with periodontal infections, while *P. nigrescens* has been detected in both healthy and diseased sites of the periodontium (Shah and Gharbia, 1992; Mättö et al., 1996b; Könönen et al., 2000; Hashimoto et al., 2003). On the other hand, *P.*

pallens seems to be associated with the healthy periodontium (Könönen et al., 1998b), while, to date, only scarce data are available in the literature regarding the occurrence and potential pathogenicity of *P. aurantiaca* orally or systemically (Sakamoto et al., 2010; Piccolo et al., 2015).

P. intermedia sensu lato is among the most frequently isolated species from subgingival plaque samples (Zambon et al., 1981; Kamma et al., 2004). However, *P. nigrescens* demonstrates a steady proportion in the subgingival pocket, which does not differ from health to disease (Abusleme et al., 2013). Thus, *P. nigrescens*, and not *P. intermedia*, is considered one of the core species, which mediates the transitions of the subgingival microbiome (Diaz et al., 2016). Yet, *P. nigrescens* occurs less frequently in periodontally diseased sites compared with *P. intermedia* (Dahlén et al., 1990; Haffajee et al., 1992). As evidence on their involvement in clinical conditions, their eradication by antibacterial treatment strategies has resulted in periodontal improvement represented by certain clinical parameters, such as less bleeding on probing, enhanced attachment levels, and shallow pocket depths (Renvert et al., 1996; Haffajee et al., 2004; Kurata et al., 2008). Besides, they have been described as "crucial substances" or predictive biomarkers in subgingival biofilm, which may provide guiding information about the microbial fluctuations in the dynamics of subgingival ecosystems (Zhang et al., 2017).

In addition to periodontal infections, *P. intermedia* and *P. nigrescens* have been regarded as potential virulent species in some other oral infections, such as pericorontitis (Wade et al., 1991), endodontic infections, and odontogenic abscesses (Haapasalo, 1989; Bae et al., 1997; Robertson and Smith, 2009), apical periodontitis (Haapasalo et al., 1986; Zakaria et al., 2015), and periimplantitis (Dingsdag et al., 2016).

2.4.2 Role in extra-oral infections

Besides being frequently and predominantly isolated from periodontal lesions, *P. intermedia* and *P. nigrescens* are also associated with various perioral, extra-oral and systemic diseases such as peritonsillar abscesses, cystic fibrosis, acute and chronic bronchitis and pulmonary infection (Shinzato and Saito, 1994; Brook and Frazier, 2003; Fernández-Canigia et al., 2015).

In a clinical study assessing the association between oral bacterial DNA and severity of coronary artery disease, bacterial DNA was measured in pericardial aspirate autopsies; 63.6% of the cases were positive for endodontic bacteria including *P. intermedia* (Louhelainen et al., 2014). While in another study where the association between the aortic aneurysm development and the occurrence of periodontopathogens were examined, *P. intermedia* and *P. nigrescens* were among the frequently detected species in saliva and aneurysm samples (Ding et al., 2014).

In a cohort study including pregnant women, the data collected have demonstrated that *P. intermedia* is one of the periodontal pathogens capable of systemically disseminating fetal cord blood and translocating to the fetus resulting in fetal prematurity (Madianos et al., 2001). Regarding *P. pallens*, its reduced numbers in the gut microbiota was associated with gastric carcinogenesis (Avilés-Jiménez et al., 2014), while the decrease of *P. pallens* in saliva was connected to immunoglobulin A nephropathy (Piccolo et al., 2015). Nevertheless, increased numbers of *P. pallens* in the female genital tract was strongly associated with genital inflammation (Lennard et al., 2017).

2.5 Impact of female sex hormones on oral conditions during pregnancy

2.5.1 Clinical considerations

The periodontium surrounding a tooth is composed of four major periodontal tissues: gingiva (epithelial and connective tissues), periodontal ligament, root cementum, and alveolar bone. These components form a functional unit by which the periodontium maintains blood supply to the teeth, controls the masticatory forces in the oral cavity, and provides a physical barrier against environmental factors and microbial burden. Balanced degradation and regeneration of these components represent a physiological process. In contrast, imbalance of the host-microbial interaction induced by systemic factors, e.g. the hormonal influence of the endocrine system on periodontal tissues, may lead to alterations in periodontal homeostasis (Mariotti, 2000). Pregnancy itself does not induce gingival inflammation, but it may exacerbate preexisting risk factors (Laine, 2002). During pregnancy, alterations in maternal hormonal levels may result in metabolic changes, hemodynamic modulations, and systemic inflammatory responses in order to safeguard the prosperity of the developing embryo (Herrera, 2000; Wu et al., 2018). Besides, increased serum levels of estrogen help to maintain a safely progressing pregnancy, while progesterone prevents preterm uterine contractions and stimulate the expression of the inflammatory mediator prostaglandin E2 (PGE2) (Magness et al., 1989; Mealey and Moritz, 2003; Wu et al., 2018).

The link between alterations in sex steroids and periodontal diseases during pregnancy is considered pregnancy-associated periodontal changes, which are classified as pregnancy-associated gingivitis or pyogenic granuloma. Both conditions were earlier classified under the plaque-induced gingival diseases modified by endocrinal systemic factors (Armitage, 1999). Nevertheless, pregnant women with regular performance of oral hygiene measures and sufficient control of dental plaque may still experience pregnancy-associated gingivitis (Hugoson, 1971;

Kornman and Loesche, 1980; Tilakaratne et al., 2000, Figuero et al., 2010). Therefore, pregnancy-associated gingivitis has been recently reclassified under the oral conditions affected by systemic risks or modifying factors (Chapple et al., 2018). Among these systemic risk factors, elevations in sex steroid hormones may exaggerate the inflammatory response to clinically insignificant amounts of dental plaque at gingival margin.

The occurrence of pregnancy-associated gingivitis worldwide diverges broadly between 35% (Hasson, 1960; Chaikin, 1977) and 100% (Löe and Silness, 1963; Hugoson, 1971). Clinically, pregnancy-associated gingivitis demonstrates a higher tendency towards gingival bleeding and periodontal pocket formation when compared with plaque-induced gingivitis in non-pregnant women (Cohen et al, 1971; Raber-Durlacher et al, 1994). As a human body strategy to avoid rejection of the genetically incompatible fetus during early stages of pregnancy, the conceived woman becomes relatively immunocompromised (Clark et al., 1999). As a systemic outcome, this immunosuppression by limiting T-cell activity reduces the risk to autoimmune diseases, e.g. rheumatoid arthritis, thyroiditis, and multiple sclerosis (Taylor et al., 2002; Kaaja and Greer, 2005). In the oral cavity, the dynamic balance between the synergistic and antagonistic microbial interactions in dental plaque maintains a relatively healthy gingival status (Roberts and Darveau, 2015; Rosier et al., 2018). However, the prolonged accumulation of dental plaque at gingival margins leads to homeostasis breakdown and initiation of gingival inflammation, which activates local innate immune responses (Christersson et al., 1991; Marsh and Zaura, 2017). These inflammatory changes are usually limited to gingival tissues, and no significant loss of clinical attachment nor alveolar bone occurs. Therefore, the entire condition is considered “clinically reversible”, since it is possible to be cured by eliminating the causative factors, i.e. bacterial biofilm and/or hormonal imbalances (Tilakaratne et al., 2000; Gürsoy et al., 2013; Wu et al., 2015). Nevertheless, chronic gingival bleeding with signs of gingival inflammation (redness, edema, bleeding, and increased exudates of GCF) are considerable predisposing factors toward an irreversible tissue destruction and progression of the condition towards periodontitis (Lang et al., 1986; Joss et al., 1994; Schätzle et al., 2003).

2.5.2 Role of female sex hormones in pregnancy-associated gingivitis

Hormones are generally divided into four major subgroups: steroids, glycoproteins, polypeptides, and amines. Cholesterol-derived steroids belong to a major group of steroid hormones, which extend to corticosteroids (glucocorticoids and mineralocorticoids) and gonadosteroids (estrogens, androgens, and progestins).

Estrogens, progesterone, and testosterone are most frequently associated with periodontal pathogenic pathways (Shapiro and Freeman, 2014; Wu *et al*., 2015). Out of the three different estrogen metabolites (estradiol, estriol, and estrone), estradiol is the dominant premenopausal estrogen secreted by the ovaries, placenta and some peripheral tissues (Weinstein et al., 1974; McCauley et al., 2002). During pregnancy, serum estrogen levels are three folds higher than during female menstruation period (Amar and Chung, 1994; Karthik et al., 2009). Although estriol is the dominantly circulating estrogen in the serum of pregnant women, estradiol is still considered the principal and the most powerful circulating estrogen during the reproductive age of women in terms of estrogenic activity and during pregnancy in terms of hormonal potency. Additionally, estradiol and estrone can be reversibly metabolized into estriol, which explains the predominance of estriol as a major estrogen in urine (Mariotti and Mawhinney, 2013).

Dental plaque is an initiating and essential factor in the etiology of periodontal disease. However, oral pathogens alone are not sufficient provokers for disease development, a susceptible host remains a requisite (Travis et al., 2000; Giannobile, 2008; Kornman, 2018). Therefore, loss of balance in the host-microbial interaction affected by other modifiable (environmental and behavioral) and non-modifiable risk factors (genetic and systemic) may initiate, develop, and exacerbate periodontal tissue destruction (Tatakis and Kumar, 2005; Tanner et al., 2006; Silva et al., 2015). Among systemic factors, elevated female sex hormones (estrogen and progesterone) during pregnancy can play a significant role in periodontal pathogenesis and wound healing exhibited by increased susceptibility to gingival inflammation. Such manifestations were described as early as 1877 when Pinard illustrated a condition associated with pregnancy and characterized by erythema, edema, hyperplasia, and increased tendency for gingival bleeding (Pinard, 1877).

Gingival tissues contain estrogen and progesterone receptors by which these hormones induce vascular permeability and enhance GCF secretion (Mealey and Moritz, 2003; Nebel, 2012). In serum, elevated levels of circulating sex hormones are related to host response modifications, which adversely affect periodontal tissues, i.e., hormone-induced vascular permeability and proliferation result in increased gingival swelling and bleeding (Hugoson, 1971; Mariotti, 1994; Mealey and Mortiz, 2003; Mariotti and Mawhinney, 2013) (Figure 5). Notably, the capacity of female sex hormones, which enhance the production of vascular endothelial growth factor (VEGF), IL-6, and IL-8 by human gingival fibroblasts (HGF), has the potential to participate in the active process of periodontal disease during pregnancy (Yokoyama et al., 2005). It seems that the increase of sex hormones in serum during pregnancy has no effect on the expression of IL-1β, TNF-α, and PGE2 levels, while IL-6 seems to raise gradually starting from the second trimester and remaining the same levels until post-partum (Carrillo-de-Albornoz et al., 2012; Wu et al., 2016) (Table 2).

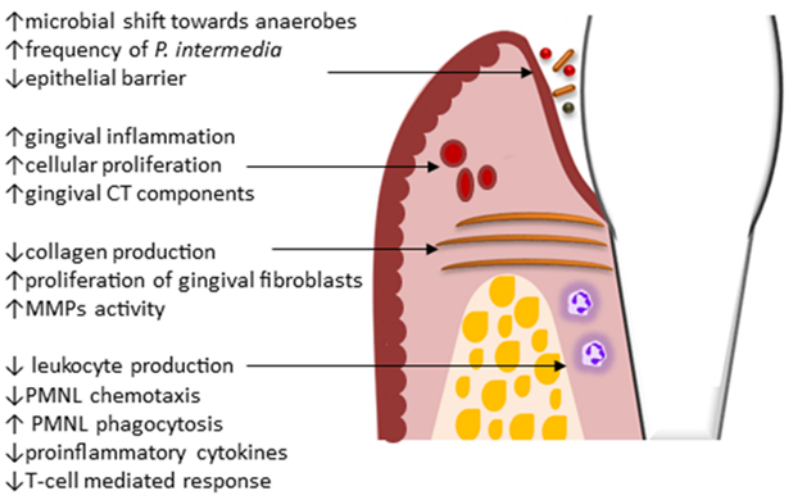


Figure 5. Effect of elevated estrogen during pregnancy on periodontal tissues (CT: connective tissue, PMNL: polymorphonuclear Leukocyte) (summarized from Hugoson, 1971; Cohen et al, 1971; Kornman and Loesche, 1980; Jensen et al., 1981; Miyagi et al., 1992; Raber-Durlacher et al., 1994; Mariotti, 1994; Güncü et al., 2005).

In primary human monocytes, β-estradiol and progesterone influence the immune response to LPS of *P. gingivalis*, the process which may indicate a clinical role for these female sex hormones in periodontal diseases associated with pregnancy (Jitprasertwong et al., 2016).

Table 2. Influence of estrogen on microbial, clinical, and immunological findings in the periodontium during pregnancy (Mariotti, 1994; Mascarenhas et al., 2003; Güncü et al., 2005).

Microbiological changes	**Clinical changes**	**Immunological changes**
microbial shifts toward Gram-negative anaerobes	↑ gingival inflammation	↓ neutrophil chemotaxis
↑ numbers of *P. gingivalis*	↑ gingival probing depths	↓ antibody production
↑ numbers of *P. intermedia*	↑bleeding on probing	↑ synthesis of PGE_2
↑ numbers of *C. rectus*	↑ flow of GCF	↑ IL-6, IL-8
↑ bacterial proteolytic enzyme activity	↑ tendency for periodontitis	↓ anti-inflammatory mechanisms
	↑ tooth mobility	
	↑tendency for pyogenic granuloma	

2.5.3 *P. intermedia* group bacteria and maternal steroids

Microbial alterations, environmental risks, genetic susceptibility, and systemic influences are thought to be principal elements in the pathogenesis of pregnancy-related gingivitis. During pregnancy, microbial shift in subgingival biofilm, favoring Gram-negative anaerobes, has been observed, along with altered host immune responses and elevated serum levels of maternal steroids (Hugoson, 1971; Kornman and Loesche, 1980; Jensen et al., 1981; Armitage, 1999; Tanner et al., 2006; Teles et al., 2010).

During the second trimester of pregnancy, gingival bleeding reaches its peak. This occurs in parallel with significantly increased proportions of *P. intermedia* in subgingival plaque. Additionally, estrogen and progesterone reach the highest levels in serum during the second or third trimesters with approximate concentrations of 20 ng/ml and 130 ng/ml, respectively (Schock et al., 2016). This is followed by an improvement in clinical manifestation related to a simultaneous reduction in serum sex hormone levels up to 3-15 months postpartum when these maternal steroids seem to drop back to steady levels observed before gestation (Kornman and Loesche, 1980; Zachariasen, 1989; Mariotti, 1994; Raber-Durlacher et al. 1994; Gürsoy et al., 2008). According to Kornman and Loesche (1982), *P. melaninogenica* group and *P. intermedia* sensu lato are able to uptake estradiol and progesterone to utilize them metabolically as vitamin K substitutes and a source of energy. The same authors claimed that the estradiol concentration of 2.9 μM (0.8 μg/ml) was sufficient for both organisms to enhance their growth properties (Kornman & Loesche, 1982).

Regardless of the amount of dental plaque at gingival margin, an increased proliferation of *P. intermedia* sensu lato by 55-fold was reported among pregnant women when compared with their non-pregnant counterparts (Jensen et al., 1981). Later, it was shown in a Finnish study, using a molecular method for distinguishing *P. intermedia* sensu stricto and *P. nigrescens*, *P. nigrescens,* and not *P. intermedia,* significantly proliferates in the oral cavity of pregnant women and was associated with pregnancy-related gingivitis (Gürsoy et al., 2009). Based on culture methods, a Spanish open cohort study on hormonal influence on subgingival biofilms during pregnancy found that *P. gingivalis* and *P. intermedia* sensu lato were indicated as subgingival dominant findings due to elevated maternal hormones (Carrillo-de-Albornoz et al., 2010). Most pregnancy-associated gingivitis studies have focused on evaluating the abundance of *Prevotella* species in subgingival biofilms. In another study, ecological shifts in supragingival biofilms during pregnancy were examined, demonstrating positive correlations between several taxonomic units and sex hormones, in which *Prevotella* and *Treponema* were the most abundant taxa (Lin et al., 2018).

3 AIMS

P. intermedia sensu lato is able to use estrogen and progesterone as an essential source of growth during pregnancy. Thus, we here hypothesized that estradiol may have an impact on the growth properties and pathogenicity of the *P. intermedia* group organisms. Since hormonal alterations by sex steroids may influence the bacterial adherence and interactions with epithelial and connective tissue cells, therefore, we also hypothesized the existence of an epithelial cytokine response against the *P. intermedia* group bacteria, in the presence of estradiol. Besides, we assumed that DPD, the universal precursor of QS molecule, AI-2, and its analogs, modulate human gingival keratinocyte cytokine response against *Prevotella* cell extracts, and may inhibit the biofilm formation of the tested *Prevotella* species, by competing with or inhibiting the native AI-2 signaling pathway of these species.

The purpose of the present doctoral thesis was to characterize the impact of estradiol on the virulence of the *P. intermedia* group organisms and the epithelial cytokine response against these species. The four *in vitro* studies were conducted using a microbiological and enzymological approach.

The specific aims (illustrated in Figure 6) were set to:

1. Examine the impact of estradiol on the growth properties of the *P. intermedia*, *P. nigrescens*, and *P. pallens* including planktonic growth, coaggregation, polysaccharide production and biofilm formation (**I**).
2. Evaluate the effect of estradiol on the DPPIV enzyme activity of the *P. intermedia*, *P. nigrescens*, *P. pallens* and *P. aurantiaca* (**II**).
3. Determine the QS role and the DPPIV dependence of the estradiol-regulated biofilm formation of *P. aurantiaca* strains (**III**).
4. Demonstrate the regulatory role of QS molecules in the epithelial cytokine response and biofilm-related virulence of the *P. intermedia*, *P. nigrescens*, and *P. pallens* (**IV**).

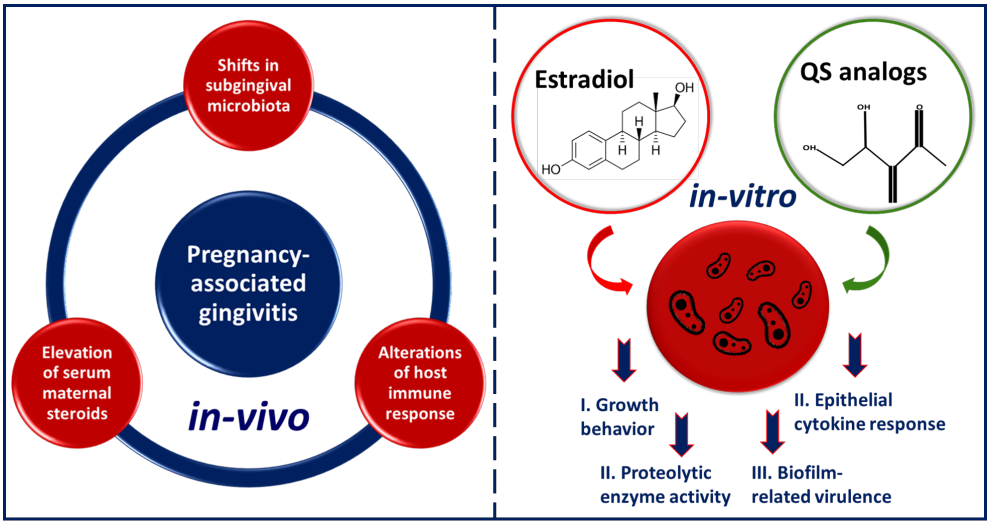


Figure 6. An illustrative diagram for study hypotheses and specific aims.

4 Materials and Methods

4.1 Bacterial strains and culture conditions

This study included 4 type strains (2 American Type Culture Collection, ATCC, 1 National Collection of Type Cultures, NCTC, and 1 Culture Collection University of Gothenburg, CCUG) and 5 clinical strains (Anaerobe Helsinki Negative, AHN) of *P. intermedia* group bacteria and 1 type strain of *F. nucleatum*. The type and clinical strains of *P. intermedia, P. nigrescens,* and *P. pallens* were provided by the National Institute for Health and Welfare, Helsinki, Finland. The strain identification description was mentioned previously (Könönen et al., 2000). The clinical strains *P. aurantiaca* AHN 37505 and AHN 37552 were isolated from the saliva of two periodontitis-free post-partum women and identified by using partial (ca. 550 bp) 16S rRNA gene sequencing (Estama et al, 2015). One type strain of *F. nucleatum* (ATCC 25586^T) was included in the conducted coaggregation assays (Table 3).

Table 3. A list of bacterial species and strains, and the source of clinical strains used in the four studies.

Species	Strain	Source of clinical isolates	Study
P. intermedia	ATCC 25611^T AHN 8290	- Periodontal pocket	I, II, IV
P. nigrescens	ATCC 33563^T AHN 8293	- Periodontal pocket	I, II, IV
P. pallens	NCTC 13042^T AHN 9283	- Saliva	I, II, IV
P. aurantiaca	CCUG 57723^T AHN 37505 AHN 37552	- Saliva Saliva	II, III
F. nucleaum	ATCC 25586^T	-	I, III

All study strains were revived from skimmed milk stocks kept in -70°C and cultivated on Brucella blood agar supplemented with hemin and vitamin K_1 in an anaerobic chamber unit (Whitley A35 Anaerobic Workstation, Don Whitely Scientific Ltd., West Yorkshire, UK), which provides an anaerobic atmosphere at 37ºC for 3-5 days. For pure culture, only distinct and well defined colonies were

passaged for another growth cycle on the same type of Brucella agar and with the same incubation conditions. Then, purely cultured bacterial colonies were collected from Brucella agar and transferred to be cultured in autoclaved Todd-Hewitt broth (Becton and Dickinson Difco™ and BBL™, USA) enriched with yeast extracts, cysteine, hemin, and menadione (Sigma Chemical Co., St. Louis, MO, USA) to allow further growth for 24-48 h (depending on experiment settings).

4.2 Bacteriological methods

4.2.1 Planktonic growth and bacterial cell viability measurements (I and III).

To standardize the bacterial cell concentration throughout all experiments, optical density (OD) adjustments were used (when needed) in all study experiments where the OD of 0.5at 490 nm of each strain was spectrophotometrically (Shimadzu Biotech, Tokyo, Japan) counted and corresponded to the logarithmic colony forming unit (log CFU/ml). Prior planktonic growth test, each bacterial strain was grown in broth for 24 h and then adjusted to the OD of 0.7 at 490 nm. Afterwards, a known amount of the OD bacterial suspensions were incubated with different known concentrations of estradiol suspensions in the anaerobic chamber for another 24 h. After incubation, 10 µl of bacterial suspension of each concentration was inoculated on a quarter surface area of Brucella agar. This was then incubated in an anaerobic atmosphere for at least 72 h prior to the counting of colony forming units (CFU).

4.2.2 Biofilm protein assays (I-IV)

In studies I, II, and IV, the protein levels produced by each *Prevotella* strain were evaluated by the Bradford method of protein determination in biological samples where the optically adjusted planktonic cells were grown in saliva-coated wells in an anaerobic atmosphere for 48 h. Then, culture media were blotted out and wells of the 96-well plate were rinsed twice with PBS to eliminate unbounded cells and retain the attached biofilm only. Afterwards, 0.2 N NaOH was loaded to each well and sonicated to power 80 for 3 s. In addition to the standard method, a step of microwave enhancement took place after sonication by heating the plate in a 600 W microwave oven for 20 s. After heating, Bradford Protein Assay (Bio-Rad, Hercules, USA) was loaded into each well and the plate was incubated for 5 min at room temperature while shaking on a vortex. Absorbance was measured at a wavelength of 595 nm with the micro-plate reader. Bovine serum albumin standard concentrations (0, 50, 125, 250, 375, and 500 mg/ml) (Sigma Aldrich, USA) were used to standardize the OD values and convert them to protein concentrations.

In study II, the Lowry method of protein concentration determination was the optimum method to evaluate the biofilm mass (in the form of static biofilm) obtained from each *Prevotella* strain (Lowry et al., 1951). In this study, biofilms were anaerobically grown on nitrocellulose membranes for 24 h. Then, the membranes carrying the biofilms were carefully transferred into Eppendorf tubes containing 1 N NaOH and heated in a boiling water bath for 1 h (tube lids were needle-punctured and tightly closed). After cooling, the tubes were centrifuged at 13,000 g for 5 min to discard the remnants of the undissolved membrane. Supernatants (test samples) as well as a known amount of blanks (sterile membranes) and standards were collected in equal amounts and the assay was performed by adding alkaline copper solution stabilized with sodium potassium tartrate to each sample as a protein pretreatment. Then, each tube underwent vortexing and a period of 10 min incubation. Protein amount in the solution was determined by measuring the absorbance values of the solution at 550 nm using a UV spectrophotometer (Schimadzu Corporation, UV-2600, Tokyo, Japan). All experiments were carried out at least twice, at different time points and all samples were measured in triplicate. Bovine serum albumin was used as protein standards, (SigmaeAldrich, USA) in the following concentrations: 0, 50, 125, 250, 375, and 500 mg/ml, to be used in the calculation of the scattered formula for the calibration curve of converting OD values into protein concentrations.

4.2.3 Biofilm polysaccharide assay (I)

As an essential part of biofilm formation, levels of biofilm polysaccharides were measured by using the phenol-sulfuric acid method (Yang et al., 2006). The OD adjusted planktonic cells of each *Prevotella* strain were grown anaerobically in a 96-well plate (in pentaplicates) for 48 h. Then, culture medium was eliminated and plates were blotted carefully and rinsed twice with PBS. Immediately after rinsing, 40 ml of de-ionized water, 40 ml of 5% phenol solution, and 200 ml of 95-97% sulfuric acid were added to each control and test well where the biofilms were grown (±estradiol). Polysaccharide levels in biofilms were determined by OD absorbance values at 490 nm using a micro-plate reader. Standard glucose concentrations of 0, 5, 10, 20, and 100 mg/ml were used in the scattering formula calculations of converting the OD readings into polysaccharide concentrations.

4.2.4 Coaggregation assay (I and III)

For the evaluation of the coaggregation ability of the *Prevotella* strains, the Kolenbrander's method with minor modifications was performed (Kolenbrander, 1995). First, each *Prevotella* strain was grown in Todd-Hewitt broth and *F.*

nucleatum ATCC 25586 in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) in anaerobic atmosphere for an overnight incubation. Each bacterial suspension was centrifuged twice at 12,000x *g* for 10 min, supernatants were discarded, and pellet resulted from the first centrifugation was washed with PBS and the same pallet obtained from the second centrifugation was resuspended in the coaggregation buffer (Tris HCl, pH 8.0), containing 0.1 mM CaCl2, 0.1 mM MgCl2, and 150 mM NaCl. The physical coaggregation process was measured spectrometrically by recording the OD of the mixed suspensions immediately (0 h), after 30 min, and after 1 h. All experiments were done in triplicates. During the incubation intervals, the test cuvettes were kept covered inside an incubator at 37 ºC.

4.2.5 Bacterial whole cell extract preparations (III)

Whole cell extract (WCE) of each *Prevotella* strain used in the study was performed according to a modified protocol from Itoh et al. (2009). Bacterial colonies were collected from one full agar plate after 72 h of anaerobic incubation and suspended in 0.3% CHAPS detergent to enhance bacterial cell membrane rupture (Thermo Fisher Scientific, USA). Suspension tubes were immediately incubated in an ice container for 30 min to ensure the stability of the enzyme structure. Each bacterial suspension was then sonicated on ice for 20 s to avoid overheating. The bacterial suspension OD of each strain was adjusted according to the lowest OD obtained at 490 nm.

4.3 Materials

4.3.1 Estradiol suspension preparations (I-IV)

Estradiol hormone was available commercially in powder form (Sigma, Poole, UK). Estradiol powder was first dissolved in 70% ethanol to ensure homogeneity of the suspension, and then distilled water was added to the concentrated suspension up to the final stock concentration required. Beside one control group with no added estradiol (distilled water only), three test groups were prepared from the stock solution with the following estradiol concentrations of 30, 90, or 120 nmol/L. Chosen concentrations simulate average clinical values of serum estradiol concentrations equivalent to the first, second, and third trimester of pregnancy (O'Leary et al., 1991).

4.3.2 Clarified saliva preparations (I-IV)

Clarified saliva was prepared by collecting saliva from healthy volunteers of average age range 25-54 years and with no history of antibiotics during the preceding month.

Stimulated saliva was collected from all volunteers by giving them paraffin wax to chew and spit the saliva into sterile Falcon tubes incubated in an ice bath for a maximum period of 10 min chewing per subject. Saliva from different subjects was collected in one container and underwent centrifugation of 12,000*g* for 40 min.

To start the pasteurization process, saliva was divided into several smaller containers and transferred into a water bath of 60 ºC for 30 min, and then was again centrifuged at 12,000*g* for 40 min. Pasteurized saliva was kept in -70 ºC for further use. For each experiment that required saliva coating, a known amount of clarified saliva (enough to cover the well bottom) was added to all wells and incubated at 37 ºC for 1 h. Then, saliva was eliminated by blotting out the well-plate without rinsing it and immediately the bacterial suspensions were loaded to prevent the drying of the saliva coating.

4.3.3 Reagents, buffers, and salt solutions (I-IV)

- Folin-Ciocalteu protein assay reagents

  A: 20g of 2% Na_2CO_3 in 0.1 N NaOH (4g/L)
  B: 0.5% $CuSo_4$ x $5H_2O$ (0,5g/100 ml) in 1% KNa-tartrate (1 g/ 100 ml)
  C: alkali Cu-solution: (50 ml A + 1 ml B)
  D: Carbonate-Cu-solution: 50 ml of 2% $NaCO_3$ + 1 ml of solution B
  E: 10 ml Flin Ciocalteu solution: 10 ml H_2O

- Coaggregation buffer

  1 mM Tris-HCl (pH 7.4) containing 0.1 mM CaCl2, 0.1 mM MgCl2, and 150 mM NaCl

- Fluorogenic substrate buffer

  0.1 mM Tris-HCl (pH 7-8) containing 0.1% Triton X-100 and 10 µM H-Ala-Pro-AFC

- Phosphate buffered saline: PBS (Gibco™, Invitrogen, Paisley, Scotland, UK)
- Trypsin deactivation buffer
- Cell lysis buffer

4.3.4 Fluorogenic substrate (H-Ala-Pro-AFC) preparations (II)

To measure the DPPIV enzyme activity of the static biofilms, a fluorogenic substrate with the specificity to access the active site of the DPPIV enzyme was purchased

(Boonacker et al., 2003). One mM of the fluorogenic substrate H-Ala-Pro-7-amido-4- trifluromethylcoumarin (H-Ala-Pro-AFC; Bachem, Bubendorf, Switzerland) was dissolved in 100 mM Tris-HCl buffer (pH 8) to facilitate the substrate penetration into the openings of the propeller and hydrolase domains of DPPIV.

4.3.5 DPPIV preparations (II and III)

The synthetic human recombinant Dipeptidyl Peptidase IV (R&D, USA) was used as a positive control in all zymography and fluorometric assays performed. The C-terminal histidine-tagged enzyme was supplied by manufacturer as a solution in 10 mM Tris-HCl, pH 7.6. In our laboratory, the synthetic enzyme was diluted into aliquots of 50 ml of Tris-HCl buffer (pH 8) containing 0.05% Triton X-100 to enhance the enzyme solubility.

4.3.6 Preparations of QS molecules and analogs (III and IV)

The synthetic molecule, 4,5-dihydroxy-2,3-pentanedione (DPD), and its C1-alkyl analogs (ethyl-DPD, butyl-DPD, and isobutyl-DPD) (Roy et al., 2010) (Figure 7) were first tested for their cytotoxicity on bacterial and epithelial cells and later for their ability to antagonize the action of the native universal autoinducer (AI-2).

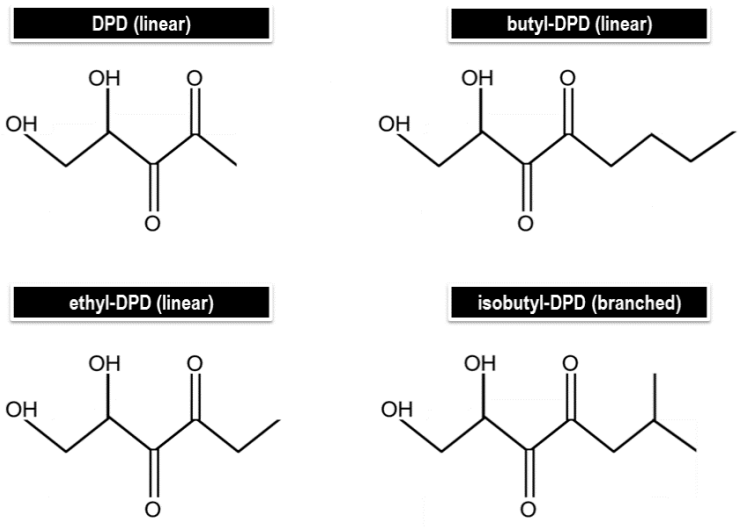


Figure 7. Chemical structure of DPD and three analogous used in this thesis work. Each structure demonstrates the linear or branched differences in the carbon side chain (Roy et al, 2010).

These antagonists were developed in the laboratory by C1-alkyl quenching of the QS response activated in multiple bacterial species through modulation of bacterial kinases, LsrK, and a specific gene transcription technique. The synthetic ecosystem comprised of three well-known species with their QS response, *Escherichia coli, Salmonella typhimurium*, and *V. harveyi* (Roy et al., 2010). In studies III and IV, stock solutions of DPD and its analogs were diluted in dimethyl sulfoxide (DMSO) and culture media and adjusted into a final molarity of 10 nM, 100 nM, 1 mM, and 10 mM. Stock solutions of each concentration were always kept at -20°C and each tube was opened to prepare a concentration, the rest was discarded afterwards in order to ensure effectiveness of the stock chemical ingredients and to avoid the unknown risk of its multiple thawing.

4.4 Functional and enzymatic methods

4.4.1 Fluorometric assay for enzyme activity detection (II and III)

In order to ensure the specific effect of DPPIV enzyme activity on bacterial biofilm formation, the DPPIV specific serine proteinase inhibitor, di-isopropylfluorophosphate, was used (Koreeda et al., 2001). In study II, the membrane holding the faced-up static biofilms of each *Prevotella* strain was placed at bottoms of a 96-well plate and loaded with an aliquot of 50 ml/well of Tris-HCl buffer containing 0.05% Triton X-100 in order to solubilize the bacterial enzymes.

To measure the DPPIV activity, the immediate addition of 1mM of the fluorogenic substrate H-Ala-Pro-7-amido-4-triflouromethylcoumarin (H-Ala-Pro-AFC; Bachem, Bubendorf, Switzerland) in 100 mM TriseHCl buffer (pH 8) prior to the measurement of all control and test wells was aimed to ensure the initiation of the reaction without losing early enzyme intensity readings. The test was repeated for several times at different time points and in quintuplicates (±estradiol). DPPIV activity measurements were recorded between 0 and 20 min (1 reading/minute) in addition to a final reading after 1 h to ensure the steadiness of the reaction curve. The loaded well plates were kept at 37ºC during the measurements and the reader was adjusted to the same temperature during the periods of incubation in the plate chamber. Since the peak of enzyme activity in all experiments has occurred always around minute 5 from starting the reaction followed by a period of steady intensity values, the average value of the minute 5 readings was selected as a standard time point for enzyme activity detection. The release of the fluorogenic part of the substrate (amido-4-triflouromethylcoumarin) was measured kinetically at 380 nm using a Synergy microplate reader and the Gen5 software (Biotek Instruments, Winooski, VT, US).

4.4.2 Analyses of enzyme activity on static biofilms (II)

The fluorogenic substrate H-Ala-Pro-7-amido-4- trifluromethylcoumarin (H-Ala-Pro-AFC) was dissolved in 100 mM Tris-HCl buffer (pH 8) to facilitate the substrate penetration to the openings of the propeller and hydrolase domains of DPPIV, and then the dissolved substrate was loaded to all control and test wells containing semipermeable membranes carrying the static biofilms facing up. The substrate was loaded followed by immediate insertion of the well-plate to the spectrophotometer in order to read the 0 min reaction between the substrate and the biofilm DPPIV enzyme. Due to light sensitivity of the used fluorogenic substrate, all steps of preparations and loading were done in dim light environment and using aluminum foil covered Eppendorf tubes.

4.5 Cell culture (IV)

Immortalized human gingival keratinocyte (HMK) (Mäkelä et al., 1998) cell lines were cultured for at least 72 h in a keratinocyte-SFM growth medium, containing human recombinant epidermal growth factor, bovine pituitary extract (Fisher Scientific, Paisley, Scotland), and antibiotics (penicillin 10.000 U/ml and streptomycin 100 mg/mL). Growth culture media were renewed every 36-48 h and the cells were passaged every 4-5 days or when the flask confluency reached 80-90%. Then, cells were trypsinized, centrifuged, and overnight incubated in 12-well plates at 37 ºC and in 5% CO_2. The HMK cells were always collected from passages 22 to 24, and incubated in co-cultures with WCE of *Prevotella*, QS molecules, and/or estradiol, depending on the aim of the experiment.

4.6 Epithelial cytokine response profile

The lysates of HMK cells were collected and centrifuged at 10000 *g* for 5 min. Lysate concentrations of IL-1β, IL- 6, and IL-8 were detected by the Luminex® xMAP™ technique (Luminex Corporation, Austin, TX) and following the commercially available Bio-Plex kits (cytokine group I assays; Bio-Rad, Santa Rosa, CA). The experimental steps were performed according to the manufacturer's protocol. All values below the detection limit were excluded and the minimal detection limit of cytokine concentrations was 0.22 pg/mL for IL-1β, 2.19 pg/mL for IL-6, and 1.27 pg/mL for IL-8.

4.7 Imaging methods

4.7.1 Transmission electron microscopy (TEM) (III, unpublished data)

The evaluation of bacterial cell morphology, intracellular inclusions, fimbria thickness and density was performed by using a high-resolution microscope, TEM. The OD-adjusted *P. aurantiaca* strains were incubated with DPD and its analogs for 24 h. Suspension were then centrifuged at 10 000*g* and 37 °C for 5 min. After discarding the supernatants, bacterial pellets were collected with a sterilized syringe and were chemically fixed to crosslink specimen proteins with adjacent molecules using 5% glutaraldehyde in s-collidine–HCl buffer, pH 7.4. To avoid sample acidity, samples were rinsed, fixed with osmium tetroxide (OsO_4) to enhance contrast, dehydrated before infiltration with epoxy resin for complete embedding, and finally, specimens were polished with abrasives and sectioned with ultramicrotome. Ready specimens were stained with heavy metals before adapting them to TEM metal grids to be visualized by a JEOL JEM-1400 Plus transmission electron microscope (JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV.

All micrographs were captured in 4 different magnifications: 1) (×2,500) to evaluate the section suitability and to ensure that the section was free of bacterial contamination or artifacts, 2) (×12,000) to detect extracellular structures such as fimbria density and bacterial cell orientation, 3) (×25,000) was mainly to run quantitative fimbria thickness calculations of the *P. aurantiaca* strains among graded concentrations of DPD and its analogs. Fimbriae thickness was measured using the software ImageJ 1.48v (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

4.7.2 Scanning electron microscopy (SEM) (IV)

SEM was used as an imaging modality to determine the cell membrane surface contours and to evaluate the density and microtopography of the biofilm mass. First, each *Prevotella* strain was OD-adjusted to 0.7 at 490 nm and then grown anaerobically on saliva-coated glass slips and incubated with DPD and its analogs and with the functioning concentration of estradiol for 24 h. After rinsing with PBS, biofilms built over the glass coverslips were chemically fixed with 5% glutaraldehyde in 0.16 mol 1-1 s collidine-HCl buffer, pH 7.4 for 20 min.

Serial dehydration was performed in a graded series of ethanol (50%, 70%, and 98%) with 5 min embedding intervals in each concentration. Afterwards, the specimens were transferred to be mounted on metal stubs and were coated by carbon thread for 1 s. The micro-topography and mass density of the biofilm was examined

by LEO 1530 Gemini scanning electron microscope. The image magnification was standardized to correspond to a Polaroid 545 print and an image size of 8.9 $_X$ 11.4 cm.

4.7.3 Micro-colony light microscopy

Beside high-resolution microscopes, a light microscope was used to image all nine strains of *Prevotella* inoculated on agar plates to visualize the shape, color, and size of their micro-colonies (unpublished data).

4.8 Statistical methods

Depending on the experiment setting, each experiment was performed in triplicate or pentaplicate and was repeated at least twice at the same conditions but at different time points. For test data comparison with the control, mean values, standard deviations, and the Student's *t*-test to detect statistical significance were the tools used in the study. A *p* value of <0.05 was considered statistically significant. The normality tests (Kolmogorov-Smirnov and Shapiro-Wilk tests) were used to determine data distribution. After confirming that all data were normally distributed. The parametric test for multiple comparisons was performed using "one way ANOVA" and a post hoc test for inter-group comparisons. Two-tailed level of significance (*P*-value) and Student's t-test *P*-values < 0.05 were accepted as statistically significant. The different statistical methods used in the four studies are illustrated in Table 4.

Table 4. Different statistical methods and analyses used in each study (I-IV)

I	• **Mean, Standard deviations** • **Student´s *t*-test; two-tailed level of significance; *P*-values: **P*<0.05 with control**
II	• Mean, Standard deviations • Normality test: Kolmogorov-Smirnov and Shapiro-Wilk tests • Parametric test: Analysis of variance (one way ANOVA) • Student´s *t*-test; two-tailed level of significance; *P*-values: **P*<0.05, ***P*<0.01, and ****P*<0.001
III	• Mean, Standard deviations • Normality test: Kolmogorov-Smirnov and Shapiro-Wilk tests • Parametric test: Analysis of variance (one way ANOVA) • Student´s *t*-test; two-tailed level of significance; *P*-values: **P*<0.05, ***P*<0.01, and ****P*<0.001
IV	• Mean, Standard deviations • Parametric test: Analysis of variance (one way ANOVA) • Student´s *t*-test; two-tailed level of significance; *P*-values: **P*<0.05, ***P*<0.01, and ****P*<0.001

5 Results and Discussion

5.1 Impact of estradiol on growth properties of the *P. intermedia* group organisms

5.1.1 Planktonic growth (I and III)

In the laboratory, growing a freely-floating community of bacteria suspended in a culture medium is often referred to as the planktonic phase of growth. During pregnancy, salivary estrogen level has been considered a determining marker of the severity of gingival inflammation against dental plaque (Gürsoy et al., 2013). Periodontitis-free pregnant and non-pregnant women had an increased frequency of *P. nigrescens*, but not *P. intermedia*, with a significant peak of increase at the second trimester of pregnancy (Gürsoy et al., 2009). In the present work, *P. nigrescens* ATCC 33563, *P. pallens* AHN 9283, and two clinical strains of *P. aurantiaca* (AHN 37505 and AHN 37552) demonstrated elevated numbers in planktonic forms with higher estradiol concentrations (I and III) (Figure 8a and b).

Simulating the *in vivo* conditions, estradiol concentrations were selected in equivalence to the serum estradiol during the three pregnancy trimesters (O'Leary et al., 1991), through which *P. intermedia* increases its numbers variably in saliva and dental plaque. In the oral cavity, pathogenic bacteria in the planktonic phase are weak to compete with the resident microbiota with established biofilms, to withstand washing by the saliva, the antimicrobials and host defenses in the surrounding environment, and to replicate and express virulence. Thus, the attachment of these pathogens to oral habitats and their adherence to host cells are rather essential requirements to maintain initial colonization and enhance their survival. In the literature, *P. intermedia* sensu lato exhibited a hormonal uptake rate of estradiol or progesterone equals to $2.6x10^{-4}$ to $5.4x10^{-4}$ µmol/µg of cell protein (Kornman & Loesche 1980; 1982).

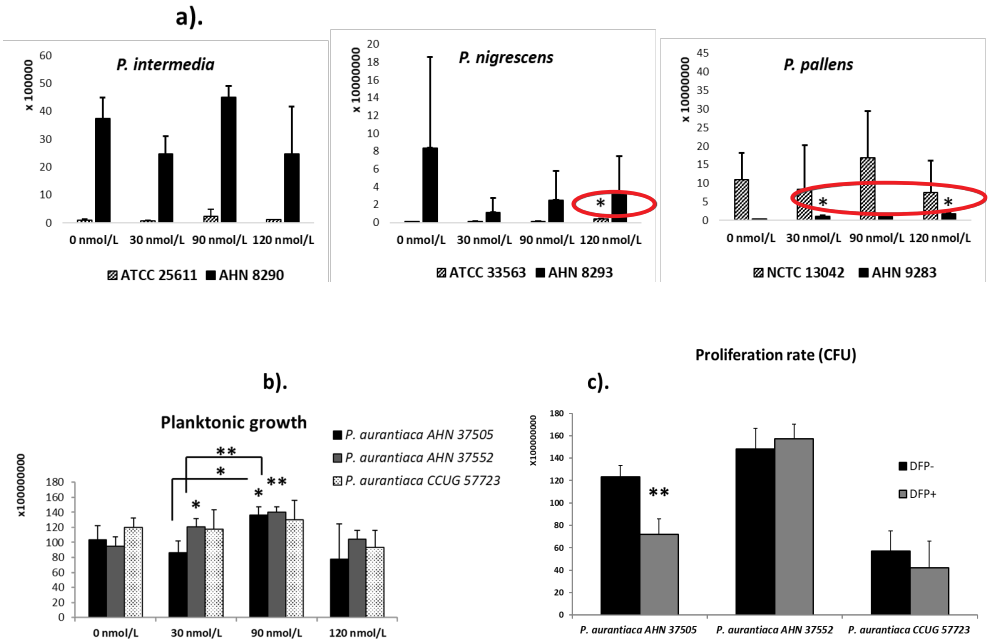


Figure 8. a) and b) Planktonic growth of the *P. intermedia* group bacteria in different estradiol concentrations (0, 30, 90, and 120 nmol/L), CFU/ml, (*$p < 0.05$) compared to control. c) Proliferation rate of *P. aurantiaca* strains ± DPPIV inhibition by di-isopropylfluorophosphate (DFP). (Modified from original publication I and III).

5.1.2 Bacterial cell viability (III)

Several bacterial cell viability assays use different approaches to determine the number of viable cells or the percentage of live to dead cells within a bacterial culture. In the present work, the serial dilution of the culture media and the plate counting method were used to evaluate the cytotoxic effect of the DPPIV enzyme inhibitor, DFP. The proliferation rate of *P. aurantiaca* strains with and without DPPIV inhibition was tested to confirm whether DPF had been cytotoxic to bacterial cells. The results indicated no significant cellular toxicity except for *P. aurantiaca* AHN 37505 (Figure 8c).

OD measurement to determine bacterial cell viability is also a convenient method in correspondence with CFU, if the bacterial growth pattern was kept linear during the test days. When growing cultivable anaerobic species, conditional limitations may exist, especially, the bacterial viability and metabolic activity may not necessarily shield bacterial cells from losing their ability to form colonies on agar (Keep et al., 2006). When bacterial cells are alive but unable to form colonies, they are in a state named as the viable but non-cultivable state (Oliver, 2005). To date, the existence of such a state has not been illustrated for the *P. intermedia* group

organisms. Thus, the CFU method was successfully used in previous studies (Gürsoy et al., 2008; 2009).

5.1.3 Biofilm formation (I-IV)

Bacterial biofilms are formed of bacterial cells and an extracellular polymeric matrix, which contains extracellular polysaccharides, proteins, lipids, and extracellular DNA. In the current work, biofilm formation was examined by measuring the protein levels in the total biofilm mass. The Bradford method of protein determination in biological samples and the Lowry method of determining protein concentration were used (Hammond and Kruger 1988; Lowry et al., 1951). By using the Bradford method, *P. intermedia* ATCC 25611 and AHN 8290, and *P. pallens* AHN 9283 exhibited a peak of elevation in biofilm formation within the 90 nmol/L of estradiol **(I)** (Figure 9a).

Confirming the results by using the Lowry method, most of the tested *Prevotella* strains showed increased protein levels with the same peak, 90 nmol/L estradiol concentration. However, *P. nigrescens* AHN 8293 and *P. pallens* AHN 9283 kept increasing their protein levels up to the highest estradiol concentration, 120 nmol/L **(II)** (Figure 9c). Thus, the results indicated a clear dose-dependent biofilm growth response.

These results were in line with a clinical study where *P. nigrescens* proliferated to a peak of growth at the second trimester of pregnancy with elevated estradiol in subgingival biofilms (Gürsoy et al., 2009). Regarding the biofilm mass of the tested *P. aurantiaca* strains, significantly elevated protein levels were observed with elevated estradiol concentrations at different peaks **(III)**. This estradiol-regulated enhancement completely vanished when a specific DDPIV enzyme inhibitor (1 mM DFP) was added to the culture medium. This depletion indicated that the growth of *P. aurantiaca* biofilm depends on its DPPIV activity **(III)** (Figure 9b).

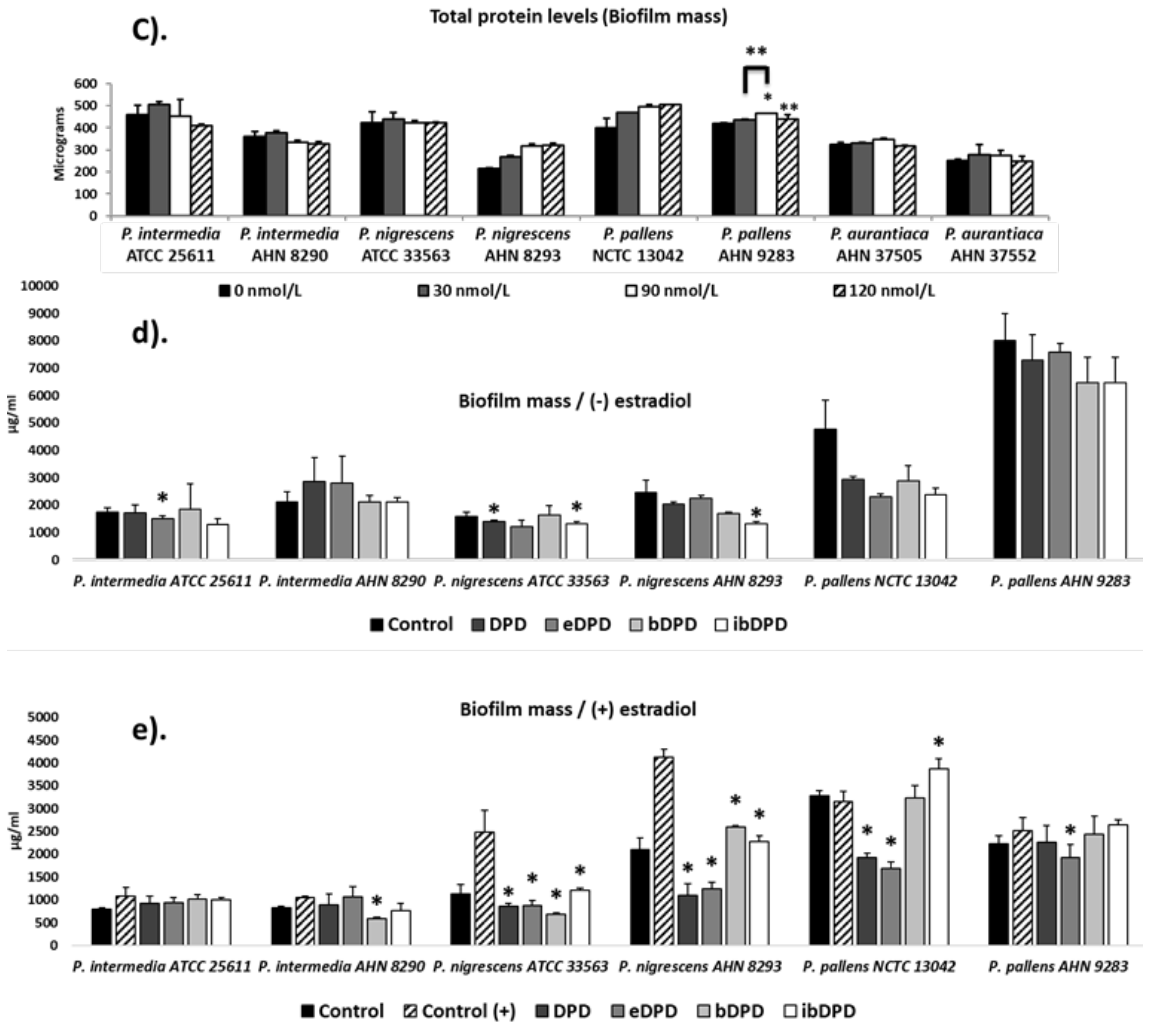


Figure 9. Biofilm mass of a) *P. intermedia*, *P. nigrescens*, and *P. pallens* strains. Data are presented in in µg and µg/ml. b) *P. aurantiaca* strains ±DPPIV inhibition. Data are presented in µg/ml. c) Static biofilms of eight *Prevotella* strains. d) and e) *P. intermedia*, *P. nigrescens*, and *P. pallens* strains in ± 90 nmol/L of estradiol and 10 µM od dihydroxy-2, 3-pentanedione (DPD, ethyl DPD, butyl DPD, and isobutyl DPD. In figures a, b, and c, the bacterial strains were incubated in different concentrations of estradiol (0, 30, 90, and 120 nmol/L), and asterisks indicate a significant difference with the control (**P*<0.05, ***P*<0.01). Underlined asterisks indicate significant difference of inter-group comparisons (Modified from original publication I, II, III, and IV).

As far as we know, the presented studies were the first to evaluate the modulatory effect of estradiol and DPPIV activity within the *P. intermedia* group bacteria. When the *Prevotella* biofilms were incubated with DPD and its analogs, a slight significant dose-dependent inhibitory effect was observed on biofilm formation and a potential

bacterial virulence inhibitor **(IV)** (Figure 9 d and e). Other *in vitro* studies have assessed the biofilm formation using other methods, such as the dynamic flow-type biofilm models, which would optimally simulate the biofilm growth in the oral cavity (Uppuluri and Lopez-Ribot, 2010; Alves et al., 2016). However, in the present thesis work, it was hard to compare the obtained results with their corresponding microbial processes occurring in the oral cavity due, in part, to the *in vitro* nature of the studies and the complexity of the oral microsystem. The latter is exemplified by the existence of the multispecies environment, the continuous flow of saliva, and the composition of the GCF which provides an important nutrient source to anaerobic bacteria. Therefore, the results of this thesis solely demonstrate the initial response characteristics of the *P. intermedia* group bacteria under the challenge of estradiol.

All experiments were performed on 48 h old biofilms. However, a 24 h biofilm formation was tested in our preliminary trials. Accurately, such a time point might not be sufficient for the tested bacteria to build up stable biofilms. Though, longer durations of incubation were not favored in order to avoid the risk of the growth media becoming lethally acidic to the tested biofilms. Besides, a 48 h time period to test biofilm formation is somehow related to the dental hygiene practice where missing proper oral hygiene measures for two days would obviously lead to biofilm formation and plaque accumulation especially in the interdental spaces. Clinically, a possible explanation for pregnant women being at higher risk to develop gingivitis is that the increase in plaque accumulation during the first trimester might be related to the fairly common pregnancy-associated nausea or morning sickness during the first weeks of pregnancy, which usually makes tooth brushing especially in premolar and molar areas difficult to perform since it may trigger gagging reflex and stimulate vomiting action (Silk et al., 2008; George et al., 2016).

5.1.4 Coaggregation capabilities (I and III)

Bacterial coaggregation is an integral mechanism by which genetically distinct bacterial species adhere and attach developing a sophisticated multispecies biofilm. In the present thesis work, estradiol had no clear impact on the coaggregation capabilities of most *P. intermedia* strains when tested with *F. nucleatum*. However, *P. intermedia* AHN 8290 showed a minor decrease in coaggregation with *F. nucleatum* at the estradiol concentrations of 30 and 90 nmol/L, while *P. aurantiaca* AHN 37505, estradiol significantly enhanced the coaggregation of the strain with *F. nucleatum* at all concentrations (30, 90, and 120 nmol/L).

In the literature, several studies have addressed the synergism between *F. nucleatum* and *Prevotella* species to form mature biofilms. For instance, plaque samples with dominating *F. nucleatum* were found to be favored by *P. intermedia* and *P. nigrescens*; they can exhibit a higher adherence activity compared with

samples not harboring *F. nucleatum* (Kobayashi et al., 2008). Nevertheless, the adhesins and other receptors involved in the coaggregation between *F. nucleatum* and *P. intermedia* or *P. nigrescens* were specific to these tested species, heat-stable, proteinase-K resistant, and can be abolished by addition of known amounts of EDTA or $_{L}$-lysine to the co-culture (Okuda et al., 2012). Interestingly, heat and proteinase K treated *P. intermedia* did not inhibit the gingipain-adhesin complex facilitating the coaggregation with *P. gingivalis*, while the reverse is true, since heat and proteinase K-treatment of *P. gingivalis* showed no coaggregation with *P. intermedia* (Kamaguch et al., 2001). Additionally, surface components such as proteins and glycoproteins on the surface of *P. intermedia* were significantly involved in the coaggregation of various Gram-positive bacteria including *Actinomyces* species through their interaction with the carbohydrates on the surface of *Actinomyces* (Nesbitt et al., 1993). Besides, QS molecules (AI-2) produced by *F. nucleatum*, *P. intermedia*, and *P. nigrescens* were relatively able to regulate gene expression involved in coaggregation. Yet, the physical interaction between species remains the key player in biofilm formation (Bakken et al., 1990; Frias et al., 2001; Okuda et al., 2012).

5.1.5 Polysaccharide production (I)

The production of exopolysaccharide in bacteria is seen as a significant virulence factor due to the rich environment the extracellular matrix provided to facilitate bacterial biofilm formation. When the expressed polysaccharides attach to an inert- or cell surfaces, the bacteria can construct a capsule for further defense.

In study I, *P. intermedia* ATCC 25611 and AHN 8290, *P. pallens* AHN 9283 and NCTC 13042, but not *P. nigrescens*, showed significantly elevated levels of polysaccharide production with increasing estradiol concentrations and at different peaks. Yamane and colleagues (2005) found that *P. intermedia* and *P. nigrescens* produce natural sugars of which 83% being mannose polysaccharide, which has a major role in the development of the chronic inflammatory lesions through the modification of human leukocyte phagocytosis (Yamane et al., 2005; Yamanaka et al., 2009). Later, the same research group showed that the capability of *P. intermedia* to form biofilm enhances its ability to produce polysaccharides, which serve as antigens resistant against host innate immunity and contribute to the capability of *P. intermedia* to evade the host defense barriers (Yamanaka et al., 2009; 2011).

5.2 Impact of estradiol on biofilm-related virulence of the *P. intermedia* group organisms

5.2.1 Dipeptidyl peptidase IV (DPPIV) enzyme activity (II and III)

Biofilm-forming bacteria are able to build surface-attached aggregations or microcolonies known as static biofilms, which technically simulate the early stages of adherence and biofilm formation (Merritt et al., 2005). In study II, the enzyme intensity of the static biofilms were significantly elevated among the tested *Prevotella* species in a strain-dependent manner. Most of the strains demonstrated their highest peak of enzyme activity at the estradiol concentration of 90 nmol/L. Whereas *P. nigrescens* and *P. pallens* had an earlier peak of increase at the estradiol concentration of 30 nmol/L. The relative enzyme activity is the enzyme intensity of the strain regardless of the mount of the biofilm formed. As a most interesting finding, the *P. aurantiaca* strains showed the highest enzyme activity and the lowest protein levels. The results may indicate a potential role for *P. aurantiaca* in the pathogenesis of periodontal diseases in general and in pregnancy-associated gingivitis specifically (Figure 10).

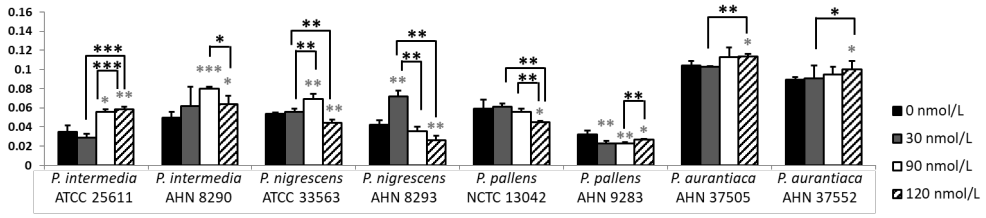


Figure 10. Relative enzyme activity expressed by the tested *Prevotella* strains in different concentrations of estradiol (0, 30, 90, and 120 nmol/L). The data represent a ratio of fluorescence excitation/emission to total biofilm mass value. (**P*<0.05, ***P*<0.01, ****P*<0.001).

In study III, the DPPIV enzyme activity was detected from fresh WCEs by both fluorometric assay and zymography. The quantification of the biofilm mass from which enzyme intensity was obtained, and the determination of the role of DPPIV in biofilm formation were performed by the Bradford protein assay (±DPPIV inhibitor, DFP). The *P. aurantiaca* strains tested with the spectrophotometer demonstrated an enhanced enzyme activity with time. The DPPIV inhibition by DFP had no cytotoxic effect on the planktonic growth of the strains except for *P. aurantiaca* AHN 37505 (Figure 11a). The zymography results confirmed the specificity of the DPPIV enzyme by clear bands of activity on a specific substrate gel. According to the

standard ladder, the bands corresponded to a molecular weight matches of the bacterial DPPIV (Figure 11b).

Several studies have demonstrated the characterization of the DPPIV enzyme from different *Prevotella* species by using different methods, e.g. ion-exchange chromatography, zymography, and SDS-PAGE bacterial cell extracts, to detect different molecular weights of the enzyme (Cookson et al., 1996; Gazi et al., 1997; Shibata et al., 2003). Gel-filtration chromatography is another method used in differentiating the host and bacterial DPPIV in GCF, produced by *Capnocytophaga* spp., *P. gingivalis*, *Prevotella* spp., and *T. denticola* (Gazi et al., 1995).

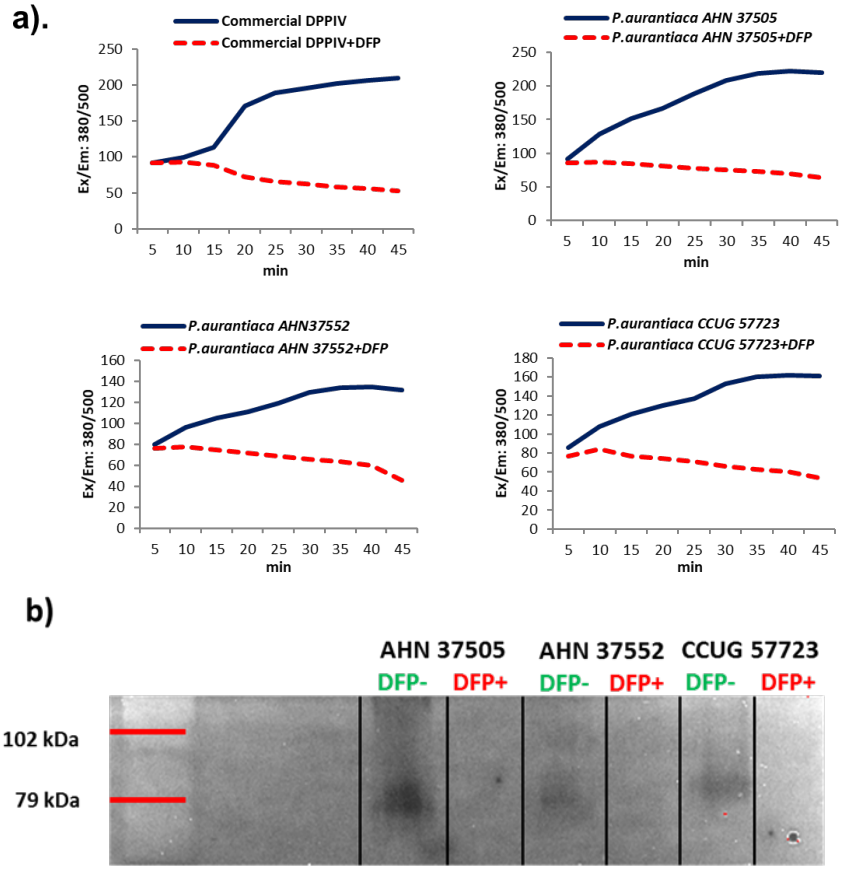


Figure 11. DPPIV enzyme activity with time (a) and zymography (b) of three *P. aurantiaca* strains ± DPPIV inhibition by DFP. For DPPIV intensity measurements, data are presented as fluorescence excitation/emission 380/500. (Modified from original publication III).

In the context of virulence, bacterial proteases expressed by periodontal pathogens exhibit common molecular mechanisms to enhance biofilm formation and

periodontal tissue breakdown. For instance, the production of the dipeptidyl aminopeptidase IV enzyme by *P. gingivalis* is an essential factor for the asaccharolytic growth of the species towards a mature biofilm (Kumagai et al., 2000; 2005).

5.2.2 Effect of QS molecules on DPPIV enzyme activity (III)

Cell-to-cell communication and bacterial behavior modulation are mediated by a variety of small molecules, known as QS molecules. To the best of our knowledge, no data exist in the literature on the role of QS in enzyme activity of oral *Prevotella* species. However, in the gut microbiota, where QS signaling masters the crosstalk between the commensals to maintain symbiosis, the bacterial DPPIV by intestinal *Prevotella* species was found to be modulated by the intestinal microbiome (Olivares et al., 2018). This novel mechanism allows an optimal protein digestion and proper host metabolic activity.

In study III, the QS molecules regulated the DPPIV activity of the three *P. aurantiaca* strains in dose- and strain-dependent manners. This study confirmed the regulatory role of estradiol on the DPPIV activity of *P. aurantiaca* at different concentrations of DPD and its analogs (100 nM and 1 μM) as described in Roy et al. (2010).

5.2.3 Effect of QS molecules on biofilm formation and coaggregation (III and IV)

In study III, QS molecules (except ethyl DPD) had a slight but significant inhibitory effect on biofilm and coaggregation of the three *P. aurantiaca* strains incubated with estradiol. In biofilm formation, elevated estradiol levels significantly enhanced biofilm formation. However, a total enhancement depletion was observed after adding the specific DPPIV inhibitor to the incubation media. Thus, biofilm formation of *P. aurantiaca* was considered to be DPPIV-dependent. Besides, the study demonstrated a novel finding regarding the disruption of native QS signaling by synthetic QS molecules, which may modify the biofilm-related virulence of *P. aurantiaca.* In study IV, DPD and its analogs in the presence of estradiol modulated the biofilm formation of the *P. intermedia*, *P. nigrescens*, and *P. pallens* in a strain-dependent manner.

The universal AI-2 are small molecules synthesized by QS bacteria and utilized by several other bacteria in the ecosystem (Bassler et al., 1997; Miller and Bassler, 2001). The synthetic analogs of AI-2 have the potential to regulate bacterial virulence competing with or inhibiting the native AI signaling pathways, and eventually act as anti-QS agents (Roy et al., 2010; Guo et al., 2012). Some studies have used the

permeation mechanisms and quenching techniques to disrupt the bacterial crosstalk by using QS analogs. For instance, by testing the permeability of the bacterial cell membrane, AI-1 and indole exhibited a high affinity to penetrate the cell membrane, while the hydrophilic AI-2 showed low affinity and, thus, it usually requires a carrier to transport the molecules through the cell membrane into the cytoplasm (Kamaraju et al., 2011). Also, DPD and isobutyl DPD have been tested for their potentials as quenchers in *E. coli* and *Salmonella typhimurium* (Guo et al., 2012).

5.2.4 Effect of QS molecules on *Prevotella* cell morphology (IV)

In the tested *P. aurantiaca* strains, clear changes in the number of intracellular inclusions, fimbriae thickness and density were observed by TEM imaging (Figure 12). When fimbriae thickness was calculated quantitatively, a significant peak of increase was observed in the strains incubated with DPD and its analogs at concentrations of 1 µM and 10 µM.

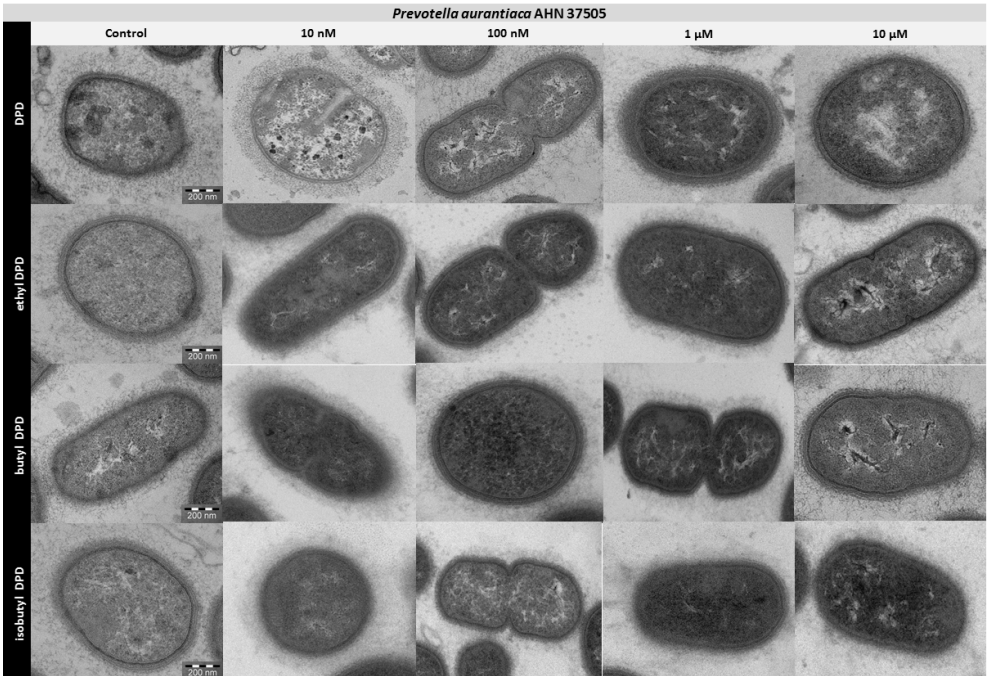
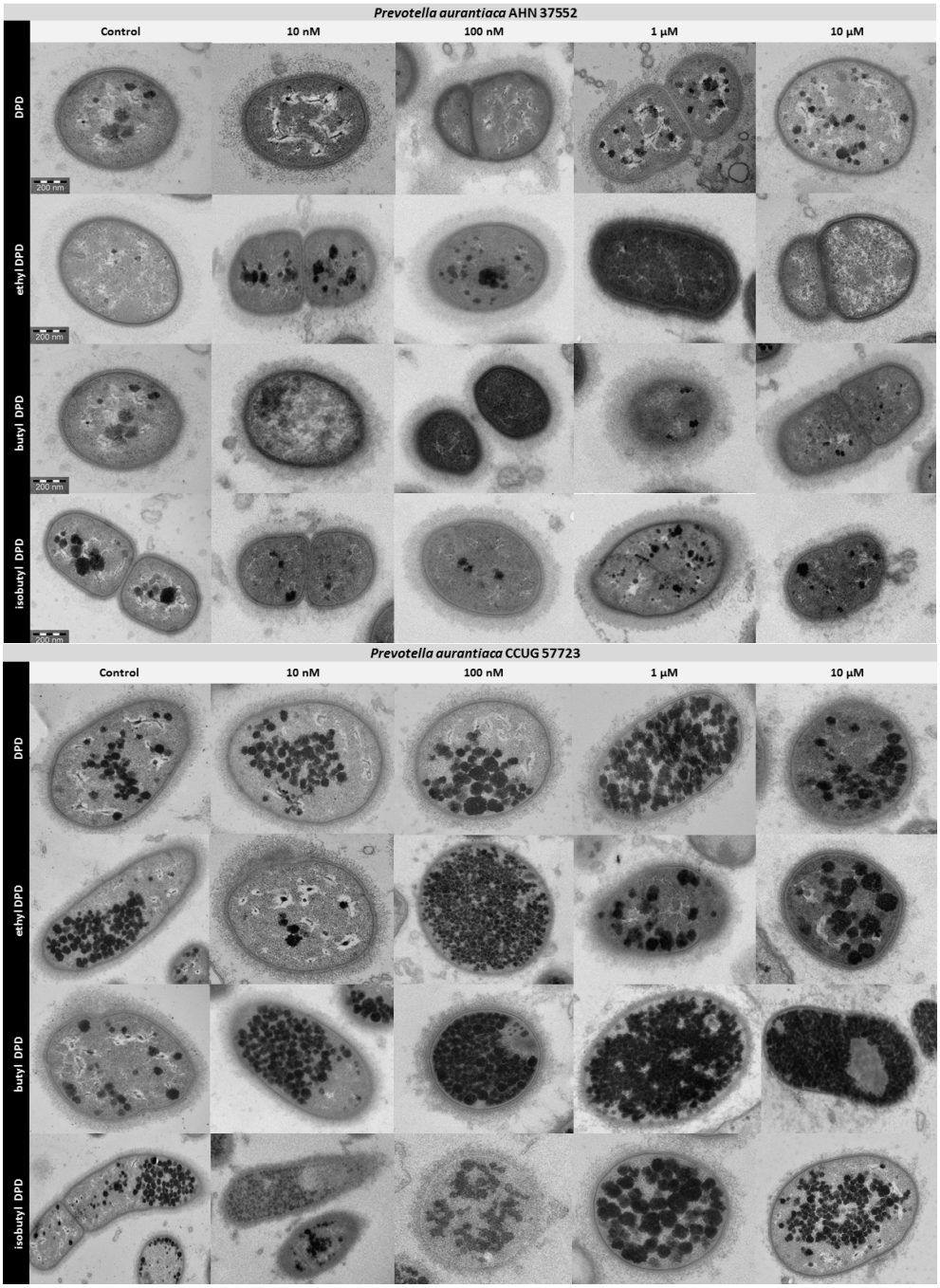


Figure 12. ▲►TEM micrographs demonstrate the changes in fimbriae thickness and intracellular inclusions between the control and different concentrations of DPD, ethyl DPD, butyl DPD, and isobutyl DPD (0, 10 nM, 100 nM, 1 µM, and 10 µM) of *P. aurantiaca* AHN 37505, *P. aurantiaca* AHN 37552, and *P. aurantiaca* CCUG 57723 (Scale bars: 200 nm) **(**From the supplementary data of the original publication IV).


Prevotella aurantiaca AHN 37552
Control
10 nM
100 nM
1 µM
10 µM
DPD
ethyl DPD
butyl DPD
isobutyl DPD
200 nm
Prevotella aurantiaca CCUG 57723
Control
10 nM
100 nM
1 µM
10 µM
DPD
ethyl DPD
butyl DPD
isobutyl DPD

In the literature, scarce data are available about bacterial morphological alterations under the effect of QS. However, the involvement of QS AI-2 and *LuxS* genes in inducing morphology variations of *Aeromonas salmonicida* has been evaluated by SEM (Meng et al., 2017). Among these variations, topographic changes on the cell surface with a noticeable reduction in cell dimensions were observed. The authors claimed that these morphological changes represent a defense mechanism by *A. salmonicida* against the host immune system attacks (Meng et al., 2017). Besides, SEM imaging has been successfully used to examine the effect of the photodynamic laser therapy on biofilm-related virulence factors and QS in *Pseudomonas aeruginosa* (Tan et al., 2018).

5.3 Impact of estradiol on cytokine expression

5.3.1 Effect of whole cell extract of *P. intermedia* group on cytokine expression of estradiol-treated gingival keratinocytes (IV)

The IL-6 and IL-8 expression of HMK cells was significantly increased when incubated with WCE of *P. intermedia* and *P. nigrescens*, and decreased when incubated with WCE of *P. pallens*. The incubation of HMK cells with estradiol and WCE of *P. intermedia* ATCC 25611 and AHN 8290 resulted in a suppressive effect on the expression of IL-1β, and the same inhibition of IL-6 and IL-8 of HMK cells incubated with WCE of *P. nigrescens* AHN 8293. While incubating the same cells with WCE of *P. pallens* AHN 8392 had an enhanced expression of IL-1β. The presented results are relatively in line with previous studies demonstrating a wider view of some *Prevotella* species as known regulators of the IL-1β and IL-23 cytokine expression through the TLR-2 pathway (de Aquino et al., 2014; Schincaglia et al., 2017), to enhance epithelial expression of IL-6, IL-8, and to stimulate the neutrophil migration towards the host inflammatory zones (Kim et al., 2007; Matsui et al., 2014).

5.3.2 Effect of QS molecules on cytokine expression of estradiol-treated gingival keratinocytes (IV)

While DPD had an enhancing effect on the expression of IL-6 and IL-8, butyl-DPD was a neutralizer for the IL-6 and IL-8 responses of the WCE-treated HMK cells. The novelty of the current study lies in being the first to present the cytokine neutralizing action by a DPD analog, the butyl-DPD. QS molecules regulate immune cells and play a key role in cytokine expression of oral fibroblasts (Scheres et al., 2015). For instance, when the ability of *LuxS*-mutant *P. gingivalis* to induce an

inflammatory response in human primary periodontal ligament fibroblasts was investigated, a massive impairment in gene-expression of IL-1β and IL-6 was detected compared with wild *P. gingivalis* (Scheres et al., 2015). Cytokines are soluble proteins with well-known specific roles in the immunopathogenesis of the periodontium and abundantly induced by specific periodontopathogenic components (Wilson et al., 1996; Okada and Murakami, 1998). The dual effect of the butyl-DPD as cytokine neutralizer and inhibitor of the estradiol-regulated biofilm formation allows this DPD analog to earn increased attention as a potential compound in the treatment choices of chronic oral infections, including hormone-modified inflammatory conditions, e.g. pregnancy-related gingivitis.

5.4 Untreated *P. intermedia* group as colonies and single cells

5.4.1 *P. intermedia* group under colony light microscope

The *P. intermedia* group organisms share the phenotypic characteristic of being pigment-producing, non-spore-forming, and non-motile rods or coccobacilli. When cultured on agar plates anaerobically incubated at 37 °C for 3-5 days, the colonies are usually 1-2 mm in diameter with color ranges between light to dark brown or black (the incubation period may affect the pigmentation intensity). Single colonies can reflect fluorescein-red boundaries when exposed to UV light. Colony shape is uniform circular with raised convex and shiny surfaces. Under TEM, cross section of the bacterial cells revealed thick outer and inner membranes with intercellular inclusions and fimbriae formation, which were more prominent in type and clinical strains of *P. pallens* and *P. aurantiaca* (Figure 13).

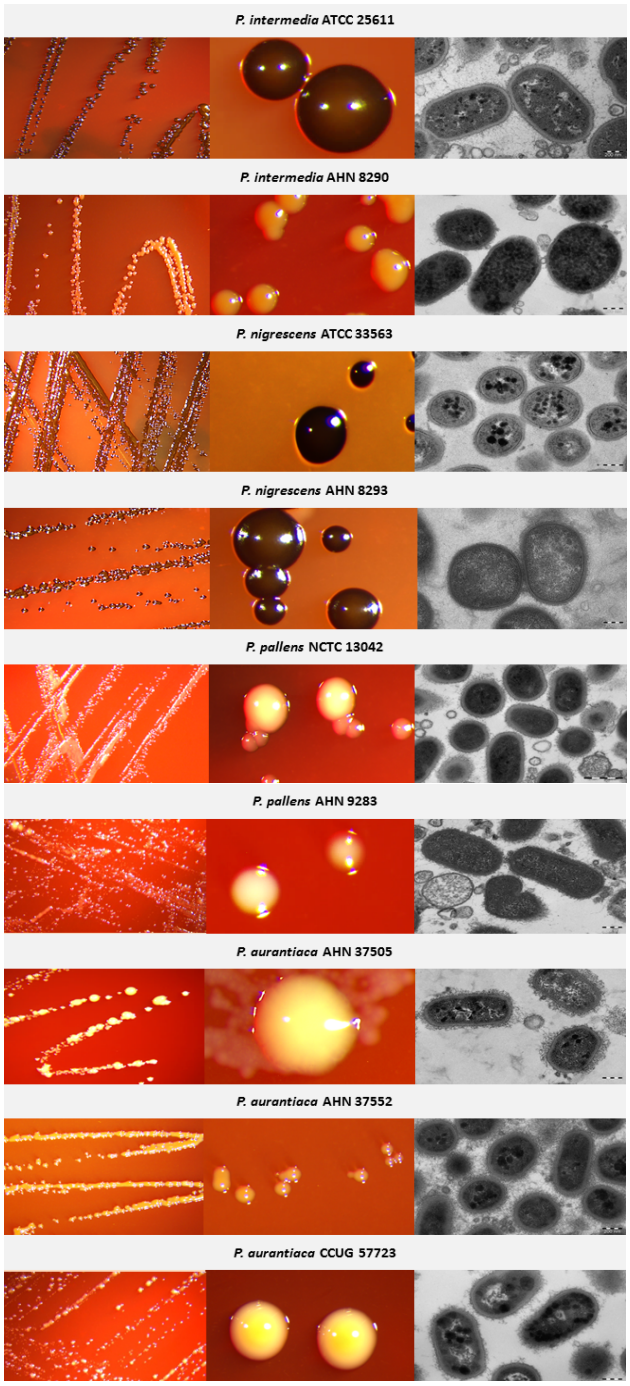


Figure 13. Human *P. intermedia* group species under colony light and transmission electron microscopes (Unpublished data).

6 Summary and Conclusions

6.1 General aspects

This study was focused on virulence mechanisms of a clinically important group of anaerobic Gram-negative bacteria, the *P. intermedia* group, and the epithelial cytokine response to this group of bacteria during pregnancy. Accordingly, the study hypotheses were addressed as: 1) estradiol has an impact on the virulence aspects and pathogenic response of the *P. intermedia* group bacteria, 2) there is an estradiol-regulated host response against the *P. intermedia* group, 3) synthetic QS molecules modulate the cytokine response of the human gingival keratinocytes against these *Prevotella* cell extracts, and 4) QS molecules inhibit their biofilm formation by competing with or inhibiting the native AI-2 signaling pathway of these species.

Based on the results of the study series, the conclusions are as follows:

1) There is a regulatory effect of estradiol on the growth properties of *P. intermedia* group organisms in terms of planktonic growth, coaggregation, polysaccharide production, and biofilm formation.

2) Elevated estradiol concentrations may enhance the relative and quantitative DPPIV proteolytic enzyme activity of *P. intermedia* group organisms.

3) The QS molecules are of a dose-dependent inhibitory effect on the DPPIV activity, coaggregation, biofilm formation and fimbria thickness of three *P. aurantiaca* strains.

4) The estradiol regulation of the proliferation and biofilm formation of *P. intermedia* group depends significantly on the DPPIV enzyme activity.

5) QS molecules have a neutralizing effect on the *Prevotella* WCE-induced cytokine expression of human gingival keratinocytes, and at the same time, they have an inhibitory effect on the estradiol-regulated biofilm formation of the *P. intermedia* group species tested.

6.2 Clinical significance

Pregnancy-related gingivitis is a common gingival inflammation that includes interactions between periodontal tissues, female sex hormones, subgingival anaerobes, and host immune response. Understanding the mechanism by which estradiol enhances the virulence of the *P. intermedia* group helps to estimate the involvement of this group of bacteria in pregnancy-associated gingivitis. It also provides preventive information, which assists in reducing the risk of developing periodontal disease.

The innovation of the current study lies in being the first to evaluate the effect of estradiol on the proteolytic enzyme activity of the *P. intermedia* group. Further, it presents new findings related to the novel species, *P. aurantiaca*, which demonstrated the highest DPPIV activity. This clinically significant finding allows *P. aurantiaca* to merit a distinct consideration related to the pathogenesis of pregnancy-associated gingivitis. The estradiol impact on the bacterial cell proliferation, cytokine expression by gingival keratinocytes, and DPPIV-dependent biofilm formation of the *P. intermedia* group organisms was partly inhibited by the disruption of QS signaling with C1-alkyl analogs of DPD.

Despite the limitation of the *in vitro* nature of the studies included, this PhD work present novel findings, which may significantly contribute to a better understanding of the *P. intermedia* group virulence aspects. From a therapeutic prospective, the project outcomes may aid to enriching the treatment options for pregnancy-related gingivitis through the multiple acting QS compounds, which demonstrated promising potential selectivity to target the pathogenicity of the tested *Prevotella* species (Figure 14).

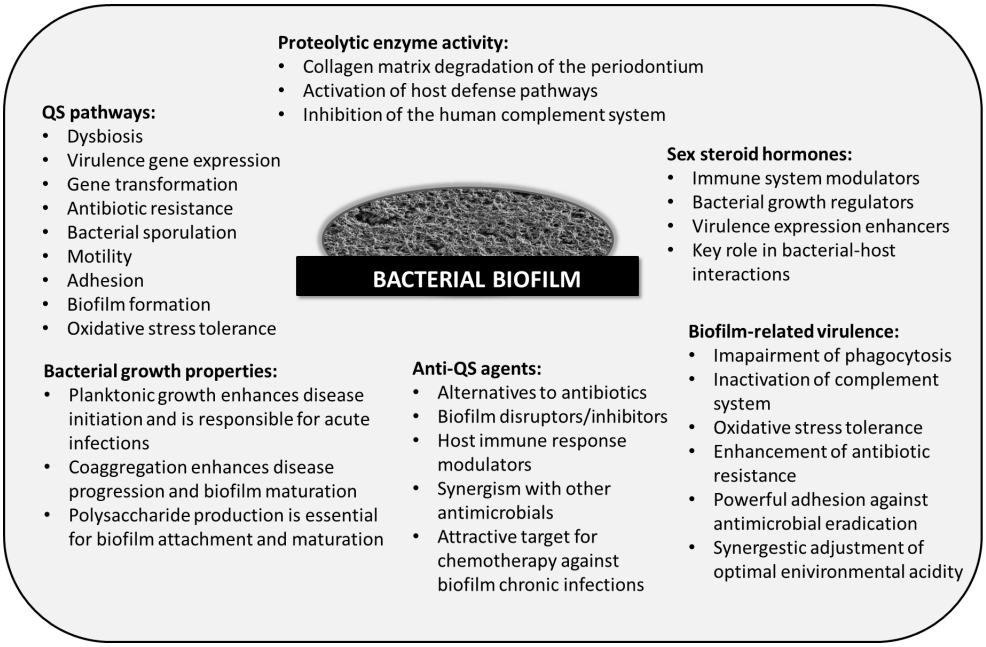


Figure 14. Summary of clinically significant roles of each tested effector on bacterial biofilms.

6.3 Strength and limitations

To the best of our knowledge, this PhD thesis is the first to investigate the impact of a major maternal hormone, estradiol, and known QS molecules on the growth behavior and virulence of the *P. intermedia* group organisms. Such an impact was demonstrated by examining the planktonic growth properties, coaggregation capabilities with *F. nucleatum*, polysaccharide production, and biofilm formation of the type and clinical strains of the following species: *P. intermedia*, *P. nigrescens*, *P. pallens*, and *P. aurantiaca*. As a result, their growth characteristics, biofilm formation, DPPIV enzyme activity, and the epithelial cytokine response to the estradiol and QS molecules varied massively and significantly. The different concentrations of estradiol tested were selected to simulate average clinical values of serum estradiol concentrations equivalent to the first, second, and third trimester of pregnancy (O'Leary et al., 1991). However, due to the *in-vitro* nature of the four studies included in the current project, an obvious limitation is that the results can be concluded solely within the laboratory conditions without the possibility to compare the outcomes with other similar clinical works.

In addition to elevated serum estradiol levels during pregnancy, several other female sex hormones, such as estriol or progesterone, increase. Nevertheless, in this doctoral thesis only estradiol was examined as an effector. Although not the predominantly circulating female sex hormone during pregnancy, estradiol remains

the predominant and most potent form of estrogen in fertile-age women in terms of serum levels and estrogenic potency. The reason why progesterone, one of the major circulating hormones in plasma during pregnancy, was not selected to be tested is that its elevated levels during pregnancy were not associated with changes in gingival inflammation and the amount of dental plaque at gingival margins.

In the context of DPPIV enzyme activity, pregnant women exhibit significantly higher DPPIV serum levels than their non-pregnant peers (Zhao et al., 2012). Another limitation is that to date, no reports on the correlation between female sex hormones and bacterial DPPIV exist in the literature. Thus, in the present work we had to hypothetically apply our enzymatic results to the pathogenesis of pregnancy-associated gingivitis.

Regarding the studies included QS molecules (III and IV), some of the analogs and concentrations tested demonstrated a significant neutralizing action on the *Prevotella* WCE-induced cytokine expression of human gingival keratinocytes. To simulate the availability of these QS compounds in natural environments, the concentrations of the QS molecules tested were taken from common thresholds of quorum occurring in established biofilms in nature and *in-vivo* (Roy et al., 2010).

6.4 Future prospective

Defining estradiol-regulated biofilm characteristics of the *P. intermedia* group organisms at a molecular level would provide a wider overview on the potential pathogenic aspects of these pregnancy-associated microorganisms. Quantitative imaging methods, e.g., the confocal microscopy or the time-lapse live imaging, and specific sugar-binding fluorescence markers could be valuable tools be used in future studies.

Under the effect of estradiol, the up- and down-regulation of the DPPIV proteolytic enzyme activity by putative genes of the *P. intermedia* group species are worth being examined at a genetic transcriptional level and through subcellular proteomic approaches.

Conservative pharmaceutical and preclinical approaches to testing synthetic QS compounds with anti-biofilm features, as well as, natural compounds with anti-QS properties in animal models during pregnancy are warranted to provide additional information about their therapeutic efficiency.

Acknowledgments

Above all, my prayers of thankfulness to Almighty God **"Allah"**, for the wisdom and constant belief He planted into my heart, for lightening up my road whenever I felt lost and hopeless, and for guiding me all the way and over every single event I passed through the whole of my life.

This study was conducted at the Department of Periodontology and the Microbiological laboratory of the Institute of Dentistry, University of Turku, Finland. The study was financially supported by a personal funding from the Finnish Doctoral Program in Oral Sciences (FINDOS-Turku), and a research funding from the Turku University Foundation, Selma and Maja-Lisa Selander's fund for research in Odontology (Minerva Foundation), and the Finnish Dental Society, Apollonia.

I wish to express my heartfelt thanks and warmest gratitude to my excellent supervisors, Professor ***Eija Könönen*** and Docent ***Ulvi Gürsoy***. Thank you ***Eija*** for keeping me on the very safe track of academic writing. Your magical touch in turning my text into a readable one, and your wise approach of teaching us with endless patience has massively inspired me to follow your way in my future academic career. Without your critical eye in revising my PhD manuscripts, side projects, and even this thesis book, I would not be now proud of the outcome of many years working and studying under your perfect supervision.

Ulvi, no words can express how much grateful and thankful I am towards your faithful practical advice, encouragement and support during the best and worst days of my PhD. Your constant belief in my abilities and skills has made me more motivated towards science and better estimating myself as a researcher. I am truly thankful to you for being always available along my thesis work step-by-step, and exactly in the right time.

I extend my sincere appreciation to Assoc. Professor ***Tülay Yucel-Lindberg*** and Professor ***Gilad Bachrach*** for thoroughly revising my PhD work and giving their invaluable and constructive criticism. Your comments and recommendations have noticeably improved and enriched my thesis book.

I wish to faithfully thank Docents: ***Mervi Gürsoy***, ***Eva Söderling***, ***Merja Laine***, and ***Vuokko Loimaranta***, the special laboratory technicians: ***Katja Sampalahti***, and ***Oona Hällfors***, as well as, ***Mariia***, ***Tatjana***, and ***Aija*** from the laboratories of the

Institute of Dentistry (***Dentalia***). Thank you all for your practical and technical assistance, your great patience introducing me into different laboratory techniques, and to your generous scientific and emotional contribution to this PhD project.

My true friends at the institute and outside the building: ***Liisa Lehto***, it is not enough to say a simple "thank you" for you or for what you have added to my life since we met each other around five years ago and how fast you became the closest to my heart and the youngest perfect "varaäiti" to my kids. So, I will just thank you for one thing, for being ***Liisa***, and not everyone can be. ***Roda***, thank you for your friendship, and for the great time we spent together in the lab and during coffee breaks, I wish you all the best in your postdoc journey. ***Samira***, when you came from my beloved Libya to join our group, you brought me "home" with you, you were a Godsend gift, to share with you my old memories, our common culture, and my thoughts and fears while feeling sure that you understand me, not only with your mind, but with your pure heart and true feelings as well.

Heba, ***Ikram***, ***Leila***, and ***Jasmina***: I am so happy and lucky to have such nice girls like you as my friends, who were always nourishing me with positive energy and lots of hope to continue diving towards success and stable career.

To ***Enas, Nazeeha, Nagat, Eman,*** and ***Fatima***, my Libyan "sisters" in Turku: our companionship from time to time despite our tight schedules, our warm calls and laughing loud chatting, the encouragement and support you provided were so much needed to motivate and inspire me with hope and faith towards working hard to achieve my goal and safely complete my PhD journey.

I also owe my honest appreciations to my so kind "brothers" in Turku: Drs. ***Ahmed***, ***Anas***, ***Tarek***, ***Khalil***, ***Thiago*** and ***Kaveh*** for the very respectful colleagueship I had with them and for their help and technical support whenever needed.

I extended my warm gratitude to my second family in Helsinki: my respectful brothers-in-low, Professor ***Mohammed*** and Dr. ***Hakim***, and their beloved families. My dearest sister-in-low ***Mabruka*** and her lovely family, and to the rest of family members in Libya, with a very special acknowledgment to my treasured father-in-low ***Salem***, from whom I learnt some wisdom and life lessons more than any knowledge book I would ever read. You have a very special place in my heart dad ***Salem*** and I am so proud that you consider me as one of your daughters. I ask God to offer you a very long happy life full of health and faith, "Amen".

To my family in Libya, my brothers ***Mohammed***, ***Jalal***, and ***Khalid***, their beloved wives and so lovely kids, I do miss you all and I am so glad that we succeeded to maintain such a warm relationship despite far distances and busy timetables of all of us.

To my beloved sister ***Bushra***, to the lightening candle in my dim nights, to the heart who gives without waiting for rewards, to the forever smiling face despite pain,

and to the only person who can read me like an open book. Thank you for being ALWAYS so close even though thousands of kilometers separate us apart. You will remain one of the most valuable gifts from our greatest **"Allah"**.

To ***mom***, who has raised me up with faithful efforts and scarifies to reach this stage, and who still gives us from her endless reserve of love and care, not only to me, but also to my own family. Thank you mom for perfectly replacing my place at home during my busiest time ever. Your heartfelt prayers and moral support value the world to me, *I love you mom*. May my Lord God bless you with health and happiness, "Amen". To my late ***dad***, although so sad that you couldn't attend my "big day", yet I am sure that your soul will be always around me, praying for me, and happy that the day you most wanted has come true. May Almighty **"Allah"** bless your soul with mercy and make His spacious paradise your forever destiny, "Amen".

The last but never the least, to the other half of my soul, my husband ***Ahmed***, thank you for every moment we shared together, for your wise advice and rich scientific discussions, for giving us your full attention and care although busy and tired of being working so hard, and for your endless and truthful emotional support you give with no price. I ask God to bless our life with peace, love and strength to raise up the most precious gift we gave each other: ***Salem***, ***Rahaf***, and ***Sujud***, the secret behind every happy day we live. To my beloved three diamonds I would say: your presence has made all the difference, thank you for everything.

Turku, 11.3.2020
Dareen Fteita

References

Abbott CA, Baker E, Sutherland GR, McCaughan GW (1994). Genomic organization, exact localization, and tissue expression of the human CD26 (dipeptidyl peptidase IV) gene. *Immunogenetics* 40:331-338.

Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* 7:1016-1025.

Aemaimanan P, Sattayasai N, Wara-aswapati N et al. (2009). Alanine aminopeptidase and dipeptidyl peptidase IV in saliva of chronic periodontitis patients. *J Periodontol* 80:1809-1814.

Alcoforado GA, McKay TL, Slots J (1987). Rapid method for detection of lactose fermenting oral microorganisms *Oral Microbiol Immunol* 2:35-38

Alves DR, Perez-Esteban P, Kot W, Bean JE, Arnot T, Hansen LH et al. (2016). A novel bacteriophage cocktail reduces and disperses *Pseudomonas aeruginosa* biofilms under static and flow conditions. Microb Biotechnol 9:61-74.

Amar S, Chung KM (1994). Influence of hormonal variation on the periodontium in women. *Periodontol 2000* 6:79-87.

Ammann TW, Belibasakis GN, Thurnheer T (2013). Impact of early colonizers on in vitro subgingival biofilm formation. *PLoS One* 8:83090.

Andrés MT, Chung WO, Roberts MC, Fierro JF (1998). Antimicrobial susceptibilities of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* spp. isolated in Spain. *Antimicrob Agents Chemother* 42:3022-3033.

Armitage GC (1999). Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 4:1-6.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR et al. (2011). Enterotypes of the human gut microbiome. Nature 473:174-180.

Ashimoto A, Chen C, Bakker I, Slots J (1996). Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 11:266-273.

Augustyns K, Bal G, Thonus G, Belyaev A, Zhang XM, Bollaert W et al. (1999).The unique properties of dipeptidyl-peptidase IV (DPP IV / CD26) and the therapeutic potential of DPP IV inhibitors. *Curr Med Chem* 6:311-327.

Avilés-Jiménez F, Vázquez-Jiménez F, Medrano-Guzmán R, Mantilla A, Torres J (2014). Stomach microbiota composition varies between patients with nonatrophic gastritis and patients with intestinal type of gastric cancer. *Sci Rep* 4:4202.

Bae KS, Baumgartner JC, Shearer TR, David LL (1997). Occurrence of *Prevotella nigrescens* and *Prevotella intermedia* in infections of endodontic origin. *J Endod* 23:620-623.

Baker JL, Bor B, Agnello M, Shi W, He X (2017). Ecology of the oral microbiome: beyond bacteria. *Trends Microbiol* 25:362-374.

Bakken V, Högh BT, Jensen HB (1990). Growth conditions and outer membrane proteins of *Fusobacterium nucleatum*. *Scand J Dent Res* 98:215-224.

Banbula A, Mak P, Bugno M et al. (1999). Prolyl tripeptidyl peptidase from *Porphyromonas gingivalis*. A novel enzyme with possible pathological implications for the development of periodontitis. *J Biol Chem* 274:9246-9252.

Banbula A, Bugno M, Goldstein J, Yen J, Nelson D, Travis J et al. (2000). Emerging family of proline-specific peptidases of *Porphyromonas gingivalis*: purification and characterization of serine dipeptidyl peptidase, a structural and functional homologue of mammalian prolyl dipeptidyl peptidase IV. *Infect Immun* 68:1176-1182.

Bassler BL, Greenberg EP, Stevens AM (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* 179:4043-4045.

Bernal LA, Guillot E, Paquet C, Mouton C (1998). beta-Lactamase-producing strains in the species *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Microbiol Immunol* 13:36-40.

Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F et al. (2006). Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci U S A*103:732-737.

Bimstein E (1991). Periodontal health and disease in children and adolescents. *Pediatr Clin North Am* 38:1183-1207.

Boonacker E, Elferink S, Bardai A, Fleischer B, Van Noorden CJ (2003). Fluorogenic substrate [Ala-Pro]2-cresyl violet but not Ala-Pro-rhodamine 110 is cleaved specifically by DPPIV activity: a study in living Jurkat cells and CD26/DPPIV-transfected Jurkat cells. *J Histochem Cytochem* 51:959-968.

Botta GA, Arzese A, Minisini R, Trani G (1994). Role of structural and extracellular virulence factors in gram-negative anaerobic bacteria. *Clin Infect Dis* 4:260-264.

Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH (2005). Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. *FEMS Immunol Med Microbiol* 45:191-199.

Bowen WH, Burne RA, Wu H, Koo H (2018). Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol* 26:229-242.

Bradshaw DJ, Marsh PD, Allison C, Schilling KM (1996). Effect of oxygen, inoculum composition and flow rate on development of mixed culture oral biofilms. *Microbiology* 142:623-629.

Bradshaw DJ, Marsh PD, Watson GK, Allison C (1997). Oral anaerobes cannot survive oxygen stress without interacting with facultative/aerobic species as a microbial community. *Lett Appl Microbiol* 25:385-387.

Brazier JS, Smith (1989). Evaluation of the Anoxomat: a new technique for anaerobic and microaerophilic clinical bacteriology. *J Clin Pathol* 42:640-644.

Brook I, Frazier EH (2003). Immune response to *Fusobacterium nucleatum* and *Prevotella intermedia* in the sputum of patients with acute exacerbation of chronic bronchitis. *Chest* 124:832-833.

Buijs J, Dofferhoff AS, Mouton JW, Wagenvoort JH, van der Meer JW (2008). Concentration-dependency of beta-lactam-induced filament formation in Gram-negative bacteria. *Clin Microbiol Infect* 14:344-349.

Byrne DP, Wawrzonek K, Jaworska A, Birss AJ, Potempa J, Smalley JW (2009). Role of the cysteine protease interpain A of *Prevotella intermedia* in breakdown and release of haem from haemoglobin. *Biochem J* 425:257-264.

Byrne DP, Manandhar SP, Potempa J, Smalley JW (2015). Breakdown of albumin and haemalbumin by the cysteine protease interpain A, an albuminase of *Prevotella intermedia*. *BMC Microbiol* 24:185.

Carrillo-de-Albornoz A, Figuero E, Herrera D, Bascones-Martínez A (2010). Gingival changes during pregnancy: II. Influence of hormonal variations on the subgingival biofilm. *J Clin Periodontol* 37:230-40.

Carrillo-de-Albornoz A, Figuero E, Herrera D, Cuesta P, Bascones-Martínez A (2012). Gingival changes during pregnancy: III. Impact of clinical, microbiological, immunological and sociodemographic factors on gingival inflammation. *J Clin Periodontol* 39:272-283.

Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J et al. (2012). *Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma. *Genome Res* 22:299-306.

Cao H, Qi Z, Jiang H, Zhao J, Liu Z, Tang Z (2012). Detection of *Porphyromonas endodontalis*, *Porphyromonas gingivalis* and *Prevotella intermedia* in primary endodontic infections in a Chinese population. *Int Endod J* 45:773-781.

Chaikin BS (1977). Incidence of gingivitis in pregnancy. *Quintessence Int Dent Dig* 8:81-89.

Chapple ILC, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P et al. (2018). Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol* 1:74-84.

Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A et al. (2011). Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 184:957-963.

Chen J, Miao X, Xu M, He J, Xie Y, Wu X, et al. (2015). Intra-Genomic Heterogeneity in 16S rRNA Genes in Strictly Anaerobic Clinical Isolates from Periodontal Abscesses. *PLoS One* 10: e0130265.

Chhour KL, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N (2005). Molecular analysis of microbial diversity in advanced caries. *J Clin Microbiol* 43:843-849.

Christersson LA, Zambon JJ, Genco RJ (1991). Dental bacterial plaques. Nature and role in periodontal disease. *J Clin Periodontol* 18:441-446.

Chow JS, Lee M, Shen Y, Khosravi A, Mazmanian SK (2010). Host–Bacterial Symbiosis in Health and Disease. *Adv Immunol* 107: 243-274.

Clark DA, Arck PC, Chaouat G (1999). Why did your mother reject you? immunogenetic determinants of the environmental selective pressure expressed at the uterine level. *Am J Reprod Immunol* 41:5-2210097783.

Cohen DW, Friedman L, Shapiro J, Kyle GC (1969). A longitudinal investigation of the periodontal changes during pregnancy. *J Periodontol* 40:563-570.

Cohen DW, Shapiro J, Friedman L, Kyle GC, Franklin S (1971). A longitudinal investigation of the periodontal changes during pregnancy and fifteen months post-partum. II. *J Periodontol* 42:653-657.

Conrads G, Flemmig TF, Seyfarth I, Lampert F, Lütticken R (1999). Simultaneous detection of *Bacteroides forsythus* and *Prevotella intermedia* by 16S rRNA gene-directed multiplex PCR. *J Clin Microbiol* 37:1621-1624.

Conrads G, Nagy E, Könönen E, Gram-Negative Rods, in: Carroll KC, Pfaller MA, Landry ML, McAdam AJ, Patel R, Richter SS, Warnock DW (Edts.), Manual of Clinical Microbiology, twelfth Ed., ASM Press, Washington DC, 2019, pp. 995-1023.

Cookson AL, Wray A, Handley PS, Jacob AE (1996). An investigation into the use of SDS-PAGE of cell surface extracts and proteolytic activity to differentiate *Prevotella nigrescens* and *Prevotella intermedia*. *FEMS Microbiol Lett* 136:109-115.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318-1322.

Dahlén G, Wikström M, Renvert S, Gmür R, Guggenheim B (1990). Biochemical and serological characterization of *Bacteroides intermedius* strains isolated from the deep periodontal pocket. *J Clin Microbiol* 28:2269-2274.

Dahlén G (1993a). Black-pigmented gram-negative anaerobes in periodontitis. *FEMS Immunol Med Microbiol* 6:181-192.

Dahlén G (1993b). Role of suspected periodontopathogens in microbiological monitoring of periodontitis. *Adv Dent Res* 7:163-174.

Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A et al. (2014). Bacterial vaginosis diagnosed by analysis of first-void-urine specimens. *J Clin Microbiol* 52:218-225.

de Aquino SG, Abdollahi-Roodsaz S, Koenders MI, van de Loo FA, Pruijn GJ, Marijnissen RJ et al. (2014). Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response. *J Immunol* 192:4103-4111.

Deacon CF (2019). Physiology and Pharmacology of DPP-4 in Glucose Homeostasis and the Treatment of Type 2 Diabetes. *Front Endocrinol* 15:10:80.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH et al. (2010). The human oral microbiome. *J Bacteriol* 192:5002-5017.

Diaz PI, Hoare A, Hong BY (2016). Subgingival Microbiome Shifts and Community Dynamics in Periodontal Diseases. *J Calif Dent Assoc* 44:421-435.

Ding F, Lyu Y, Han X, Zhang H, Liu D, Hei W et al. (2014). Detection of periodontal pathogens in the patients with aortic aneurysm. *Chin Med J* (Engl) 127:4114-4118.

Wusdag S, Nelson S, Coleman NV (2016). Bacterial communities associated with apical periodontitis and dental implant failure. *Microb Ecol Health Dis* 8:31307.

Domenech M, Ramos-Sevillano E, García E, Moscoso M, Yuste J (2013). Biofilm formation avoids complement immunity and phagocytosis of *Streptococcus pneumoniae*. *Infect Immun* 81:2606-2615.

Dorn BR, Leung KL, Progulske-Fox A (1998). Invasion of human oral epithelial cells by *Prevotella intermedia*. *Infect Immun* 66:6054-6057.

Doung-udomdacha S1, Rawlinson A, Douglas CW (2000). A novel closed-tube quantitative--PCR method for enumerating *Porphyromonas gingivitis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*. *J Periodontal Res* 35:247-258.

Drawz SM, Bonomo RA (2010). Three decades of beta-lactamase inhibitors. *Clin Microbiol Rev* 23:160-201.

Dzink JL, Socransky SS, Haffajee AD (1988). The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15:316-323.

Elgün S, Ozmeriç N, & Demirtaş S (2000). Alanine aminopeptidase and dipeptidylpeptidase IV in saliva: the possible role in periodontal disease. *Clin Chim Acta 2000* 298:187-191.

Estama S, Gürsoy M, Könönen E (2015). Identification of oral *Prevotella* spp. from periodontitis-free mothers. *J Dent Res* 94: Spec Iss A:4218.

Fernández-Canigia L, Cejas D, Gutkind G, Radice M (2015). Detection and genetic characterization of β-lactamases in *Prevotella intermedia* and *Prevotella nigrescens* isolated from oral cavity infections and peritonsillar abscesses. *Anaerobe* 33:8-13.

Figuero E, Carrillo-de-Albornoz A, Herrera D, Bascones-Martínez A (2010). Gingival changes during pregnancy: I. Influence of hormonal variations on clinical and immunological parameters. *J Clin Periodontol* 37:220-229.

Fosse T, Madinier I, Hitzig C, Charbit Y (1999). Prevalence of beta-lactamase-producing strains among 149 anaerobic gram-negative rods isolated from periodontal pockets. *Oral Microbiol Immunol* 14:352-357.

Fosse T, Madinier I, Hannoun L, Giraud-Morin C, Hitzig C, Charbit Yet al. (2002). High prevalence of cfxA beta-lactamase in aminopenicillin-resistant *Prevotella* strains isolated from periodontal pockets. *Oral Microbiol Immunol* 17:85-88.

Frandsen EV, Poulsen K, Kilian M (1995). Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. *Int J Syst Bacteriol* 45:429-435.

Frias J, Olle E, Alsina M (2001). Periodontal pathogens produce quorum sensing signal molecules. *Infect Immun* 69:3431-3434.

García L, Tercero JC, Legido B, Ramos JA, Alemany J, Sanz M (1998). Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromona gingivalis* by multiplex PCR. *J Periodontal Res* 33:59-64.

Gazi MI, Cox SW, Clark DT, Eley BM (1995). Comparison of host tissue and bacterial dipeptidyl peptidases in human gingival crevicular fluid by analytical isoelectric focusing. *Arch Oral Biol* 40:731-736.

Gazi MI, Cox SW, Clark DT, & Eley BM (1997). Characterization of protease activities in Capnocytophaga spp., *Porphyromonas gingivalis*, *Prevotella* spp., *Treponema denticola* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol* 12:240-248.

George A, Dahlén HG, Blinkhorn A, Ajwani S, Bhole S, Ellis S et al. (2016). Measuring oral health during pregnancy: sensitivity and specificity of a maternal oral screening (MOS) tool. *BMC Pregnancy Childbirth* 16:347.

George M, Pierce G, Gabriel M, Morris C, Ahearn D (2005). Effects of quorum sensing molecules of *Pseudomonas aeruginosa* on organism growth, elastase B production, and primary adhesion to hydrogel contact lenses. *Eye Contact Lens* 31:54-61.

Gharbia SE, Haapasalo M, Shah HN, Kotiranta A, Lounatmaa K, Pearce MA (1994). Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *J Periodontol* 65:56-61.

Gharbia SE, Williams JC, Andrews DM, Shah HN (1995). Genomic clusters and codon usage in relation to gene expression in oral Gram-negative anaerobes. *Anaerobe* 1:239-262.

Giannobile WV (2008). Host-response therapeutics for periodontal diseases. *J Periodontol* 79:1592-600.

Gölz L, Buerfent BC, Hofmann A, Hübner MP, Rühl H, Fricker N et al. (2016). Genome-wide transcriptome induced by *Porphyromonas gingivalis* LPS supports the notion of host-derived periodontal destruction and its association with systemic diseases. *Innate Immun* 22:72-84.

Grant MM, Kolamunne RT, Lock FE, Matthews JB, Chapple IL, Griffiths HR (2010). Oxygen tension modulates the cytokine response of oral epithelium to periodontal bacteria. *J Clin Periodontol* 37(:1039-1048.

Graves D (2008). Cytokines that promote periodontal tissue destruction. *J Periodontol* 79:1585-1591.

Guillot E, Mouton C (1997). PCR-DNA probe assays for identification and detection of *Prevotella intermedia* sensu stricto and *Prevotella nigrescens*. *J Clin Microbiol* 35:1876-1882.

Guo M, Gamby S, Nakayama S, Smith J, Sintim HO (2012). A pro-drug approach for selective modulation of AI-2-mediated bacterial cell-to-cell communication. *Sensors (Basel)* 3:3762-7672.

Guo M, Fang Z, Sun L, Sun D, Wang Y, Li C et al. (2018). Regulation of Thermostable Direct Hemolysin and Biofilm Formation of *Vibrio parahaemolyticus* by Quorum-Sensing Genes luxM and luxS. *Curr Microbiol* 75:1190-1197.

Güncü GN, Tözüm TF, Cağlayan F (2005). Effects of endogenous sex hormones on the periodontium-review of literature. *Aust Dent J* 50:138-145.

Gürsoy M, Pajukanta R, Sorsa T, Könönen E (2008). Clinical changes in periodontium during pregnancy and post-partum. *J Clin Periodontol* 35:576-583.

Gürsoy M, Haraldsson G, Hyvönen M, Sorsa T, Pajukanta R, Könönen E (2009). Does the frequency of *Prevotella intermedia* increase during pregnancy?. *Oral Microbiol Immunol* 24:299-303.

Gürsoy M, Gürsoy UK, Sorsa T, Pajukanta R, Könönen E (2013). High salivary estrogen and risk of developing pregnancy gingivitis. *J Periodontol* 84:1281-1289.

Gürsoy M, Harju I, Matomäki J, Bryk A, Könönen E (2017). Performance of MALDI-TOF MS for identification of oral *Prevotella* species. *Anaerobe* 47:89-93.

Haapasalo M, Ranta H, Ranta K, Shah H (1986). Black-pigmented *Bacteroides* spp. in human apical periodontitis. *Infect Immun* 53:149-153.

Haapasalo M (1989). *Bacteroides* spp. in dental root canal infections. *Endod Dent Traumatol* 5:1-10.

Hajishengallis G, Darveau RP, Curtis MA (2012). The keystone-pathogen hypothesis. *Nat Rev Microbiol* 10:717-725.

Haffajee AD, Socransky SS, Smith C, Dibart S (1992). The use of DNA probes to examine the distribution of subgingival species in subjects with different levels of periodontal destruction. *J Clin Periodontol* 19:84-91.

Haffajee AD, Uzel NG, Arguello EI, Torresyap G, Guerrero DM, Socransky SS (2004). Clinical and microbiological changes associated with the use of combined antimicrobial therapies to treat "refractory" periodontitis. *J Clin Periodontol* 31:869-877.

Hammond JB, Kruger NJ (1988). The bradford method for protein quantitation . *Methods Mol Biol* 3:25-32.

Hanioka T, Matsuse R, Shigemoto Y, Ojima M, Shizukuishi S (2005). Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid. *J Periodontal Res* 40:331-338.

Haraldsson G, Meurman JH, Könönen E, Holbrook WP (2005). Properties of hemagglutination by *Prevotella melaninogenica*. *Anaerobe* 11:285-289.

Harper-Owen R, Dymock D, Booth V, Weightman AJ, Wade WG (1999). Detection of unculturable bacteria in periodontal health and disease by PCR. *J Clin Microbiol* 37:1469-1473.

Harvey JD (2017). Periodontal Microbiology. *Dent Clin North Am* 61:253-269.

Hashimoto M, Asai Y, Tamai R, Jinno T, Umatani K, Ogawa T (2003). Chemical structure and immunobiological activity of lipid A from *Prevotella intermedia* ATCC 25611 lipopolysaccharide. *FEBS Lett* 543:98-102.

Hasson E (1960). Pregnancy gingivitis. *Harefuah* 58:224-226.

Herrera E (2000). Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus. *Eur J Clin Nutr* 1:47-51.

Hieke C, Kriebel K, Engelmann R, Müller-Hilke B, Lang H, Kreikemeyer B (2016). Human dental stem cells suppress PMN activity after infection with the periodontopathogens *Prevotella intermedia* and *Tannerella forsythia. Sci Rep* 6: 39096.

Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C et al. (2010). Disordered microbial communities in asthmatic airways. *PLoS One* 5:e8578.

Holbrook WP, Duerden BI (1974). A comparison of some characteristics of reference strains of *Bacteroides oralis* with *Bacteroides melaninogenicus*. *Arch Oral Biol* 19:1231-1235.

Holdeman LV, Moore WEC (1970). *Bacteroides*. In: Cato EP, Cummins CS, Holdeman LV, Johnson JL, Moore WEC, Smibert RM, Smith LDS, eds. Outline of clinical methods in anaerobic bacteriology. 2nd ed. Blacksburg, VA: Virginia Polytechnic Institute and State University 34-44.

Holdeman LV, Johnson JL (1982). Description of *Bacteroides loesscheii* sp. nov. and emendation of the description of *Bacteroides melaningenicus* (Oliver and Wherry) Roy and Kelly 1939 and *Bacteroides denticola* Shas and Collins 1981. *Int J Syst Bacteriol* 32:399-409.

Huang K, Zhang XX, Shi P, Wu B, Ren H (2014). A comprehensive insight into bacterial virulence in drinking water using 454 pyrosequencing and Illumina high-throughput sequencing. *Ecotoxicol Environ Saf* 109:15-21.

Hugoson A (1971). Gingivitis in pregnant women. A longitudinal clinical study. *Odontol Revy* 22:65e84.

Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT et al. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486:207-214.

Hyvärinen K, Laitinen S, Paju S, Hakala A, Suominen-Taipale L, Skurnik M et al. (2009). Detection and quantification of five major periodontal pathogens by single copy gene-based real-time PCR. *Innate Immun* 15:195-204.

Jakubovics NS, Kolenbrander PE (2010). The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis* 16:729-739.

Jang YJ, Choi YJ, Lee SH, Jun HK, Choi BK (2013). Autoinducer 2 of *Fusobacterium nucleatum* as a target molecule to inhibit biofilm formation of periodontopathogens. *Arch Oral Biol* 58:17-27.

Jensen J, Liljemark W, Bloomquist C (1981). The effect of female sex hormones on subgingival plaque. *J Periodontol* 52:599-602.

Jensen A, Fagö-Olsen H, Sørensen CH, Kilian M (2013). Molecular mapping to species level of the tonsillar crypt microbiota associated with health and recurrent tonsillitis. *PLoS One* 8:e56418.

Jentsch HF, März D, Krüger M (2013). The effects of stress hormones on growth of selected periodontitis related bacteria. *Anaerobe* 24:49-54.

Jitprasertwong P, Charadram N, Kumphune S, Pongcharoen S, Sirisinha S (2016). Female sex hormones modulate *Porphyromonas gingivalis* lipopolysaccharide-induced Toll-like receptor signaling in primary human monocytes. *J Periodontal Res* 51:395-406.

Johnson JL, Holdeman LV (1983). *Bacteroides intermedius* comb. nov. and descriptions of *Bacteroides corporis* sp. nov. and *Bacteroides levii* sp. nov. *Int J Syst Bacteriol* 33:15-25.

Joss A, Adler R, Lang NP (1994). Bleeding on probing. A parameter for monitoring periodontal conditions in clinical practice. *J Clin Periodontol* 21:402-408.

Jousimies-Somer H, Summanen P, Citron DM, Baron EJ, Wexler H, Finegold SM (2002). Wadsworth-KTL Anaerobic bacteriology manual, 6th edition, Belmot, CA: Star Publishing Company.

Jousimies-Somer H, Summanen P (2002). Recent taxonomic changes and terminology update of clinically significant anaerobic gram-negative bacteria (excluding spirochetes). *Clin Infect Dis* 35:17-21.

Kaaja RJ, Greer IA (2005). Manifestations of chronic disease during pregnancy. *JAMA* 294:2751-2757.

Kamaguch A, Nakayama K, Ohyama T, Watanabe T, Okamoto M, Baba H (2001). Coaggregation of *Porphyromonas gingivalis* and *Prevotella intermedia*. *Microbiol Immunol* 45:649-656.

Kamaraju K, Smith J, Wang J, Roy V, Sintim HO, Bentley WE, et al. (2011). Effects on membrane lateral pressure suggest permeation mechanisms for bacterial quorum signaling molecules. *Biochemistry* 32:6983-6993.

Kamma JJ, Nakou M, Gmur R, Baehni PC (2004). Microbiological profile of early onset/aggressive periodontitis patients. *Oral Microbiol Immunol* 19:314-321.

Karthik SJ, Arun KV, Sudarsan S, Talwar A, James JR (2009). Evaluation of estrogen receptor and circulating estradiol levels in pre- and postmenopausal women with periodontal disease. *J Int Acad Periodontol* 11:202-205.

Keijser BJ, Zaura E, Huse SM, van der Vossen JM, Schuren FH, *et al*. (2008). Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res* 87:1016-1020.

Keep NH, Ward JM, Robertson G, Cohen-Gonsaud M, Henderson B (2006). Bacterial resuscitation factors: revival of viable but non-culturable bacteria. *Cell Mol Life Sci* 63:2555-2559.

Kim SJ, Choi EY, Kim EG, Shin SH, Lee JY, Choi JI, Choi IS (2007). *Prevotella intermedia* lipopolysaccharide stimulates release of tumor necrosis factor-alpha through mitogen-activated protein kinase signaling pathways in monocyte-derived macrophages. *FEMS Immunol Med Microbiol* 51:407-413.

Kim MJ, Hwang KH, Lee YS, Park JY, Kook JK (2011a). Development of *Prevotella intermedia*-specific PCR primers based on the nucleotide sequences of a DNA probe Pig27. *J Microbiol Methods* 84:394-397.

Kim MJ, Lee YS, Park JY, Kook JK (2011b). Development of *Prevotella nigrescens*-specific PCR primers based on the nucleotide sequence of a Pn23 DNA probe. *Anaerobe* 17:32-35.

Koreeda Y, Hayakawa M, Ikemi T, Abiko Y (2001). Isolation and characterisation of dipeptidyl peptidase IV from *Prevotella loescheii* ATCC 15930. *Arch Oral Biol* 46:759-766.

Kolenbrander PE, Andersen RN, Moore LV (1990). Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. *Appl Environ Microbiol* 56:3890-3894.

Kolenbrander PE, London J (1993). Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 175:3247-3252.

Kolenbrander PE (1995). Coaggregations among oral bacteria. *Methods Enzymol* 253:385-397.

Kolenbrander PE (2000). Oral microbial communities: Biofilms, interactions, and genetic systems. *Annu Rev Microbiol* 54:413-437.

Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ (2002). Communication among oral bacteria. *Microbiol Mol Biol Rev* 66:486-505.

Kolenbrander PE, Palmer RJ, Periasamy S, Jakubovics NS (2010). Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 8:471-480.

Kornman KS, Loesche WJ (1980). The subgingival microbial flora during pregnancy. *J Periodontal Res* 15:111-122.

Kornman KS, Loesche WJ (1982). Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. *Infect Immun* 35:256-263.

Kornman KS (2018). Contemporary approaches for identifying individual risk for periodontitis. *Periodontol 2000* 78:12-29

Kobayashi N, Ishihara K, Sugihara N, Kusumoto M, Yakushiji M, Okuda K (2008). Colonization pattern of periodontal bacteria in Japanese children and their mothers. *J Periodontal Res* 43:156-161.

Kuhnert P, Frey J, Lang NP, Mayfield L (2002). Phylogenetic analysis of *Prevotella nigrescens*, *Prevotella intermedia* and *Porphyromonas gingivalis* clinical strains reveals a clear species clustering. *Int J Syst Evol Microbiol* 52:1391-1395.

Kumagai Y, Konishi K, Gomi T, Yagishita H, Yajima A, Yoshikawa M (2000). Enzymatic properties of dipeptidyl aminopeptidase IV produced by the periodontal pathogen *Porphyromonas gingivalis* and its participation in virulence. *Infect Immun* 68:716-724.

Kumagai Y, Yagishita H, Yajima A, Okamoto T, & Konishi K (2005). Molecular mechanism for connective tissue destruction by dipeptidyl aminopeptidase IV produced by the periodontal pathogen *Porphyromonas gingivalis*. *Infect Immun* 73:2655-2664.

Kurata H, Awano S, Yoshida A, Ansai T, Takehara T (2008). The prevalence of periodontopathogenic bacteria in saliva is linked to periodontal health status and oral malodour. *J Med Microbiol* 57:636-642.

Kuriyama T, Karasawa T, Nakagawa K, Yamamoto E, Nakamura S (2001). Incidence of beta-lactamase production and antimicrobial susceptibility of anaerobic gram-negative rods isolated from pus specimens of orofacial odontogenic infections. *Oral Microbiol Immunol* 16:10-15.

Könönen E, Jousimies-Somer H, Asikainen S (1992). Relationship between oral gram-negative anaerobic bacteria in saliva of the mother and the colonization of her edentulous infant. *Oral Microbiol Immunol* 7:273-276.

Könönen E, Mättö J, Väisänen-Tunkelrott ML, Frandsen EV, Helander I, Asikainen S et al. (1998a). Biochemical and genetic characterization of a *Prevotella intermedia/nigrescens*-like organism. *Int J Syst Bacteriol* 48:39-46.

Könönen E, Eerola E, Frandsen EV, Jalava J, Mättö J, Salmenlinna S et al. (1998b). Phylogenetic characterization and proposal of a new pigmented species to the genus *Prevotella*: *Prevotella pallens* sp. nov. *Int J Syst Bacteriol* 48:47-51.

Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H (1999). Establishment of oral anaerobes during the first year of life. *J Dent Res* 78:1634-1639.

Könönen E, Wolf J, Mättö J, Frandsen EV, Poulsen K, Jousimies-Somer H et al. (2000). The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. *J Periodontal Res* 35:329-334.

Könönen E, Paju S, Pussinen PJ, Hyvönen M, Di Tella P, Suominen-Taipale L *et al.* (2007). Population-based study of salivary carriage of periodontal pathogens in adults. *J Clin Microbiol* 45:2446-2451.

Laine MA (2002). Effect of pregnancy on periodontal and dental health. *Acta Odontol Scand* 60:257-264.

Lang NP, Joss A, Orsanic T, Gusberti FA, Siegrist BE (1986). Bleeding on probing - a predictor for the progression of periodontal disease? *J Clin Periodontol* 13:590-596.

Larsen JM (2017). The immune response to *Prevotella* bacteria in chronic inflammatory disease. *Immunology* 151:363-374.

Lazarevic V, Whiteson K, Huse S, Hernandez D, Farinelli L, Osterås M, et al. (2009). Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *J Microbiol Methods* 79:266-271.

Lazarevic V, Whiteson K, Hernandez D, François P, Schrenzel J (2010). Study of inter- and intra-individual variations in the salivary microbiota. *BMC Genomics* 28:523.

Lee KJ, Kim JA, Hwang W, Park SJ, Lee KH (2013). Role of capsular polysaccharide (CPS) in biofilm formation and regulation of CPS production by quorum-sensing in *Vibrio vulnificus*. *Mol Microbiol* 90:841-857.

Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ (2017). Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infect Immun* 19:410-417.

Lin W, Jiang W, Hu X, Gao L, Ai D, Pan H et al. (2018). Ecological Shifts of Supragingival Microbiota in Association with Pregnancy. *Front Cell Infect Microbiol* 8:24.

Loesche WJ, Syed SA, Laughon BE, Stoll J (1982).The bacteriology of acute necrotizing ulcerative gingivitis. *J Periodontol* 53:223-230.

Louhelainen AM, Aho J, Tuomisto S, Aittoniemi J, Vuento R, Karhunen PJ, et al. (2014). Oral bacterial DNA findings in pericardial fluid. *J Oral Microbiol* 6:25835.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.

Löe H, Silness J (1963). Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 21:533–551.

Madianos PN, Lieff S, Murtha AP, Boggess KA, Auten RL Jr, Beck JD, et al. (2001). Maternal periodontitis and prematurity. Part II: Maternal infection and fetal exposure. *Ann Periodontol* 6:175-182.

Magness RR, Rosenfeld CR (1989). Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. *Am J Physiol* 256:536-542.

Marcotte H, Lavoie MC (1998). Oral microbial ecology and the role of salivary immunoglobulin A. *Microbiol Mol Biol Rev* 62:71-109.

Mariotti A (1994). Sex steroid hormones and cell dynamics in the periodontium. *Crit Rev Oral Biol Med* 5:27-53.

Mariotti A (2000). The ambit of periodontal reproductive endocrinology. *Periodontol 2000* 61:7-15.

Mariotti A, Mawhinney M (2013). Endocrinology of sex steroid hormones and cell dynamics in the periodontium. *Periodontol 2000* 61:69-88.

Marsh PD, Hunter JR, Bowden GH, Hamilton IR, McKee AS, Hardie JM, *et al.* (1983). The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. *J Gen Microbiol* 129:755-770.

Marsh PD (2003). Are dental diseases examples of ecological catastrophes? *Microbiology* 149:279-294.

Marsh PD, Zaura E (2017). Dental biofilm: ecological interactions in health and disease. *J Clin Periodontol* 18:12-22.

Mascarenhas P, Gapski R, Al-Shammari K, Wang HL (2003). Influence of sex hormones on the periodontium. *J Clin Periodontol* 30:671-681.

Matsui A, Jin J-O, Johnston CD, Yamazaki H, Houri-Haddad Y, Rittling SR (2014). Pathogenic bacterial species associated with endodontic infection evade innate immune control by disabling neutrophils. *Infect Immun* 82:4068-4079.

McCauley LK, Tözüm TF, Rosol TJ (2002). Estrogen receptors in skeletal metabolism: lessons from genetically modified models of receptor function. *Crit Rev Eukaryot Gene Expr* 12:89-100.

Mealey BL1, Moritz AJ (2003). Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium. *Periodontol 2000* 32:59-81.

Meng L, Du Y, Liu P, Li X, Liu Y (2017). Involvement of LuxS in *Aeromonas salmonicida* metabolism, virulence and infection in Atlantic salmon (Salmo salar L). *Fish Shellfish Immunol* 64:260-269.

Merritt JH, Kadouri DE, O'Toole GA (2005). Growing and analyzing static biofilms. *Curr Protoc Microbiol* Chapter 1:Unit 1B.1.

Metzemaekers M, Van Damme J, Mortier A, Proost P (2016). Regulation of Chemokine Activity - A Focus on the Role of Dipeptidyl Peptidase IV/CD26. *Front Immunol* 11:7:483.

Miller MB, Bassler BL (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.

Milsom SE, Sprague SV, Dymock D, Weightman AJ, Wade WG (1996). Rapid differentiation of *Prevotella intermedia* and *P. nigrescens* by 16S rDNA PCR-RFLP. *J Med Microbiol* 44:41-43.

Miyagi M, Aoyama H, Morishita M, Iwamoto Y. Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *J Periodontol* 63:28-32

Moon JH, Kim C, Lee HS (2013). Antibacterial and antibiofilm effects of iron chelators against *Prevotella intermedia*. *J Med Microbiol* 62:1307-1316.

Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Palcanis KG et al., (1985). Comparative bacteriology of juvenile periodontitis. *Infect Immun* 48: 507-519.

Mäkelä M, Sorsa T, Uitto VJ, Salo T, Teronen O, Larjava H (1998). The effects of chemically modified tetracyclines (CMTs) on human keratinocyte proliferation and migration. *Adv Dent Res* 12:131-135.

Mättö J, Saarela M, von Troil-Lindén B, Könönen E, Jousimies-Somer H, Torkko H, *et al*. (1996a). Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Microbiol Immunol* 11: 96–102.

Mättö J, Saarela M, von Troil-Lindén B, Alaluusua S, Jousimies-Somer H, Asikainen S (1996b). Similarity of salivary and subgingival *Prevotella intermedia* and *Prevotella nigrescens* isolates by arbitrarily primed polymerase chain reaction. *Oral Microbiol Immunol* 11:395-401.

Mättö J, Asikainen S, Väisänen ML, Rautio M, Saarela M, Summanen P, et al. (1997). Role of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* in extraoral and some odontogenic infections. *Clin Infect Dis* 2:194-198.

Mättö J, Asikainen S, Väisänen ML, Von Troil-Lindén B, Könönen E, Saarela M, et al. (1999). Beta-lactamase production in *Prevotella intermedia*, *Prevotella nigrescens*, and *Prevotella pallens* genotypes and in vitro susceptibilities to selected antimicrobial agents. *Antimicrob Agents Chemother* 43:2383-2388.

Nagaoka S, Tokuda M, Sakuta T, Taketoshi Y, Tamura M, Takada H, *et al*. (1996). Interleukin-8 gene expression by human dental pulp fibroblast in cultures stimulated with *Prevotella intermedia* lipopolysaccharide. *J Endod* 22:9-12.

Nagashima S, Yoshida A, Suzuki N, Ansai T, Takehara T (2005). Use of the genomic subtractive hybridization technique to develop a real-time PCR assay for quantitative detection of *Prevotella* spp. in oral biofilm samples. *J Clin Microbiol* 43:2948-2951.

Nagy E, Becker S, Kostrzewa M, Barta N, Urbán E (2012). The value of MALDI-TOF MS for the identification of clinically relevant anaerobic bacteria in routine laboratories. *J Med Microbiol* 61:1393-1400.

Nakao M, Yukishige K, Kondo M, Imada A (1986). Novel morphological changes in gram-negative bacteria caused by combination of bulgecin and cefmenoxime. *Antimicrob Agents Chemother* 30: 414-417.

Nebel D (2012). Functional importance of estrogen receptors in the periodontium. *Swed Dent J Suppl* 221:11-66.

Nemoto E, Sugawara S, Takada H, Shoji S, Horiuch H (1999). Increase of CD26/dipeptidyl peptidase IV expression on human gingival fibroblasts upon stimulation with cytokines and bacterial components. *Infect Immun* 67:6225-6233.

Nesbitt WE, Fukushima H, Leung KP, Clark WB (1993). Coaggregation of *Prevotella intermedia* with oral *Actinomyces* species. *Infect Immun* 61:2011-2014.

Nomura Y, Takeuchi H, Okamoto M, Sogabe K, Okada A, Hanada N (2017). Chair-side detection of *Prevotella Intermedia* in mature dental plaque by its fluorescence. *Photodiagnosis Photodyn Ther* 18:335-341.

Nonnenmacher C, Dalpke A, Mutters R, Heeg K (2004). Quantitative detection of periodontopathogens by real-time PCR. *J Microbiol Methods* 59:117-125.

Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, et al. (1995). The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol Gen Genet* 248:446-458.

Nyvad B, Kilian M (1987). Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand J Dent Res* 95:369-380.

Okamoto M, Maeda N, Kondo K, Leung KP (1999). Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. *FEMS Microbiol Lett* 178:299-304.

Okada H, Murakami S (1998). Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 9:248-266.

Okuda T, Kokubu E, Kawana T, Saito A, Okuda K, Ishihara K (2012). Synergy in biofilm formation between *Fusobacterium nucleatum* and *Prevotella* species. *Anaerobe* 18:110-116.

O'Leary P, Boyne P, Flett P, Beilby J, James I (1991). Longitudinal assessment of changes in reproductive hormones during normal pregnancy. *Clin Chem* 37:667-672.

Olivares M, Schüppel V, Hassan AM, Beaumont M, Neyrinck AM, Bindels LB, et al. (2018). The Potential Role of the Dipeptidyl Peptidase-4-Like Activity From the Gut Microbiota on the Host Health. *Front Microbiol* 22:9:1900.

Oliver WW, Wherry WB (1921). Notes on some bacterial parasites of the human mucous membranes. *J Infec Dis* 28:341-345.

Oliver JD (2005). The viable but nonculturable state in bacteria. *J Microbiol* 43:93-100.

Olsen GJ, Woese CR, Overbeek R (1994). The winds of (evolutionary) change: breathing new life into microbiology. *J Bacteriol* 176:1-6.

Olsen I, Chen T, Tribble GD (2018). Genetic exchange and reassignment in *Porphyromonas gingivalis*. *J Oral Microbiol* 10:1457373.

Pearce MA, Dixon RA, Gharbia SE, Shah HN, Devine DA (1996). Characterization of *Prevotella intermedia* and *Prevotella nigrescens* by enzyme production, restriction endonuclease and ribosomal RNA gene restriction analyses. *Oral Microbiol Immunol* 11:135-141.

Pearson JP, Van Delden C, Iglewski BH (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* 181:1203-1210.

Piccolo M, De Angelis M, Lauriero G, et al. (2015) Salivary microbiota associated with immunoglobulin A nephropathy. *Microb Ecol* 70:557-565.

Pinard A (1877). Gingivitis in pregnancy. *Dent Regist* 31:258-259.

Potempa J, Banbula A, Travis J (2000). Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000* 24:153-192.

Quince C, Lanzén A, Curtis TP, Davenport RJ, Hall N, Head IM, et al., (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. *Nat Methods* 6:639-641.

Raber-Durlacher JE, van Steenbergen TJ, Van der Velden U, de Graaff J, Abraham-Inpijn L (1994). Experimental gingivitis during pregnancy and post-partum: clinical, endocrinological, and microbiological aspects. *J Clin Periodontol* 21:549-558.

Rams TE, Sautter JD, Hsiao CY, van Winkelhoff AJ (2018). Phenotypic identification of periodontal *Prevotella intermedia*/*nigrescens* group isolates validated by MALDI-TOF mass spectrometry. *Anaerobe* 54:201-204.

Renvert S, Dahlén G, Wikström M (1996). Treatment of periodontal disease based on microbiological diagnosis. Relation between microbiological and clinical parameters during 5 years. *J Periodontol* 67:562-571.

Riggio MP, Lennon A, Rolph HJ, Hodge PJ, Donaldson A, Maxwell AJ, et al. (2008). Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis. *Oral Dis* 14:251-258.

Roberts FA, Darveau RP (2015). Microbial protection and virulence in periodontal tissue as a function of polymicrobial communities: symbiosis and dysbiosis. *Periodontol 2000* 69:18-27.

Robertson KL, Blinkhorn AS, Davies RM, Drucker DB (2000). An examination of 'unidentified' *Prevotella* (formerly PINLO) using RAPD-PCR and partial 16S rRNA gene sequencing. *Anaerobe* 6:249-256.

Robertson D, Smith AJ. The microbiology of the acute dental abscess (2009). *J Med Microbiol* 58:155-162.

Rodríguez-Cavallini E, Vargas P, Rodríguez C, Quesada-Gómez C, Gamboa-Coronado MM (2011). Phenotypic identification of over 1000 isolates of anaerobic bacteria recovered between 1999 and 2008 in a major Costa Rican hospital. *Clin Microbiol Infect* 17:1043-1047.

Rosier BT, Marsh PD, Mira A (2018). Resilience of the oral microbiota in health: Mechanisms that prevent dysbiosis. *J Dent Res* 97:371-380.

Rosan B and Lamont RJ (2000). Dental plaque formation. *Microbes Infect* 2:1599-1607.

Roy V, Smith JA, Wang J, Stewart JE, Bentley WE, Sintim HO (2010). Synthetic analogs tailor native AI-2 signaling across bacterial species. *J Am Chem Soc* 132: 11141-11150.

Sakamoto M, Kumada H, Hamada N, Takahashi Y, Okamoto M, Bakir MA et al. (2009). *Prevotella falsenii* sp. nov., a *Prevotella intermedia*-like organism isolated from monkey dental plaque. *Int J Syst Evol Microbiol* 59:319-322.

Sakamoto M, Ohkuma M (2010). Usefulness of the hsp60 gene for the identification and classification of Gram-negative anaerobic rods. *J Med Microbiol* 59:1293-1302.

Sakamoto M, Suzuki N, Okamoto M (2010). *Prevotella aurantiaca* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* 60:500-503.

Sakamoto M, Ohkuma M (2012). Reclassification of *Xylanibacter oryzae* Ueki et al. 2006 as *Prevotella oryzae* comb. nov., with an emended description of the genus *Prevotella*. *Int J Syst Evol Microbiol* 62:2637-2642.

Scheres N, Lamont RJ, Crielaard W, Krom BP (2015). LuxS signaling in *Porphyromonas gingivalis*-host interactions. *Anaerobe* 35:3-9.

Schincaglia GP, Hong BY, Rosania A, Barasz J, Thompson A, Sobue T et al. (2017). Clinical, immune, and microbiome traits of gingivitis and peri-implant mucositis. *J Dent Res* 96:47–55.

Schock H, Zeleniuch-Jacquotte A, Lundin E, Grankvist K, Lakso HÅ, Idahl A et al. (2016). Hormone concentrations throughout uncomplicated pregnancies: a longitudinal study. *BMC Pregnancy Childbirth* 16:146.

Schätzle M, Löe H, Bürgin W, Anerud A, Boysen H, Lang NP (2003). Clinical course of chronic periodontitis. I. Role of gingivitis. *J Clin Periodontol* 30:887-901.

Shah HN, Collins MD (1988). Proposal of reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. *Int J Syst Bacteriol* 38:128-131.

Shah HN, Collins DM (1990). *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int J Syst Bacteriol* 40(2):205-208.

Shah HN. Gharbia SE (1992). Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. *Int J Syst Bacteriol* 42:542-546.

Shapiro LF, Freeman K (2014). The relationship between estrogen, estrogen receptors and periodontal disease in adult women: a review of the literature. *N Y State Dent J* 80:30-34.

Shenker B J, and Slots J (1989). Immunomodulatory effects of Bacteroides products on in vitro human lymphocyte functions. *Oral Microbiol Immunol* 4:24-29.

Shenker BJ, Vitale L, Slots J (1991). Immunosuppressive effects of *Prevotella intermedia* on in vitro human lymphocyte activation. *Infect Immun* 59: 4583-4589.

Shibata Y, Fujimura S, Nakamura T (1993). Purification and partial characterization of an elastolytic serine protease of *Prevotella intermedia*. *Appl Environ Microbiol* 59:2107-2111.

Shibata Y, Miwa Y, Hirai K, Fujimura S (2003). Purification and partial characterization of a dipeptidyl peptidase from *Prevotella intermedia*. *Oral Microbiol Immunol* 18:196-198.

Shin JM, Luo T, Lee KH, Guerreiro D, Botero TM, McDonald NJ, et al. (2018). Deciphering Endodontic Microbial Communities by Next-generation *Sequencing. J Endod* 44:1080-1087.

Shinzato T, Saito A (1994). A mechanism of pathogenicity of "Streptococcus milleri group" in pulmonary infection: synergy with an anaerobe. *J Med Microbiol* 118-123.

Silk H, Douglass AB, Douglass JM, Silk L (2008). Oral health during pregnancy. *Am Fam Physician* 77:1139-1144.

Silness J, Loe H (1964). Periodontal disease in pregnancy. II. Correlation between oral hyigene and periodontal condition. *Acta Odontol Scand* 22:121-135.

Signoretto C, Marchi A, Bertoncelli A, Burlacchini G, Tessarolo F, Caola T, et al. (2011). Effects of Mushroom and Chicory Extracts on the Physiology and Shape of *Prevotella intermedia*, a Periodontopathogenic Bacterium. *J Biomed Biotechnol* 635348.

Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R, et al. (2015). Host response mechanisms in periodontal diseases. *J Appl Oral Sci* 23:329-355.

Siqueira JF Jr, Rôças IN (2013). As-yet-uncultivated oral bacteria: breadth and association with oral and extra-oral diseases. *J Oral Microbiol* 23:5.

Slots J, Bragd L, Wikström M, Dahlén G (1986). The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. *J Clin Periodontol* 13:570-577.

Slots J, Ashimoto A, Flynn MJ, Li G, Chen C (1995). Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 2:304-307.

Smalley JW, Silver J, Birss AJ, Withnall R, Titler PJ (2003). The haem pigment of the oral anaerobes *Prevotella nigrescens* and *Prevotella intermedia* is composed of iron(III) protoporphyrin IX in the monomeric form. *Microbiology* 149:1711-1718.

Socransky SS, Manganiello SD (1971). The oral microbiota of man from birth to senility. *J Periodontol* 42:485-496.

Socransky SS, Manganiello AD, Propas D, Oram V, van Houte J (1977). Bacteriological studies of developing supragingival dental plaque. *J Periodontal Res* 12:90-106.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134-144.

Stubbs S, Park SF, Bishop PA, Lewis MA (1999). Direct detection of *Prevotella intermedia* and *P. nigrescens* in suppurative oral infection by amplification of 16S rRNA gene. *J Med Microbiol* 48:1017-1022.

Sun J, Song X (2011). Assessment of antimicrobial susceptibility of *Enterococcus faecalis* isolated from chronic periodontitis in biofilm versus planktonic phase. J Periodontol 82:626-631.

Takahashi N (2003). Acid-neutralizing activity during amino acid fermentation by *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum. Oral Microbiol Immunol* 18(2):109-13.

Takahashi N, Ishihara K, Kimizuka R, Okuda K, Kato T (2006). The effects of tetracycline, minocycline, doxycycline and ofloxacin on *Prevotella intermedia* biofilm. *Oral Microbiol Immunol* 21:366-371.

Takahashi N, Nyvad B (2011). The role of bacteria in the caries process: ecological perspectives. *J Dent Res* 90:294-303.

Talan DA, Abrahamian FM, Moran GJ, Citron DM, Tan JO, Goldstein EJ (2003). Clinical presentation and bacteriologic analysis of infected human bites in patients presenting to emergency departments. *Clin Infect Dis* 37:1481-1489.

Tan Y, Cheng Q, Yang H, Li H, Gong N, Liu D3 et al. (2018). Effects of ALA-PDT on biofilm structure, virulence factor secretion, and QS in *Pseudomonas aeruginosa*. *Photodiagnosis Photodyn Ther* 24:88-94.

Tanner AC, Haffer C, Bratthall GT, Visconti RA, Socransky SS (1979). A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol* 6:278-307.

Tanner A (1992). Microbial etiology of periodontal diseases. Where are we? Where are we going? *Curr Opin Dent* 2:12-24.

Tanner A, Maiden MF, Paster BJ, Dewhirst FE (1994). The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. *Periodontol 2000* 5:26-51.

Tanner AC, Paster BJ, Lu SC, Kanasi E, Kent R Jr, Van Dyke T et al. (2006). Subgingival and tongue microbiota during early periodontitis. *J Dent Res 85:318-323.*

Tatakis DN, Kumar PS (2005). Etiology and pathogenesis of periodontal diseases. *Dent Clin North Am* 49:491-516.

Taylor DD, Sullivan SA, Eblen AC, Gercel-Taylor C (2002). Modulation of T-cell CD3-zeta chain expression during normal pregnancy. *J Reprod Immunol* 54:15-31.

Teanpaisan R, Douglas CW, Eley AR, Walsh TF (1996). Clonality of Porphyromonas gingivalis, *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontally diseased and healthy sites. *J Periodontal Res* 31:423-432.

Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S et al. (2010). Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *J Periodontol* 81:89-98.

Tilakaratne A, Soory M, Ranasinghe AW, Corea SM, Ekanayake SL, de Silva M (2000). Periodontal disease status during pregnancy and 3 months post- partum, in a rural population of Sri-Lankan women. *J Clin Periodontol* 27:787-792.

Travis J, Banbula A, Potempa J (2000). The role of bacterial and host proteinases in periodontal disease. *Adv Exp Med Biol* 477:455-65.

Uematsu T, Tanaka H, Yamaoka M, Furusawa K (2004). Effects of oral squamous cell carcinoma-derived TGF-beta1 on CD26/DPPIV expression in T cells. *Anticancer Res* 24:619-624.

Uppuluri P, Lopez-Ribot JL (2010). An easy and economical in vitro method for the formation of *Candida albicans* biofilms under continuous conditions of flow. *Virulence* 1:483-487.

Uriarte SM, Edmisson JS, Jimenez-Flores E (2016). Human neutrophils and oral microbiota: a constant tug-of-war between a harmonious and a discordant coexistence. *Immunol Rev* 273:282–298.

van Steenbergen TJ, Bosch-Tijhof CJ, Petit MD, Van der Velden U (1997). Intra-familial transmission and distribution of *Prevotella intermedia* and *Prevotella nigrescens*. *J Periodontal Res* 32:345-350.

Van Winkelhoff AJ, Van der Velden U, Clement M, De Graaff J (1988a). Intraoral distribution of black-pigmented *Bacteroides* species in periodontitis patients. *Oral Microbiol Immunol* 3: 83-85.

Van Winkelhoff AJ, Van Steenbergen TJM, de Graaff J (1988b). The role of black-pigmented *Bacteroides* in human oral infections. *J Clin Periodontol* 15: 145-155.

van Winkelhoff AJ, Herrera Gonzales D, Winkel EG, Dellemijn-Kippuw N, Vandenbroucke-Grauls CM, et al. (2000). Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. A comparison between The Netherlands and Spain. *J Clin Periodontol* 27:79-86.

Vartoukian SR, Palmer RM, Wade WG (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol Lett* 309:1-7.

Vartoukian SR, Adamowska A, Lawlor M, Moazzez R, Dewhirst FE, Wade WG (2016). In Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by Community Culture and Media Supplementation with Siderophores. *PLoS One* 14:e0146926.

Vollaard EJ, Clasener HA (1994). Colonization resistance. *Antimicrob Agents Chemother* 38:409-414.

Wade WG, Gray AR, Absi EG, Barker GR (1991). Predominant cultivable flora in pericoronitis. *Oral Microbiol Immunol* 6:310-312.

Wade WG (1999). Unculturable bacteria in oral biofilms. In: Newman HN, Wilson M, eds. Dental plaque revisited. *Cardiff:BioLine* 313-322.

Wade WG (2002). Unculturable bacteria--the uncharacterized organisms that cause oral infections. *J R Soc Med* 95:81-83.

Wade WG (2013). The oral microbiome in health and disease. *Pharmacol Res* 69:137-143.

Wang BY, Alvarez P, Hong J, Kuramitsu HK (2011). Periodontal pathogens interfere with quorum-sensing-dependent virulence properties in *Streptococcus mutans*. *J Periodontal Res* 46:105-110.

Walker CB (1996). The acquisition of antibiotic resistance in the periodontal microflora. *Periodontology 2000* 10:79-88.

Walker CB, Bueno LC (1997). Antibiotic resistance in an oral isolate of *Prevotella intermedia*. *Clin Infect Dis* 25:281-283.

Waters CM, Bassler BL (2005). Quorum sensing: cell-to-cell communication in bacteria. *Anal Rev Cell Dev Biol* 21: 319-346.

Weinstein RL, Kelch RP, Jenner MR, Kaplan SL, Grumbach MM (1974). Secretion of unconjugated androgens and estrogens by the normal and abnormal human testis before and after chorionic gonadotropin. *J Clin Invest* 53:1-6.

Wilson M, Reddi K, Henderson B (1996). Cytokine-inducing components of periodontopathogenic bacteria. *J Periodontal Res* 31:393-407.

Wu M, Chen SW, Jiang SY (2015). Relationship between gingival inflammation and pregnancy. *Mediators Inflamm* 623427.

Wu M, Chen SW, Su WL, Zhu HY, Ouyang SY, Cao YT, et al. (2016). Sex Hormones Enhance Gingival Inflammation without Affecting IL-1β and TNF-α in Periodontally Healthy Women during Pregnancy. *Mediators Inflamm* 2016:4897890.

Wu SP, Li R, DeMayo FJ (2018). Progesterone Receptor Regulation of Uterine Adaptation for Pregnancy. *Trends Endocrinol Metab* 29:481-491.

Xie H, Gibbons RJ, Hay DI (1991). Adhesive properties of strains of *Fusobacterium nucleatum* of the subspecies *nucleatum*, *vincentii* and *polymorphum*. *Oral Microbiol Immunol* 6:257-263.

Yamanaka T, Furukawa T, Matsumoto-Mashimo C, Yamane K, Sugimori C, Nambu T et al. (2009). Gene expression profile and pathogenicity of biofilm-forming *Prevotella intermedia* strain 17. *BMC Microbiol* 9:11.

Yamanaka T, Yamane K, Furukawa T, Matsumoto-Mashimo C, Sugimori C, Nambu T et al. (2011). Comparison of the virulence of exopolysaccharide-producing *Prevotella intermedia* to exopolysaccharide non-producing periodontopathic organisms. *BMC Infect Dis* 11: 228.

Yamane K, Yamanaka T, Yamamoto N, Furukawa T, Fukushima H, Walker CB et al. (2005). A novel exopolysaccharide from a clinical isolate of *Prevotella nigrescens*: purification, chemical characterization and possible role in modifying human leukocyte phagocytosis. *Oral Microbiol Immunol* 20:1-9.

Yanagisawa M, Kuriyama T, Williams DW, Nakagawa K, Karasawa T (2006). Proteinase activity of *Prevotella species* associated with oral purulent infection. *Curr Microbiol* 52:375-378.

Yang Y, Sreenivasan PK, Subramanyam R, Cummins D (2006). Multiparameter assessments to determine the effects of sugars and antimicrobials on a polymicrobial oral biofilm. *Appl Environ Microbiol* 72:6734-6742.

Yokoyama M, Hinode D, Masuda K, Yoshioka M, Grenier D (2005). Effect of female sex hormones on *Campylobacter rectus* and human gingival fibroblasts. *Oral Microbiol Immunol* 20:239-243.

Yoshida A, Tachibana M, Ansai T, Takehara T (2005). Multiplex polymerase chain reaction assay for simultaneous detection of black-pigmented *Prevotella* species in oral specimens. *Oral Microbiol Immunol* 20:43-46.

Yost S, Duran-Pinedo AE (2018). The contribution of *Tannerella forsythia* dipeptidyl aminopeptidase IV in the breakdown of collagen. *Mol Oral Microbiol* 33:407-419.

Yu F, Anaya C, Lewis JP (2007). Outer membrane proteome of *Prevotella intermedia* 17: identification of thioredoxin and iron-repressible hemin uptake loci. *Proteomics* 7:403-412.

Zachariasen RD (1989). Ovarian hormones and oral health: pregnancy gingivitis. *Compendium* 10:508-512.

Zakaria MN, Takeshita T, Shibata Y, Maeda H, Wada N, Akamine A, Yamashita Y (2015). Microbial community in persistent apical periodontitis: a 16S rRNA gene clone library analysis. *Int Endod J* 48:717-728.

Zambon JJ, Reynolds H S, Slots J (1981). Black-pigmented *Bacteroides* spp. in the human oral cavity. *Infect Immun* 32:198-203.

Zaura E, Keijser BJ, Huse SM, Crielaard W (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol* 15:259.

Zhang Y, Zhen M, Zhan Y, Song Y, Zhang Q, Wang J (2017). Population-Genomic Insights into Variation in *Prevotella intermedia* and *Prevotella nigrescens* Isolates and Its Association with Periodontal Disease. *Front Cell Infect Microbiol* 21:409.

Zhao S, Song M, Fan Y, Chang Q, Yi W, Li P et al. (2012). Elevation of plasma soluble CD26 levels during pregnancy. *J Obstet Gynaecol Res* 38:272-279.

Zhu J, Winans SC (2001). The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc Natl Acad Sci USA* 98:1507-1512.

UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-8009-3 (PRINT)
ISBN 978-951-29-8010-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)