PÂMELA PONTES PENAS AMADO

ANÁLISE DA DIVERSIDADE DA MICROBIOTA SUPRA E SUBGENGIVAL E PERFIL DE PROTEÍNAS DA SALIVA E PELÍCULA ADQUIRIDA DE INDIVÍDUOS COM PERIODONTITE AGRESSIVA

Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para a obtenção do Título de Doutora em Ciências.

São Paulo
2019

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DIVERSITY ANALYSIS OF THE SUPRA AND SUBGINGIVAL MICROBIOTA AND PROTEINS PROFILE OF SALIVA AND ACQUIRED ENAMEL PELLICLE OF INDIVIDUALS WITH PERIODONTITIS

Thesis presented to the Graduate Program in Microbiology of the Institute of Biomedical Sciences of the University of São Paulo, to obtain the title of Doctor of Science.

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São Paulo, 19 de setembro de 2014.

PARECER 1197/CEPSH

A Comissão de *Ética em Pesquisas em Seres Humanos* do ICB, nesta data, **APROVOU** o projeto intitulado: " *Análise da diversidade da microbiota supra e subgengival e perfil de proteínas da saliva de indivíduos com periodontite agressiva*" da pesquisadora MÁRCIA PINTO ALVES MAYER e aluna PÂMELA PONTES PENAS AMADO.

Cabe ao pesquisador elaborar e apresentar a este Comitê, relatórios anuais (parciais e final), de acordo com a Resolução nº 466/12, item II, II.19 e II.20, do Conselho Nacional de Saúde, conforme modelo constante no site: **icb.usp.br.**

Ao pesquisador cabe também finalizar o processo junto à Plataforma Brasil quando do encerramento deste.

O primeiro relatório deverá ser encaminhado à Secretaria deste CEP em **19.09.2015**.

Atenciosamente,

Profa. Dra. PAOLO M.A.ZANOTTO
Coordenador da Comissão de Ética em
Pesquisas com Seres Humanos - ICB/USP

Dedico este trabalho com muito amor e gratidão...

A Deus, primeiramente. Sem Ele, eu nada seria.

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ABSTRACT

AMADO, P. P. P. **Diversity analysis of the supra and subgingival microbiota and proteins profile of saliva and acquired enamel pellicle of individuals with periodontitis**. 2019. 132 p. PhD thesis (Microbiology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019.

Localized aggressive periodontitis (LAgP) is still a poorly understood disease. The subgengival microbiota of LAgP is characterized by the presence of periodontopathogens such as *Aggregatibacter actinomycetemcomitans* (*Aa*), and by the reduction of beneficial bacteria, however, the microbiome associated with LAgP has not yet been described. Since the initial adhesion of bacteria to the dental surfaces is dependent on the composition of the acquired enamel pellicle (PAE) formed by proteins present in the whole saliva (WS), the present study evaluated the PAE and WS proteome of LAgP, compared with healthy patients (HLAgP). In addition, the salivary cytokines/chemokines profile was evaluated in both groups. We also aimed to determine the composition of the oral and gut microbiota in patients with LAgP, and compare to the microbiota of HLAgP. Seven women with LAgP, aged between 19-26 years, Afro-descendants, and 8 healthy controls with the same profile were selected. Samples of supra (SP) and subgingival biofilme of shallow sites (SH) and affected medium/deep sites (MD), WS, AEP and feces were collected. The oral and gut microbiome were analyzed by sequencing of *16S rRNA*, and the presence of the *Aa* JP2 clone was determined in oral biofilme (OB) samples. WS and AEP proteome was analyzed by mass spectrometry, levels of NO in WS were dosed by Griess colorimetric reaction, levels of cytokines/chemokines in WS were quantified in multiplex assay. The proteolytic activity of WS was evaluated through the degradation of histatins 1 and 5 in different time-points. Spearman's correlation was applyed to evaluate correlations between different variables. qPCR analysis revealed that all LAgP patients harbored *Aa*, unlike HLAgP, however, the JP2 clone was detected in only 1 patient. Oral and gut microbiome analysis revealed no differences in the alpha diversity indexes. Beta-diversity analysis revealed that samples of OB and MD of LAgP were different from OB and SH of HLAgP, respectively. In LAgP, there was a reduction of the abundance of benefical bacteria, and increase of putative pathogens such as *Aa*, *Porphyromonas*, *Tannerela* and *Treponema*. The oral dysbiosis was acompained by imbalance in the gut microbiota, with higher abundance of *Desulfovibrio* in LAgP than in HLAgP. The WS of LAgP patients presented lower levels of CCL2 and CCL25, and higher levels of CCL17 and CCL27 than HLAgP, correlated to levels of *Aa*, *Acidovorax ebreus* and *Helicobacter pylori* in the OB. The AEP of LAgP-affected individuals presented undetectable levels of some proteins involved in immune response, antimicrobial activity and anti-inflammatory molecules, differing from the PAE of the HLAgP. AEP proteins such as ALMS1 and Cystatin-S and in WS such as alpha-enolase, profilin-1, dystonin, A2ML1, alpha-actinin-4 and IGHA1 were present differentially between LAgP and HLAgP. The proteolytic activity of WS was more intense in LAgP than in HLAgP. The data revealed new aspects of LAgP and collaborate with the understanding of mechanisms that may be involved in the development and progression of the disease, and indicate that new treatment strategies aiming at reestablishing the balance of the microbiota could be developed.

Keywords: Localized aggressive periodontitis. Oral microbiome. Gut microbiome. Salivary proteome. Saliva. Acquired enamel pellicle. Chemokines.

RESUMO

AMADO, P. P. P. **Análise da diversidade da microbiota supra e subgengival e perfil de proteínas da saliva e película adquirida de indivíduos com periodontite agressiva**. 2019. 132 f. Tese de doutorado (Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019.

A Periodontite agressiva localizada (PAgL) é ainda uma doença pouco compreendida. A microbiota subgengival da PAgL é caracterizada pela presença de patógenos periodontais como *Aggregatibacter actinomycetemcomitans* (*Aa*), e pela redução de bactérias benéficas, mas o microbioma associado a PAgL ainda não foi descrito. Tendo em vista que a adesão inicial de bactérias às superfícies dentais é dependente da composição da película adquirida do esmalte (PAE) formada por proteínas presentes na saliva total (ST), o presente estudo avaliou o proteoma de PAE e ST de PAgL e comparou-o com o de pacientes saudáveis (S). Além disso, o perfil de citocinas/quimiocinas salivares foi avaliado em ambos os grupos. Também visamos determinar a composição da microbiota oral e intestinal em pacientes com PAgL, e compara-las à microbiota de pacientes S. Foram selecionadas 7 mulheres com PAgL, com idade entre 19-26 anos, afrodescendentes e 8 controles saudáveis com mesmo perfil. Amostras de biofilme de sítios supra (SP) e subgingival de sítios rasos (SR) e médio/profundos (MP), ST, PAE e fezes foram coletadas. O microbioma oral e intestinal foi analisado por sequenciamento de*16S rRNA*, e a presença do genótipo de *Aa* clone JP2 foi determinado em amostras de biofilme oral (BO). O proteoma da ST e PAE foi analisado por espectrometria de massa, níveis de NO na ST foram dosados por reação colorimétrica de Griess, níveis de citocinas/quimiocinas na ST foram quantificados em ensaio multiplex. A atividade proteolítica da ST foi avaliada pela degradação de histatinas 1 e 5 em diferentes intervalos de tempo. Teste de correlação de Spearman foi empregado para avaliar correlações entre diferentes variáveis. A análise de qPCR revelou que todos os pacientes com PAgL albergavam *Aa*, ao contrário de S, mas o clone JP2 foi detectado em apenas 1 paciente. A análise de beta diversidade do microbioma oral e de fezes revelou que amostras de BO e MP de PAgL se diferenciam do BO e SR de S, respectivamente. Na PAgL, houve redução da abundância de bactérias benéficas, e aumento de patógenos putativos como *Aa*, *Porphyromonas*, *Tannerela* e*Treponema*. A disbiose oral foi acompanhada de desequilíbrio no microbioma intestinal, com maior abundância de *Desulfovibrio* em PAgL do que em S. A ST de pacientes LAgP apresentou níveis de CCL2 e CCL25 menores e de CCL17 e CCL27 maiores do que S, e estes foram correlacionados com os níveis de *Aa*, *Acidovorax ebreus* e *Helicobacter pylori* no BO.A PAE de indivíduos com PAgL apresentou níveis indetectáveis de algumas proteínas envolvidas na resposta imune, atividade antimicrobiana e moléculas anti-inflamatórias, diferindo da PAE dos controles S. Proteínas como ALMS1 e Cistatina-S na PAE e como alfa-enolase, profilina-1, distonina, A2ML1, alfa-actinina-4 e IGHA1 na ST estavam presentes de maneira diferencial entre PAgL e S. A atividade proteolítica da ST foi mais intensa em PAgL do que em S. Os dados revelaram novos aspectos da PAgL e colaboram com o entendimento de mecanismos que podem estar envolvidos no desenvolvimento e progressão da doença, e indicam que novas estratégias de tratamento visando o restabelecimento do equilíbrio da microbiota poderiam ser desenvolvidas.

Palavras-chave: Periodontite agressiva localizada. Microbioma oral. Microbioma intestinal. Proteoma salivar. Saliva. Película adquirida do esmalte. Quimiocinas.

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List of abbreviations

A2ML1 – Alpha-2-macroglobulin-like protein 1

Aa – A. actinomycetemcomitans

AAs – African-Americans

AC – accession number

AEP – acquired enamel pellicle

AgP – Aggressive periodontitis

BCA – bicinchoninic acid

BoP – bleeding on probing

bp – base pairs

CAL – clinical attachment level

Cationic-PAGE – native cationic polyacrylamide gel electrophoresis

CDT – cytolethal distending toxin

ChP – Chronic periodontitis

CM – chemokines

CRC – colorectal cancer

CSS – cumulative sum scaling

CT – cytokines

DS – Down Syndrome

DStSS – diluted stimulated saliva supernatant

DTT – dithiothreitol

ECM – extracellular matrix derived proteins

ENO1 – alpha-enolase, also known as enolase 1

F/B ratio – Firmicutes/Bacteroidetes ratio

Fe-S – iron–sulfur

GAgP – Generalized aggressive periodontitis

GCF – gingival crevicular fluid

GO – Gene Ontology

H_2O_2 – hydrogen peroxide

H_2S – hydrogen sulphide

His1 – histatin 1

His5 – histatin 5

HLAgP - healthy subjects

HOMD – Human Oral Microbiome Database

HPLC – high performance liquid chromatography

Ig – immunoglobulins

IGHA1 – immunoglobulin heavy constant alpha 1

IGHG1 – immunoglobulin heavy constant gamma 1

iNOS – inducible NO synthase

LAgP – Localized aggressive periodontitis

LC-ESI-MS/MS – liquid chromatography-electrospray ionization-tandem mass spectrometry

LIP – molar incisor pattern periodontitis

log2FC – log2 fold change

LPS – lipopolysaccharide

MAPK – map kinase

M-CSF – macrophage colony–stimulation factor

MD – biofilm of medium/deep sites

mg – milligram

Micro BCA – micro bicinchoninic acid

min – minute

mL – milliliter

MWM – molecular weight marker

NDFO – nitrate-dependent iron oxidation

NGS – next generation sequencing

nm – nanometer

NO – nitric oxide

NO_2^- – nitrite

NO_3^- – nitrate

NRB – nitrate reducing bacteria

$ONOO^-$ – peroxynitrite

OUT – operational taxonomic unit

PCoA – Principal Coordinates Analysis

PD – probing depth

pIgR – polymeric immunoglobulin receptor

PIP – prolactin-inducible protein.

PMNs – polymorphonuclear neutrophils

PRPs – proline-rich proteins

QIIME – Quantitative Insights Into Microbial Ecology

qPCR – quantitative PCR

RA – relative abundance

RAR – rheumatoid arthritis

SC – secretory component

SD – standard deviation

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH – biofilm of healthy shallow sites

SIgA – secretory IgA

SMR3B – submaxillary gland androgen-regulated protein 3B

SP – biofilm of supragingival sites

SPRR3 – small proline-rich protein 3

SRB – sulfate reducing bacteria

SS – saliva supernatant

StS – stimulated saliva

TECK – thymus-expressed chemokine

Tregs – regulatory T cells

TSAT – transferrin saturation

UtS – unstimulated saliva

WS – whole saliva

μL – microliter

μM – micromolar

SUMMARY

1 INTRODUCTION

Periodontitis is multifactorial inflammatory disease affecting the supporting tissues of the teeth, with progressive attachment loss and bone destruction (ARMITAGE, 1999). It is one of the most prevalent diseases in developed and developing countries, affecting about 20-50% of global population. Periodontitis has become a public health concern due to its high prevalence in adolescents, adults, and older individuals (NAZIR, 2017) and due to its associations with other inflammatory conditions such as type 2 diabetes, cardiovascular disease, respiratory tract infection, adverse pregnancy outcomes, neurodegenerative disease and cancer (BUI et al., 2019; KIM; AMAR, 2006; WHITMORE; LAMONT, 2014).

The inflammatory process observed in this disease is induced by a dysbiotic microbiota (HAJISHENGALLIS, 2014) and the continuous local chronic host response may alter the inflammatory profile resulting in deleterious effects in these tissues (BERGLUNDH; DONATI, 2005; GRAVES; COCHRAN, 2003).

Periodontitis was classified, from 1999 to November of 2017, as chronic (ChP) and aggressive periodontitis (AgP), localized or generalized, and necrotizing and as a manifestation of systemic diseases (ARMITAGE, 1999), based on clinical parameters extension and rate of disease progression. Recently, a new classification was proposed, classifying the disease in necrotizing periodontitis (HERRERA et al., 2018), periodontitis as a manifestation of systemic diseases (ALBANDAR; SUSIN; HUGHES, 2018) and “periodontitis”, which is further characterized by severity, progression rate, systemic effects and extension (PAPAPANOU et al., 2018; TONETTI; GREENWELL; KORNMAN, 2018).

The previously defined ChP was characterized by its slow progression, whereas AgP consisted of rapidly progressing disease, and both were further defined by their extension as generalized and localized (ARMITAGE, 1999). However, there are still no biomarkers allowing the differentiation among different clinical features of periodontitis (PAPAPANOU et al., 2018; TONETTI; GREENWELL; KORNMAN, 2018), reinforcing the need of studies on the pathobiology of this disease. The previously described localized AgP (LAgP) exhibits an early onset and affects mostly incisors and molars (ARMITAGE, 1999), and is currently classified as molar incisor pattern periodontitis (LIP) (CATON et al., 2018; FINE; PATIL; LOOS, 2018).

For more than 30 years, *Aggregatibacter actinomycetemcomitans* was considered the etiological agent of AgP, mainly in its localized form (NEWMAN et al., 1976; NEWMAN;

SOCRANSKY, 1977; SLOTS, 1976; SLOTS; REYNOLDS; GENCO, 1980). Six serotypes of *A. actinomycetemcomitans* are known (a, b, c, d and f), and serotype b is more frequently associated with the disease (KAPLAN et al., 2001; ZAMBON; SLOTS; GENCO, 1983). Currently, *A. actinomycetemcomitans* is seen mainly as an opportunistic pathogen of the resident oral microbiota, whereas its clone JP2, belonging to serotype b, may be considered a true exogenous pathogen (HAUBEK, 2010). The endemic presence of the highly leukotoxic JP2 clone was associated with the high prevalence of AgP, whereas a substantial role of non-JP2 clones in the disease was not established (HAUBEK et al., 2001).

Several studies supported the hypotheses that the presence *A. actinomycetemcomitans* would be required to initiate LAgP, which occurs with a higher frequency in African children or Hispanic descendants (HAUBEK et al., 1997; SHADDOX et al., 2010). However, the association between *A. actinomycetemcomitans* and AgP was based on target-directed methods, such as culture in selective media (SLOTS et al., 1982; ZAMBON; CHRISTERSSON; SLOTS, 1983), DNA hybridization (FAVERI et al., 2009; SHADDOX et al., 2012), and quantitative PCR (SARAIVA et al., 2014). The role of *A. actinomycetemcomitans* in LAgP was evidenced by the correlation of its elimination in response to periodontal treatment (MANDELL; EBERSOLE; SOCRANSKY, 1987; VAN WINKELHOFF; TIJHOF; DE GRAAFF, 1992) and by the high antibody response to this microorganism in patients with LAgP (ALBANDAR et al., 2001; ANDO et al., 2010; EBERSOLE; CAPPELLI, 1994; SARAIVA et al., 2014). In addition, its pathogenic role was suggested by the production of the cytolethal distending toxin (CDT), an exotoxin with immunemodulatory activity (ANDO et al., 2010; FERNANDES et al., 2008; SHENKER et al., 1999) and by the production of leukotoxin (LALLY; GOLUB; KIEBA, 1994).

Previous data using cloning and 16SrDNA sequencing, Faveri et al. (2008) could not detect *A. actinomycetemcomitans* in samples from patients with GAgP. However, it should be mentioned that the method could only detect species in proportion higher than 1% (personal information). *A. actinomycetemcomitans* can be detected in healthy individuals or affected by ChP (HÖLTTÄ et al., 1994; YANG et al., 2004), but its serotype b is more associated with AgP both in studies with culture-dependent methods (YANG et al., 2004), and indirectly by measuring serum antibody levels (ANDO et al., 2010).

A. actinomycetemcomitans pathogenic potential may be questioned especially due to the lack of experimental animal models of periodontitis (SCHREINER et al., 2003). Moreover, recognized periodontopathogens such as *Porphyromonasgingivalis*, *Treponema denticola* and

Tannerela forsythia are not only associated to ChP, but also to GAgP and LAgP (CHAHBOUN et al., 2015; FAVERI et al., 2009; SARAIVA et al., 2014; SOCRANSKY; HAFFAJEE, 1994).

It is currently accepted that periodontitis is the result of a microbial community and not exclusively by the presence of a single pathogen (FINE et al., 2013a) and the subgingival dysbiotic microbiota can be evidenced not only by the presence of periodontal pathogens but also by the low proportion of beneficial bacteria (VAN ESSCHE et al., 2013). Among individuals with *A. actinomycetemcomitans*, those with healthy periodontium showed a higher abundance of *Actinomyces* species (FINE et al., 2013b). Other data showed that the oral biofilm of LAgP patients present low levels of *Actinomyces naeslundi*, *A. gerencsiae*, *S. gordonii* and *S. oralis* when compared to healthy control patients (FAVERI et al., 2009). Furthermore, healthy patients harboring *A. actinomycetemcomitans* had a higher proportion of *Streptococcus* and *Actinomyces* species, whereas patients with bone loss had a higher proportion of *Parvimonas micra, Filifactor alocis*, *A. actinomycetemcomitans* and *Peptostreptococcus* sp. human oral taxon 113 (HOT-113). In addition, the presence of a bacterial consortium formed by *A. actinomycetemcomitans, S. parasanguinis* and *F. alocis* was strongly associated with bone loss in LAgP (FINE et al., 2013b). These data raised the hypothesis that *A. actinomycetemcomitans* is a keystone pathogen, by its ability to alter the subgingival environment by the production of toxins (leukotoxin and CDT), leading to an immunological paralysis and allowing the proliferation of pathobionts. Thus, data on the whole microbial community in LAgP are needed through the application of "open-end" methods, such as new generation sequencing (FINE et al., 2013b).

Due to the absence of evidence of a correlation among the microbial composition of the subgingival sites and the rate and severity of the periodontal tissue destruction, it was postulated that genetically-driven host factors would predispose to dysbiotic changes in the subgingival microbiota and consequently the rapid destruction observed in LAgP (NIBALI, 2015).

AgP seems to be inherited in an autosomal dominant way in Afro-descendants' families (MARAZITA et al., 1994). The incidence of AgP is often very high among certain families, with the percentage of affected siblings and affected lineage members reaching 40-50%, suggesting that genetic factors may be important in susceptibility to AgP (MENG et al., 2011). Few studies on family aggregation in ChP have been conducted so far, but there is evidence that parents with periodontal poor health tend to have children with periodontal disease, suggesting that family history is a valid representation of shared genetic and

environmental factors that contribute to the individual's periodontal condition (SHEARER et al., 2011).

The composition of the gut and oral microbiota differs, but the types of communities observed in one site may be predictive of the communities observed in the other (DING; SCHLOSS, 2014). Both communities are influenced by host factors, and the oral microbiota serves as an inoculum for the intestine. It is interesting to note that oral inoculation with *P. gingivalis* in experimental models leads to alteration of the intestinal microbiota, suggesting that this could be a possible mechanism for the association of other inflammatory diseases with periodontitis, such as cardiovascular diseases (ARIMATSU et al., 2014).

Recently, the gut microbiome of individuals with gingivitis, ChP and periodontal health was evaluated by *16S rRNA* sequencing (LOURENÇO et al., 2018). Abundance of Firmicutes, Proteobacteria, Verrucomicrobia and Euryarchaeota were increased, whereas abundance of Bacteroidetes were decreased in patients with ChP compared to healthy controls. *Mogibacteriaceae*, *Ruminococcaceae* and the genus *Prevotella* were able to discriminate between ChP and healthy individuals. Moreover, significant correlations between OTUs (operational taxonomic unit) representative of periodontopathogens and attachment loss were demonstrated. These findings suggested a relationship between oral and gut dysbiosis in affected individuals (LOURENÇO et al., 2018).

Differences in host factors are likely to play a determinant role on the balance between oral tissues and the microbiota. Populational studies have shown that subgroups of the population show differences in susceptibility to inflammatory diseases, including periodontitis (GORR, 2009). Saliva is a rich reservoir of different proteins and peptides and has been used as a powerful and accessible instrument in the search for host factors that can contribute to the understanding and diagnosis of systemic and oral diseases (ROI et al., 2019).

In the oral cavity, several proteins are released through secretions of different salivary glands and their proportional contribution to saliva varies depending on sympathetic and parasympathetic stimulation, circadian rhythm, health-illness spectrum, and personal habits such as diet and drug intake (LORENZO-POUSO et al., 2018; SIQUEIRA; DAWES, 2011). The total protein concentration in the secretion of each gland varies considerably depending on factors such as flow rate, duration of stimulation, nature of the stimulus and circadian rhythm (SIQUEIRA; DAWES, 2011). Whole saliva (WS) is composed mainly by secretions of three major glands (parotid, submandibular and sublingual) together with the minor salivary glands, but may also contain plasma proteins (such as albumin, transferrin and immunoglobulins) coming from the intimate contact between saliva and gingival crevicular fluid (GCF), which

achieves the oral cavity through the gap between the tooth and the overlying gingiva (LORENZO-POUSO et al., 2018; SIQUEIRA; DAWES, 2011). The contribution of GCF to the WS composition is higher in individuals affected by gingivitis and periodontitis (SIQUEIRA; DAWES, 2011). This complex mixture of fluids is composed by a variety of electrolytes (i.e., Ca^{2+}, Cl^{-}, $H_2PO_4^{-}$, HCO_3^{-}, I^{-}, K^{+}, Mg^{2+}, Na^{+}, and SCN^{-}), proteins, glycoproteins, lipids, desquamated epithelial cells, immune cells and microbial products that can impact the oral homeostasis (HUMPHREY; WILLIAMSON, 2001; SIQUEIRA; DAWES, 2011).

The major families of salivary proteins (structurally related proteins) include acidic and basic proline-rich proteins (PRPs), amylase, high-molecular-weight glycoprotein MUC5B, low-molecular-weight glycoprotein MUC7, aglutinin, cystatin, histatin and statherin (SIQUEIRA; DAWES, 2011). However, through the application of more sensitive analytical techniques (i. g., proteomic analyzes), a large number of other proteins have been detected (LOO et al., 2010), comprising more than 3,652 proteins and 12,562 peptides (LORENZO-POUSO et al., 2018).

The biosynthesis of salivary proteins begins into the salivary glands, followed by the post-translational process within the acinar cells, involving protein glycosylation, phosphorylation, sulfation and proteolysis (HELMERHORST; OPPENHEIM, 2007). When salivary proteins achieve the oral cavity, they can suffer other structural modifications, leading to a formation of a heterogenous protein-protein complex, proteolysis and deglycosylation, mainly by the activity of enzymes produced by oral bacteria (SIQUEIRA; DAWES, 2011). WS contain approximately 700 different species of microorganisms and the total number varies between 10^6-10^9/ml (CHEN et al., 2010). These microorganisms produce a variety of proteolytic enzymes and other enzymes (HELMERHORST; OPPENHEIM, 2007; ITO; HIROSE; TAKEUCHI, 1959). Many of these enzymes have not yet been characterized and they can quickly break down several proteins in the saliva (SIQUEIRA; DAWES, 2011).

Saliva also contains a large number of epithelial cells desquamated from the oral mucosa. The release of proteolytic enzymes by these cells is still uncertain, but they express a transglutaminase that can catalyze the formation of a g-glutamyl-g-lysine bound between glutamine and lysine residues (BRADWAY et al., 1989). In addition, leukocytes derived from GCF, especially during inflammation in periodontal diseases, when in contact with the hypotonic saliva (which has about one-sixth of the osmotic pressure of the plasma) suffer rupture and release many enzymes, including proteolytic ones (SIQUEIRA; DAWES, 2011).

Proteolysis and deglycosylation processes may alter the profile of salivary proteins by altering the composition of the acquired enamel pellicle (AEP) and, consequently, the receptors for bacterial adhesins. In periodontitis, there is greater neutrophil output from the gingival fluid and, possibly, the proteolysis of the salivary proteins will be greater. In addition, bacteria associated with ChP, such as *P. gingivalis, T. denticola* and *T. forsythia* are extremely proteolytic, unlike the bacteria that characterize the biofilm associated with health. On the other hand, some salivary proteins have mechanisms that hinder their degradation. Binding of histamin 1 to hydroxyapatite provides its resistance to proteolytic degradation by salivary enzymes and this binding may serve as a mean of maintaining other proteins in a stable form within the oral cavity (MCDONALD et al., 2011).

Hydroxyapatite has the ability to attract salivary phosphoproteins that bind strongly to the surface of the enamel, forming a protein film called AEP. A study of the total saliva phosphoproteome led to the identification of 65 salivary phosphoproteins (SALIH et al., 2010). Microbial colonization on tooth surfaces can occur by microbial recognition of specific receptors of the adsorbed proteins within the AEP. AEP is a thin acellular film predominantly composed by salivary proteins, but also by non-salivary-derived proteins, carbohydrates, and lipids (SIQUEIRA; CUSTODIO; MCDONALD, 2012). Among 130 proteins forming AEP, the majority is derived from epithelial cells (67.8%) and serum/gingival crevicular fluid (17.8%), and only 14.4% come from salivary glands secretions (SIQUEIRA et al., 2007).

The formation of the AEP is dependent on three major groups of proteins: proteins that bind to calcium ions (18% of the already identified AEP proteins) such as acidic proline-rich proteins (PRPs) and histatins, derived from exocrine salivary secretions; proteins that show a high tendency to bind to phosphate ions (15% of the AEP) such as elongation factor 2 and myosin 9, derived from epithelial cells; and proteins that interact with other proteins (28% of the AEP) (LEE et al., 2013). As an example, MUC5B form complexes with several other salivary proteins, including salivary α-amylase, histatin and statherin (IONTCHEVA et al., 2000; IONTCHEVA; OPPENHEIM; TROXLER, 1997). *In vivo*, the AEP is created by the formation of successive layers of proteins, based initially on binding to the mineral surface of the teeth (calcium and phosphate) and subsequently on protein-protein interactions (LEE et al., 2013).

The oral biofilm is initiated by the adhesion of early colonizers, which present adhesins that interact with saliva, serum, extracellular matrix compounds, host cells and other microorganisms (NOBBS; LAMONT; JENKINSON, 2009). Streptococci are the most successful early colonizers of the tooth surface, by binding to AEP proteins. For instance,

Streptococcus sanguinis, *S. gordonii* and *S. oralis* possess adhesins which recognize statherin, PRPs, amylase and salivary agglutinin (KOLENBRANDER et al., 2010). *S. gordonii* express surface adhesins, such as antigen I/II(AgI/II), SspA and SspB, CshA and CshB which mediate interactions with the salivary agglutinin gp340 (JAKUBOVICS et al., 2005), AbpA which binds to amylase (ROGERS et al., 1998), GspB and Hsa which binds to MUC7 (KESIMER et al., 2009), secretory IgA (S-IgA) (TAKAMATSU et al., 2005), and platelet glycoprotein Ibα (BENSING; LÓPEZ; SULLAM, 2004). The putative pathogens *A. actinomycetemcomitans* have the ability to adhere to AEP (RUDNEY; STAIKOV, 2002) by fimbrial binding to low-molecular-weight salivary mucin (MG2), lactoferrin, and S-IgA (GROENINK et al., 1998). Furthermore, certain salivary proteins can modulate the oral microbiome by their antimicrobial features, affecting bacteria distribution on/in dental biofilms, such as lactoferrin, lysozyme, histatin 1, 3 and 5, statherin, and others (DAWES et al., 2015).

Histatin 1, 3, and 5 are multifunctional proteins involved in the formation of AEP (OPPENHEIM et al., 2007), buffering process and can exhibit antibacterial and antifungal activities (HELMERHORST et al., 2005, 2006; OPPENHEIM et al., 2007; PURI; EDGERTON, 2014). Histatin 1 (his1) inhibits crystal growth of calcium phosphate salts (OPPENHEIM et al., 1988) and is the only phosphorylated histatin in the oral cavity (OPPENHEIM et al., 2007), whereas histatin 5 (his5) is active against pathogenic fungi, such as *Candida albicans* (HELMERHORST et al., 2006; OPPENHEIM et al., 1988; PURI; EDGERTON, 2014). *In vitro* studies revealed that histatins can also present antimicrobial effect against *Streptococcus mutans* (HELMERHORST et al., 1997; PAYNE et al., 1991) and *P. gingivalis* (BORGWARDT et al., 2014; GUSMAN et al., 2001b).

Histatins seems to present a modulatory effect, hindering biofilm formation. Clinical trials demonstrated that histatins can reduce gingivitis severity (PAQUETTE et al., 2002; VAN DYKE et al., 2002). Several human oral antimicrobial peptides play important roles including maintenance, repairing of oral tissues (hard or soft) and defense against oral microbes and do not present adverse effects to the host and/or tissues, indicating their great innovative potential for the development of new therapies against oral biofilm associated diseases, such as periodontitis (KHURSHID et al., 2017). The limitation of the use of these peptides/proteins involves the inconsistency between the presence of salivary proteins in glandular secretions and the absence of some of these proteins in WS (JENSEN et al., 1994; HELMERHORST et al., 2006). For example, histatins concentration in glandular secretion varies between 43-120 μg/ml, dropping to 2-8 μg/ml in WS (HELMERHORST, 2007). Thus, several studies aimed to explain these differences and evaluated histatins proteolysis (HELMERHORST, 2007;

HELMERHORST et al., 2006; OPPENHEIM et al., 1988; SIQUEIRA et al., 2012a; TAKAMATSU et al., 2005). In the oral cavity, histatins proteolysis occurs extremely fast (HELMERHORST, 2007; HELMERHORST et al., 2006; PAYNE et al., 1991), being triggered by bacterial and host derived enzymes (HELMERHORST, 2007).

The arsenal of salivary proteins and compounds with antimicrobial properties is huge and includes products of immune defense cells such as IgA, defensins, and nitric oxide (NO) (DAWES et al., 2015; FANG; VAZQUEZ-TORRES, 2002). The total concentration and relative proportion of salivary proteins are dependent on several factors (CABRAS et al., 2009; DAWES, 1987; RAYMENT et al., 2001), including age, since hormonal factors were associated with stabilization of the microbiota in adults (CASTAGNOLA et al., 2011). The concentration and activity of antimicrobial components could affect the distribution of bacterial species in the dental biofilm, and levels of these components may vary between individuals, also according to resistance to infection, or even physical exercise (GILLUM et al., 2014).

An early study, evaluating salivary proteins by using two-dimensional gel electrophoresis, revealed that the levels of serum albumin, immunoglobulin (Ig) gamma2 chain C region, Ig alpha2 chain C region, vitamin D-binding protein, salivary alpha-amylase and zinc-alpha2 glycoprotein were increased in whole unstimulated saliva of GAgP subjects when compared to healthy controls, while those of lactotransferrin, elongation factor 2, 14-3-3 sigma, short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor and carbonic anhydrase 6 were decreased (WU et al., 2009). Inflammation influences the WS composition, and gingivitis subjects present increased amounts of blood proteins (serum albumin and hemoglobin), Ig peptides and keratins than healthy controls, assessed by two-dimensional gel electrophoresis and liquid chromatography (GONÇALVES et al., 2011). Although some studies have demonstrated slight differences in cytokines levels such as IL-29 in GCF and serum of GAgP compared to ChP subjects (SHIVAPRASAD; PRADEEP, 2015), there are still conflicting data on differences in cytokines/chemokines profiles between AgP and ChP subjects (DUARTE et al., 2015). For instance, higher GCF levels of IL-8 were reported in GAgP (ERTUGRUL et al., 2013; FINE et al., 2014), whereas others reported reduced IL-8 levels in the GCF of sites with *A. actinomycetemcomitans* infection (SHADDOX et al., 2012). Furthermore, MIP-1α levels in both saliva and GCF were consistently elevated in AgP, and considered as a biomarker for bone loss in adolescents, independently on infection by *A. actinomycetemcomitans* (FINE et al., 2014).

Thus, the identification of saliva and AEP components, by proteomic analysis and immunedetection of chemokines and cytokines, as well as the characterization of the oral and

gut microbiota associated with LAgP, could facilitate the understanding of the pathogenesis of the disease and to clarify the role of salivary proteins in survival and emergence of the dysbiotic microbiota. This knowledge would allow the development of strategies aimed to recover the balance of the microbiota, in homeostasis with the host, useful for the prevention and control of LAgP. Moreover, the analysis of saliva components could help to establish the factors associated with LAgP risk, and healthy associated proteins could be used to modify the AEP or increase saliva antimicrobial properties in order to prevent and control periodontitis, applied as complementary therapy. The analysis of the salivary proteolytic activity of individuals affected by periodontitis against salivary proteins opens avenues for a comprehensive investigation of how these salivary proteins could be used as an additional diagnostic method for the prevention and early treatment of periodontitis.

2 OBJECTIVES

General objective: To test the hypothesis that patients with LAgP present differences in the oral and gut microbiome, as well as in the composition of whole saliva and AEP in relation to healthy individuals.

Specific objectives: To determine differences between subjects with LAgP and subjects with healthy periodontium in the following aspects:

- oral microbiome of supra and subgingival biofilms, and gut microbiome;
- genotyping of *Aggregatibacter actinomycetemcomitns* in oral biofilm samples;
- proteome analysis of stimulated whole saliva and acquired enamel pellicle;
- levels of nitrite (i. e., NO) in saliva through Griess colorimetric reaction;
- levels of cytokines and chemokines in saliva;
- Correlation between abundance of oral bacteria, salivary levels of cytokines/chemokines, and between oral bacteria and cytokines/chemokines;
- proteolytic activity of saliva using histatin 1 and 5, as indicators.

3 METHODS

3.1 Study subjects and clinical assessments

The Research Ethics Committee of the Biomedical Sciences Institute of University of São Paulo (number 1.119.953) and associated institutions provided the approval of this study. The subjects were informed about the study objectives and signed the ‘‘Informed and Free Consent Form”. Subjects were selected from 2015 to September of 2017, thus the term LAgP will be used, since patients were diagnosed according to the clinical criteria established by the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions (ARMITAGE, 1999).

Calibrated periodontists performed the clinical measurements. The following clinical parameters were evaluated: clinical attachment level (CAL, in mm), probing depth (PD) and, bleeding on probing (BoP) (no = 0/ yes = 1), measured at six sites per tooth in all teeth (excluding third molars), using a periodontal probe (Hu-Friedy®, Chicago, IL, USA).

LAgP subjects (n= 7) were females of Afro-descendant, aged between 19-26 years, with interproximal loss of attachment and destruction of alveolar bone in at least two permanent teeth (CAL ≥3 mm and PD ≥ 4 mm), one of which is the first molar, and involving no more than two other permanent teeth than first molars and incisors. Periodontally healthy age/race/and gender-matched subjects were selected as controls (n=8).

Periodontally healthy subjects (HLAgP) presented no sites with CAL and PD measurements < 3 mm, < 20% of sites exhibiting BoP, no extensive caries lesions and at least 28 permanent teeth (JOSS; ADLER; LANG, 1994). Exclusion criteria included pregnancy, smoking, current or previous periodontal treatment, presence of systemic diseases, use of medications that could affect the periodontium, and use in the previous three months of systemic antibiotics and/or mouthwashes containing antimicrobials. All subjects diagnosed with periodontitis received the required periodontal treatment after sample collection.

Differences in age mean and clinical parameters between subjects with LAgP and controls (HLAgP) were determined by Mann-Whitney U Comparison. The normality of the residue was evaluated by Shapiro-Wilk test and data were analyzed using BIOESTAT 5.0 (AYRES et al., 2007).

3.2 Genotyping of *Aggregatibacter actinomycetemcomitns* in the oral biofilm of LAgP and HLAgP

The levels of *A. actinomycetemcomitans* (*Aa*) JP2-like strains in oral samples of LAgP and HLAgP subjects were determined by quantitative PCR (qPCR) using *Taqman*. To detect the *orfX'* region, the primers sequences were JP2-F3 5'-TCT ATG AAT ACT GGA AAC TTG TTC AGA AT-3' and JP2-R2 5'-GAA TAA GAT AAC CAA ACC ACA ATA TCC-3' and the probe was 5'-FAM-ACA AAT CGT TGG CAT TCT CGG CGA A-TAMRA-3' (YOSHIDA et al., 2012). The number of copies of *Aa* JP2-like was calculated comparing the results of each sample to the standard curve containing 10 to 10^8 copies of *Aa* JP2 *orfX'* of 151bp clonned in a recombinant plasmid (pPCR 2.1 TOPO TA® vector, Invitrogen, Carlsbad, CA, EUA). SP and subgingival biofilm samples of each subject were pooled. The reaction was performed as follows: 1 µL of DNA template, 0.1 µL of probe, 0.12 µL of primers, 10 µL of *TaqMan* Master Mix and 8.75 µL of H_2O. The amplification cycle was 50ºC/2´, 95ºC/10´, followed by 40 cycles of 95ºC/15´´ and 60ºC/1´. qPCR reactions were performed in triplicate in a thermocycler Step One Plus Real-Time PCR System (Applied Biosystem, Foster City, CA, EUA).

3.3 Oral and Gut Microbiome analysis

3.3.1 Oral biofilm samples collection

Dental biofilm from supragingival (SP) and subgingival healthy (SH) sites (probing depth ≤3 mm) were collected from LAgP and HLAgP. Biofilms from periodontal pockets (probing depth >3 mm) were collected from LAgP subjects, named medium/deep (MD) sites. Subgingival samples were collected from one site per quadrant using sterile periodontal curettes and were pooled according to its periodontal condition (SH or MD). Biofilm samples were added to Tris EDTA buffer (10mM Tris-HCl, 0,1mM EDTA, pH 7.6). Fecal samples were self-collected by subjects using a sterilized recipient. Subjects were asked to store the specimen at -20°C and transport in Styrofoam box with recyclable ice. All samples were stored at -80°C until manipulation.

3.3.2 Oral biofilm samples processing and sequencing

DNA from oral dental biofilm samples was extracted using the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). DNA from fecal samples was extracted using the QIAamp® DNA Stool Mini Kit (QIA) (Qiagen, Hilden, Germany). All DNA extractions were performed according to the manufacturer's recommended protocol. The quality of the purified DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific) and Qubit 2.0 fluorometer (Life Technologies).

A barcoded primer set based on universal primers Bakt_341F CC TAC GGG NGG CWG CAG and Bakt_805R GAC TAC HVG GGT ATC TAA TCC (HERLEMANN et al., 2011) was used to amplify the hypervariable V4–V5 region of the 16S rRNA gene. DNA samples were shipped to Macrogen (Seoul, Republic of Korea) for high-throughput sequencing using Illumina MiSeq 2 x 250 platform according to the manufacturer's instructions.

3.3.3 Sequencing Data Analyses

Raw sequencing reads were filtered for length (>440 bp), quality score (mean >30) using USEARCH tools (EDGAR, 2010). Reads were assembled using PEAR software (ZHANG et al., 2014), with a minimum overlap of 20 bp and with an e-value cutoff of 4e-10. Sequences were clustered at 97% similarity, chimera filtered and singleton reads were removed using USEARCH (EDGAR, 2010). Reads were filtered only for bacterial sequences for further analyses in the Quantitative Insights Into Microbial Ecology (QIIME) 1.8.0 pipeline (CAPORASO et al., 2010). Representative sequences for each OTU were further subjected to taxonomic analysis using the BLAST method against SILVA 128 database (YILMAZ et al., 2014) for faecal samples and the Human Oral Microbiome Database (HOMD) version 15.1, a curated dataset for oral taxa (DEWHIRST et al., 2010), was used to classify the oral biofilms. Phylum with a relative abundance (RA) below 0.15%, as well as unclassified and unknown Bacteria were collapsed and named as "others" to generate relative abundance plots. Statistical analysis was performed on all taxonomic levels for RA data using the nonparametric t test in QIIME.

Alpha diversity was determined by Chao 1 (estimates the richness), ACE (Abundance-based Coverage Estimator), Shannon (estimates the diversity and evenness) and observed species (estimates the amount of unique OTUs found in each sample). At the same time,

Simpson's Index was calculated to visualize evenness. Student t test was applied to compare all parameters between groups of samples.

OTU tables of each pair groups were normalized to the RA using the cumulative sum scaling (CSS) normalization method (PAULSON et al., 2013). Beta diversity was determined by Weighted UniFrac (LOZUPONE et al., 2011), which takes into consideration the genetic distance of the community members (OTUs) in each sample to the members in the other samples and adds information about the relative abundance of each OTU to every genetic distance. Principal Coordinates Analysis (PCoA) was used in order to visualize the generated distance matrices, which helps to get the principal coordinates and allows visualization of complex, multidimensional data. PERMANOVA test (vegan::adonis) was performed in each case to determinate if the separation between samples groups is significant (ANDERSON, 2001).

Spearman's Rank correlation test was determined between species RA in oral biofilm samples of LAgP and HLAgP, considering only strong correlations (positive correlation: $R \geq 0.97$, $p<0.001$; negative correlation: $R \leq -0.97$, $p<0.001$), with the aid of the package "corrplot" (WEI; SIMKO, 2017) in RStudio version 3.4.4 (Integrated Development for R. RStudio, Inc., Boston, MA, USA).

3.3.4 Core Microbiome

Core microbiomes data consisting of the OTUs detected in 50% of samples from each site of LAgP and healthy subjects were obtained using QIIME. SP, SH and MD oral biofilm phylotypes at the species level and gut phylotypes at the genus level were plotted in a Venn diagram using Venny 2.1 (OLIVEROS, 2015-2017).

3.3.5 Firmicutes/Bacteroidetes ratios

Firmicutes/Bacteroidetes ratios were calculated based on the total amount of Firmicutes and Bacteroidetes present in samples from each site (gut, SP, SH and MD) compared between LAgP and HLAgP. The non-parametric Wilcoxon statistical test was performed using BioEstat 5.3 software (AYRES et al., 2007).

3.4 Samples collection and preparation

3.4.1 Saliva collection and preparation

To minimize circadian effects, saliva samples were collected between 9:00 and 11:00 A.M. Unstimulated saliva (UtS) was collected by passively drooling into a chilled centrifuge tube for 5-10 min (NAVAZESH; CHRISTENSEN, 1982). Stimulated saliva (StS) was collected under mastication of Parafilm, 25 cm^2 ~1 g. Approximately 5 mL of saliva was collected by each method. Saliva samples were kept on ice during the collection procedure. Afterwards, samples were centrifuged at 14,000×g for 20 min at 4°C (SIQUEIRA et al., 2012b). Saliva supernatant (SS) was separated and kept at -80°C until use. The total protein concentration of SS was measured by the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin used as the standard. Aliquots of 20µg of UtS and StS protein from each individual and pooled samples from each group (HAgP and LAgP) were prepared and dried (VacufugeTM Eppendorf AG, Barkhausenweg, HH, Germany) to check for proteins presence, quality of the samples in SDS-PAGE and for proteomic analysis.

3.4.2 AEP collection and preparation

The collection of *in vivo* AEP was carried out as described previously (SIQUEIRA; OPPENHEIM, 2009). Samples were also collected in the morning to avoid circadian effects on pellicle composition. Each donor was subjected to a dental prophylaxis employing coarse pumice containing no additives. AEP was then allowed to form on the enamel surfaces over a 2 h period in order to have a pellicle comprised by both precursor proteins and proteins clusters. During this time span, the participants were asked to refrain from any consumption of food or beverages, other than water. After 2 h, teeth from each quadrant were isolated with cotton rolls, washed with distilled water and dried by air. For the actual removal of AEP from the enamel surface, collection strips of 0.5 cm × 1.0 cm (electrode wick filter paper, Bio-Rad, Hercules, CA, USA) pre-soaked in 3% citric acid were folded so that one half could be held using a dental forceps (Hu-Friedy, Chicago, IL, USA) and the other half could be brought in contact with the tooth surface. To avoid any contamination emanating from the gingival margin, only the coronal two thirds of the labial/buccal surfaces were swabbed. One collection strip was used per quadrant, starting with the buccal area of the central incisor and ending at the buccal surface

of the first molar. The collection was carried out in both dental arches. A total of four collection strips from each participant were obtained and placed into a polypropylene microcentrifuge tube. The collection strips were then kept frozen at −20 °C until used.

To extract the AEP proteins from the collection strips, 200 µL of 50 mM ammonium bicarbonate, pH 7.8 were added to each polypropylene micro centrifuge tube, containing the four collection strips from each subject. Each microcentrifuge tube was then sonicated for 1 min, and the recovered solution was then collected and placed into a new microcentrifuge tube for each subject. This procedure was repeated for a total of 4 times. The extracted solution was then centrifuged at 14,000× g for 15 min and the supernatant was extracted. This centrifugal procedure was carried out to prevent the debris from the collection strip that could be released into the solution during the sonication step. The supernatant was dried using a rotary evaporator (Eppendorf, Parkway, NY, USA), and then resuspended in 100 µL of distilled water. Protein concentrations were determined calorimetrically by Micro bicinchoninic acid (Micro BCA) assay. The remaining volume of each sample was dried using a rotary evaporator and stored at 4 °C until manipulation.

3.4.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A total of 20µg of UtS and StS protein from each individual and pooled samples from each group (HAgP and LAgP) were re-suspended in 20µL of sample buffer (0.4 M Tris−HCl pH 6.8, 2% SDS, 20% glycerol, 165 mM dithiothreitol (DTT), 0.4% bromophenol blue, 2% 2-mercaptoethanol). After boiling for 5 min, samples were loaded directly in the wells of the 12% SDS-PAGE gel. Five microliters of protein standard (Precision Plus Protein™ All Blue Pre-stained Protein Standards, BioRad, Hercules, CA, USA) were also loaded as molecular weight marker (MWM) control. Gel electrophoresis was carried out at a constant voltage of 100 V. After staining overnight with Coomassie Blue (40% methanol, 10% acetic acid, 2g Coomassie blue), gels were distained (40% methanol, 10% acetic acid, 50% water) for 2 hours with shaking and stored in ultrapure water until image analysis. Gel images were obtained by a densitometer Bio-Rad ChemiDrop MP (Bio-Rad Inc., Hercules, USA), connected to a computer using the Image Lab 5.2 software (Bio-Rad Inc., Hercules, USA).

3.5 Levels of nitrite in saliva

The SS of UtS saliva samples of LAgP and HLAgP subjects were used to determined the levels of nitrite (NO_2^-) to investigate nitric oxide (NO) formation. The quantification is based on the Griess colorimetric reaction described by Han et al. (2013). Griess reagent is a 1:1 misture of 1% sulfanilamide in 5% phosphoric acid and 0.1% of N-1-napthylethylenediamine dihydrochloride in distilled water (v/v). This reagent reacts with nitrite and produces a purple dye as final product, which can be measured by spectrometry based in maximum absorbance of 570nm. Fifty μL of SS of UtS samples were mixed with equal volumns of the Griess reagent in triplicate in 96 wells plate. The absorbance was compared to the standard curve consisting in triplicates of sodium nitrate ($NaNO_2$) in PBS (pH of 7.2) in diferent concentrations (100-50-25-12,6-6,25-3,12-1.56 e 0,78 μM) After 10 min, the optical density was measured in a ELISA reader applying 540nm filter. Nitrite quantification was performed in three independent assays. Statistical analysis was performed based on the non-parametric Mann-Whitney test in BIOESTAT 5.0 (AYRES et al. 2007). The significance level was set at 5% ($p<0.05$).

3.6 Levels of chemokines and cytokines in saliva

Chemokines (CM), cytokines (CT) and growth factor levels (pg/mL) in SS of StS samples were evaluated by a Bio-Plex Pro™ Human Chemokine assay kit (Bio-Rad, Hercules, CA, USA) as described on http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6499.pdf and following the manufacturer's instructions, using Bio-Plex analyzer (Luminex Corp., Austin, TX, USA) by comparison with a standard curve (mean of fluorescence intensity versus pg/mL), with the aid of Bio-Plex software manager 4.0.

Differences in age mean, clinical parameters, CT and CM levels between LAgP and HLAgP were determined by Mann-Whitney U Comparison. The normality of the residue was evaluated by Shapiro-Wilk test and data were analyzed using GraphPad Prism version 4.0 (La Jolla, CA, USA). Correlations between clinical parameters (PD and CAL) and mediators' levels were determined by Spearman's Rank correlation test with the aid of the Statistical Package for the Social Sciences v17.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at 5% ($p<0.05$).

Spearman's Rank correlation test was also used to determine correlations between CT and CM levels in saliva, as well as the salivary levels of CT and CM were correlated to species abundance in oral biofilm samples of LAgP and HLAgP, considering only strong correlations (positive correlation= $R \geq 0.97$, $p<0.001$; negative correlation= $R \leq -0.97$, $p<0.001$), with the aid of the package "corrplot" (WEI; SIMKO, 2017) in RStudio version 3.4.4 (Integrated Development for R. RStudio, Inc., Boston, MA, USA).

3.7 Proteolytic activity analysis

3.7.1 Histatins degradation assay

In order to evaluate the saliva proteolytic activity of subjects affected by LAgP and compare healthy controls, we analyzed the degradation of histatins in StS samples of both groups. The protein concentration in the StS of each individual and pooled samples were adjusted to the average protein concentration of the group (LAgP= 1075.5µg/mL, HLAgP= 834.5µg/ml, ChP= 1170.9µg/ml, HChP= 846µg/ml) in a final volume of 100µL. Synthetic his1 and 5 (American Peptide Company, Sunnyvale, CA, USA) were added in diluted stimulated SS (DStSS) (1:10 in sterile distilled water) to a final concentration of 100 µg/mL. The characterization of his1 and his5 degradation products were conducted according to Helmerhorst et al. (2006) and samples were incubated at 37 ºC in a water bath under different time-points: 0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours. Immediately after the addition of proteins (t = 0), and after different incubation times, two 50 µl aliquots were removed and boiled to abolish proteolytic activity. The aliquots were dried (VacufugeTM Eppendorf AG, Barkhausenweg, HH, Germany) and stored at -20 ºC. Next, samples were re-suspended and were submitted to native cationic polyacrylamide gel electrophoresis.

3.7.2 Native cationic polyacrylamide gel electrophoresis (Cationic-PAGE)

Cationic polyacrylamide gel electrophoresis was performed as described by Baum et al. (1976) and Oppenheim et al. (1988). The degradation aliquots of DStSS from each subject added with his1 or his5 at each time-point (0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours) and histatins

standards (4 µg of each histatin 1, 3 and 5) were resuspended in 20 µL of sample buffer (4 g sucrose, 4mg methyl green in a 10 mL final volume). Protein separation was performed by electrophoresis in 20% acrylamide gel at a constant voltage of 120 V. Gels were stained (0.1% Coomassie blue, 8% methanol, 7% acetic acid) overnight with shaking, and distained with 40% methanol, 10% acetic acid and 50% water. Gels images were obtained as described for SDS-PAGE. Pixel intensity was measured using Image Lab 5.2 software (Bio-Rad Inc., Hercules, USA). All values were corrected for background intensity. The pixel intensity of the protein band at t=0 (immediately after mixing his1 or 5 to DStSS) was set to 100%.

3.7.3 Extraction of histatin degradation peptides from stained polyacrylamide gel

DStSS pooled samples from each group (HAgP and LAgP) added with his1 or his5 at each time-point (0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours) were submitted to Cationic-PAGE and the bands containing the degradation peptides were excised from the gel and were cut into 1×1 to 2×2 mm pieces. Gel pieces were distained (200 µl of 25 mM ammonium bicarbonate in 50% acetonitrile) at 37°C for 30 minutes with shaking. Peptides were removed by shrinking the gel pieces with 50 µl of solution 1 (acetonitrile + 0.1% of trifluoroacetic acid - TFA), rehydrating with 20 µl of solution 2 (0.1% of TFA in water), and final shrinking with solution 3 (acetonitrile + 0.5% of TFA). Samples were dried and submitted to mass spectrometry, as reported in item 4.7.5.

3.7.4 In-Solution Digestion

Dried aliquots of AEP and StS prepared for mass spectrometry analysis were resuspended in 50 µL of 4 M urea, 10 Mm DTT and 50 mM ammonium bicarbonate at pH 7.8 and incubated for 1 hour at room temperature. Afterwards, 150 µL of 50 mM ammonium bicarbonate was added to the samples, followed by 2% (w/w) trypsin (Promega, Madison, WI, USA). Samples were then incubated overnight at 37 °C. Finally, the samples were dried in a rotary evaporator, de-salted by C-18 ZipTip® Pipette Tips (Millipore, Billerica, MA, USA), and subjected to mass spectrometry (LC-ESI-MS/MS).

3.7.5 LC-ESI-MS/MS analyses

Mass spectrometric analyses were carried out with a LTQ-Velos (Thermo Scientific, San Jose, CA, USA) which allows for in-line liquid chromatography with the capillary fused silica column (column length 10 mm, column ID 75 μm) packed in-house using C-18 resin of 5μm spherical beads and 200 Å pores size (Michrom BioResources, Auburn, CA, USA) linked to the mass spectrometer using an electrospray ionization in a survey scan in the range of m/z values 390–2000 tandem MS/MS. A dynamic exclusion criterion was established as a repeat count of 1 and a repeat duration of 30 s. All samples were dried by rotary evaporator and re-suspended in 15 μL of 0.1% formic acid then subjected to reversed-phase nLC-ESI-MS/MS. The nano-flow reversed-phase HPLC was developed with linear 85-minute gradient ranging from 50% to 100% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 200 nL/min with a maximum pressure of 280 bar. Electrospray voltage and the temperature of the ion transfer capillary were 1.9 kV and 250 °C respectively. Each survey scan (MS) was followed by automated sequential selection of seven peptides for CID, with dynamic exclusion of the previously selected ions.

3.7.6 Identification in MS/MS

For the identification of proteins spectra obtained from MS/MS were searched databases of human proteins (UniProt TrEMBL and, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot) using the algorithm Sequest software Proteome Discoverer 1.3 (Thermo Scientific, San Jose, CA, USA). Search results were filtered by a false detection rate of 1% using a search strategy using a database of decoy reverse. A protein which passes beyond the inclusion of the filter arrangement at least in three different MS analyzes of the same group in a total of four analyzes by MS group.

3.7.7 Protein Annotation

The identified proteins were classified and assigned by origin, molecular interaction and biological function using two web-based applications: Uniprot database

(https://www.uniprot.org/) and PANTHER Classification System (http://pantherdb.org/). Protein lists were plotted in a Venn diagram using Venny 2.1 (OLIVEROS, 2007-2015).

3.7.8 Statistical analysis

Student t Test was performed to determine differences in the protein composition by important salivary processes and function between groups.

To evaluate the influence of studied groups on histatins degradation upon different incubation periods, ANOVA with repeated measures was applied. To identify pairs of means that differed from each other, a multiple comparison of means with Bonferroni correction was performed.

The significance level of all tests was set at 5% ($p < 0.05$).

4 RESULTS

4.1 Clinical data

The total sample consisted of 7 LAgP-affected subjects and 8 healthy controls. Demographic and periodontal clinical data of the volunteers are shown in Table 1. PD, CAL and BoP were significantly higher in LAgP than in HLAgP.

In Appendix 1 are listed the samples used in each experiment, since it was not possible to obtain all samples of each subject.

Table 1. Demographic and periodontal clinical parameters of the studied population. *Difference between LAgP and the healthy control group (HLAgP) by Mann-Whitney test ($p < 0.05$).

Variables	LAgP (n=7)	HLAgP (n=8)
Age (mean ± SD)	21.29 ± 2.29	21.25 ± 2.12
Gender (%) **Female**	100	100
PD (mm ± SD)	2.25 ± 0.65*	1.71 ± 0.31
Affected sites	4.8±0.8	
CAL (mm ± SD)	2.85 ± 0.3*	1.91 ± 0.43
Affected sites	4.9±1.0	
BoP (mean % ± SD)	43.54 ± 16.58*	15.00 ± 5.05

4.2 Microbiome Analysis

4.2.1 Bacterial community profiling

The oral and gut microbiomes of patients with LAgP and HLAgP were determined by *16S rRNA* sequencing in a total of 51 samples, which generated a total of 3,604,164 high quality paired-end reads, and the average number of 70,670 reads per sample (the minimum and maximum numbers of reads from 51 samples were 56,631 and 83,131, respectively). A total of 3,315 OTUs were classified among oral and feces samples. Statistical analyzes were performed in order to compare oral sites altogether of LAgP (oral_LAgP) to oral sites altogether of HLAgP (oral_HLAgP), SP sites of LAgP (SP_LAgP) to SP sites of HLAgP (SP_HLAgP), SH and MD sites of LAgP (SH_LAgP and MD_LAgP, respectively) to SH sites of HLAgP (SH_HLAgP).

4.2.2 Taxonomical analyses of the oral biofilm microbiome

OTUs detected in oral samples were distributed in 11 phyla, 75 families and 513 species, classified by HOMD database. The most abundant phyla detected in all samples are listed in Figure 1. Taxa belonging to phyla Epsilonbacteraeota, Lentisphaerae, Tenericutes, Verrucomicrobia were not detected in oral sites. In descending order, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Actinobacteria were the predominant phyla in oral sites

altogether, SP and SH sites of LAgP and HLAgP subjects (abundance >7%). The relative RA of Actinobacteria was increased in oral_HLAgP compared to oral_LAgP ($p<0.05$), while Spirochaetes and Synergistetes were more abundant in oral_LAgP than in oral_HLAgP, and in MD_LAgP when compared to SH_HLAgP ($p<0.01$). No statisticaly significant differences were observed between SP and SH sites of both groups at the phylum level.

Statistical analyzes were performed to determine significant differences between sites of the same group at the phylum level. Synergistetes was more abundant in MD_LAgP ($p<0.05$) when compared to SH_LAgP. The comparison between SP_LAgP and MD_LAgP revealed that the RA of Synergistetes and Spirochaetes was increased in MD_LAgP ($p<0.05$ and $p<0.001$, respectively). No statisticaly significant differences were observed in the RA of any detected phyla between SP and SH sites of LAgP and HLAgP.

At the family level, *Micrococcaceae* (Actinobacteria), *Flavobacteriaceae* (Bacteroidetes), *Gemellaceae*, *Carnobacteriaceae*, and *Streptococcaceae* (Firmicutes) were more abundant in oral_HLAgP than in oral_LAgP ($p<0.05$), whereas *Porphyromonadaceae* (Bacteroidetes), *Peptococcaceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Selenomonadaceae* (Firmicutes), *Rhodocyclaceae*, *Desulfobulbaceae*, *Desulfovibrionaceae* (Proteobacteria), *Spirochaetaceae* (Spirochaetes), and *Synergistaceae* (Synergistetes) were more abundant in oral_LAgP ($p<0.05$). The analyzes per site revealed that *Xanthomonadaceae* (Proteobacteria) was more abundant in SP_LAgP, whereas the abundance of *Selenomonadaceae* (Firmicutes) was increased in SP and SH sites of LAgP when compared to SP and SH sites of HLAgP, respectively. *Commamonadaceae* and *Rhodocyclaceae* (Proteobacteria) were more abundant in SP and MD sites of LAgP when compared to SP_HLAgP and SH_HLAgP, respectively. *Pasteurellaceae* (Proteobacteria) was more abundant in SH_HLAgP, whereas *Pseudomonadaceae* was increased in SH_LAgP ($p<0.05$). *Cardiobacteriaceae* (Proteobacteria), *Gemellaceae* and *Streptococcaceae* (Firmicutes) were more abundant in SH_HLAgP when compared to MD_LAgP. *Micrococcaceae* (Actinobacteria) abundance was higher in SH_HLAgP than in SH and MD sites of_LAgP. On the other hand, *Desulfovibrionaceae* (Proteobacteria) abundance was increased in SH and MD_LAgP compared to SH_HLAgP. *Porphyromonadaceae* (Bacteroidetes), *Peptostreptococcaceae* (Firmicutes), *Helicobacteraceae, Desulfobulbaceae* (Proteobacteria), *Spirochaetaceae* (Spirochaetes), and *Synergistaceae* (Synergistetes) were more abundant in MD_LAgP when compared to SH_HLAgP.

At the species level, the abundance of 81 species differed between oral_LAgP and oral_HLAgP, 21 between SP_LAgP and SP_HLAgP, 16 between SH_LAgP and SH_HLAgP, and 78 between MD_LAgP and SH_HLAgP ($p<0.05$) (Appendix 1).

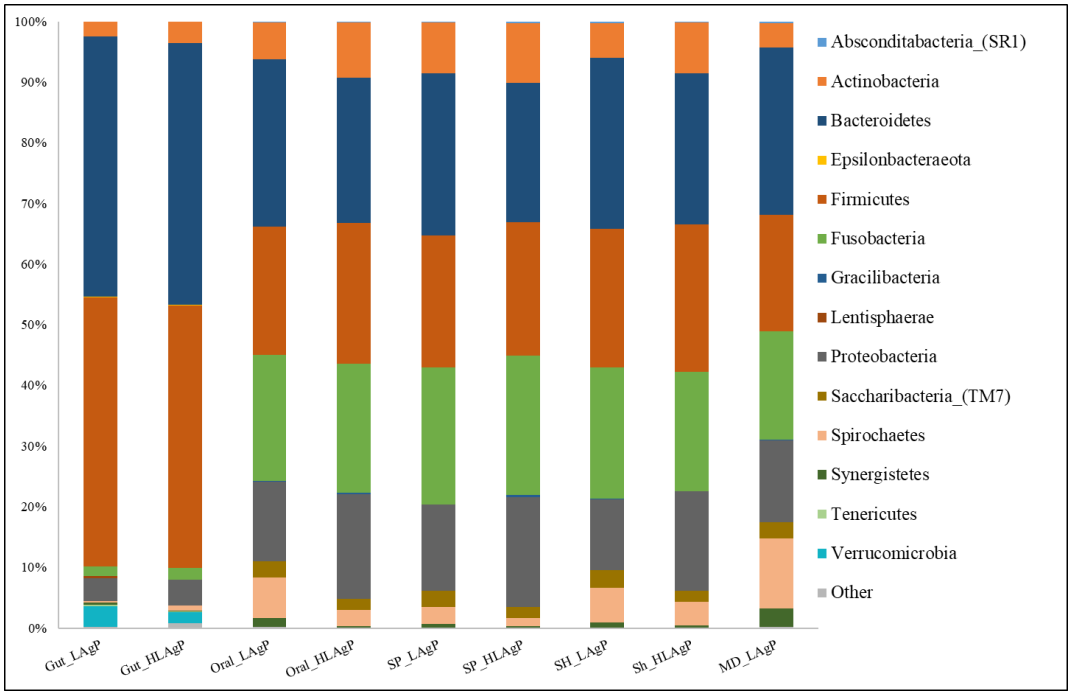

Figure 1. Bacteria relative abundance plots at the phylum taxonomic level per site: gut, supragingival (SP), shallow (SH) and medium/deep (MD) subgingival biofilms of LAgP and HLAgP groups.

The RAs of species with lower (negative values) or higher abundance (positive values) in SP, SH and MD sites of LAgP compared to HLAgP ($p<0.05$, non parametric t test) are presented as $\log_2$ fold change ($\log_2$FC) in figure 2. Species detected only in one of the groups were not included in the graphics, however, they are listed in Appendix 2 (marked with asterisk).

In the SP biofilm (Figure 2A), the RA of 18 species differed between the groups, 13 were in higher abundance whereas 5 were in lower abundance in LAgP when compared to HAgP. Species that were more abundant ($\log_2$FC >4) in SP_LAgP than in SP_HLAgP were *Johnsonella* sp._HMT_166, *Sneathia sanguinegens* and *Acidovorax ebreus*, whereas the RA of *Gemella haemolysans* was decreased ($\log_2$FC <-3) in SP_LAgP. In the SH biofilm (Figure 2B), of 14 species, the RAs of 11 were increased and 3 were decreased in LAgP. Species that were more abundant ($\log_2$FC >4) in LAgP than in HLAgP were Absconditabacteria_(SR1)_[G-1] bacterium_HMT_875 and *Selenomonas* sp._HMT_936, and the RAs of any of them were

reduced with log_2FC <-3. In affected sites (Figure 2C), of 73 species, the RAs of 44 were increased and 29 were decreased in MD_LAgP compared to SH_HLAgP. Species that were more abundant (log_2FC >4) in MD_LAgP than in SH_HLAgP were *Aggregatibacter actinomycetemcomitans*, *Treponema* sp._HMT_249, *Veillonellaceae*_[G-1] bacterium_HMT_135, *Veillonellaceae*_[G-1] bacterium_HMT_145, *Lachnoanaerobaculum* sp._HMT_496, *Treponema* sp._HMT_490, *Helicobacter pylori*, *Desulfobulbus* sp._HMT_041, *Peptostreptococcaceae*_[XI][G-3] bacterium_HMT_950, *Fretibacterium* sp._HMT_361, *Treponema* sp._HMT_238 and *Acidovorax ebreus*, whereas the RAs of *Prevotella* sp._HMT_376, *Eggerthella lent*a and *Veillonella atypica* were decreased (log_2FC <-3).

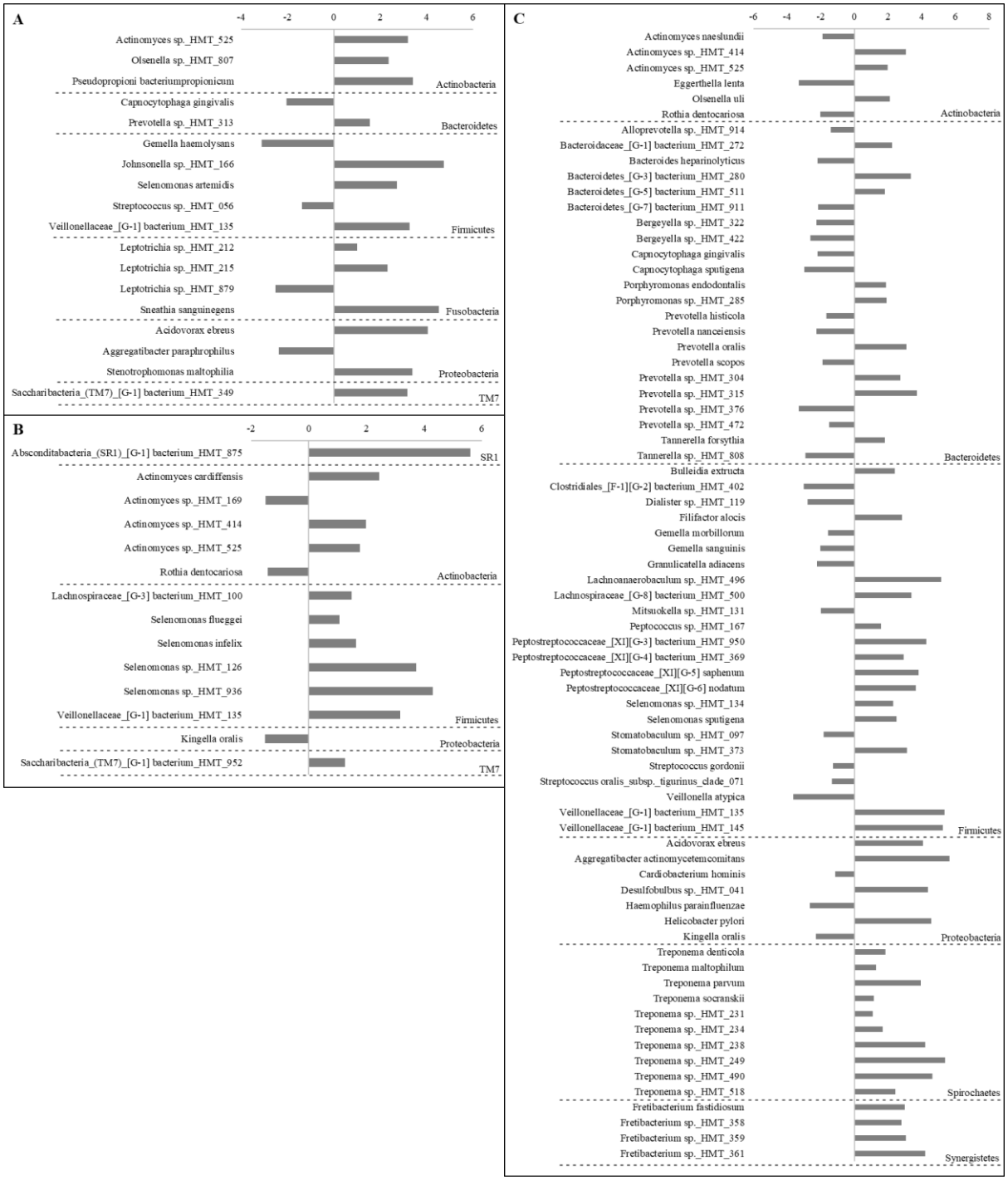

Figure 2. Relative abundance of species with lower (negative values) or higher abundance (positive values) in: supragingival (A), shallow (B) and medium/deep vs. shallow (C) sites of LAgP compared to HLAgP (non-parametric t test, $p<0.05$). Results are presented as $\log_2$fold change.

4.2.3 Taxonomical analyses of the gut microbiome

OTUs detected in faecal samples were distributed in 15 phyla, 98 families and 312 genera, classified by SILVA database. Firmicutes and Bacteroidetes were the predominant phyla (Figure 1). No statistically significant differences were observed between groups at the phylum level. At the family level, *Desulfovibrionaceae* was more abundant in LAgP than in HLAgP ($p<0.01\%$). The genus *Shuttleworthia* (Firmicutes) was detected only in LAgP samples ($p<0.01\%$), whereas *Desulfovibrio* (Proteobacteria) was more abundant in LAgP than in HLAgP samples ($p<0.05$).

4.2.4 Core microbiome

The core microbiome was calculated based on the OTUs present in 50% of the subjects of the same group and shared OTUs. The absolute numbers and percentages are demonstrated on Venn diagrams (Figure 3). Several OTUs were common between 50% of the subjects of each group, however the core microbiome of HLAgP was composed by a greater number of OTUs than in LAgP, specially in SP and when comparing SH_HLAgP to MD_LAgP.

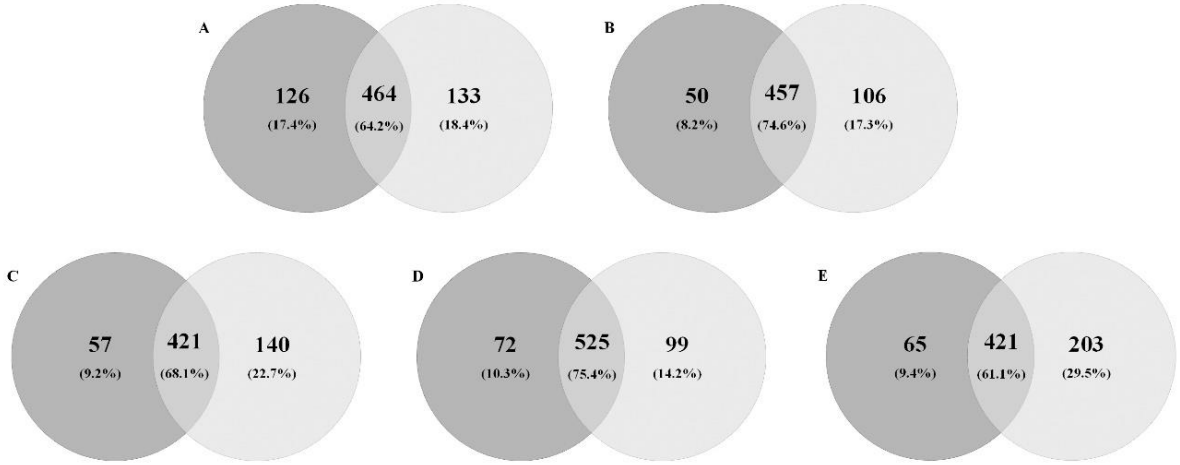

Figure 3. Venn diagram of the core microbiome based on the absolute number and percentage of OTUs present in 50% of the subjects of LAgP (dark grey circle) and HLAgP (light grey circle) and overlaps in each site: gut (A), oral (B), supragingival (C), shallow (D) and medium/deep vs. shallow (E).

The core microbiome was also evaluated at the specie and genus level of taxonomy for oral altogether and faecal samples, respectively. It was revealed common and exclusive taxa between LAgP and HLAgP. Figure 4 shows the species present in 50% of the subjects of each group and common in the oral biofilm of LAgP and HLAgP. A total of 291 species were common between oral_LAgP and oral_HLAgP, while 24 species were exclusively detected in LAgP and 23 in HLAgP. In "Common species" box are species that were more abundant in oral_LAgP (left column), and species that were more abundant in oral_HLAgP (right column). Due to the large number of common species observed in oral samples, only those who presented a statistically significant difference between the groups are shown in figure 4. The complete list of species is in appendix 3.

Among faecal samples, 50% of the subjects of the same group presented a total of 160 genera in common between LAgP and HLAgP, while 13 were exclusively detected in LAgP and 17 in HLAgP (Appendix 4). Of the common genera, the RA of *Desulfovibrio* was higher in LAgP than in HLAgP ($p<0.05$), whereas *Lachnospiraceae* CAG-56 was more abundant in HLAgP ($p<0.05$). Among the exclusive genera, only *Shuttleworthia* was detected in 50% of the subjects of LAgP and it was more abundant in this group than in HLAgP ($p<0.01$).

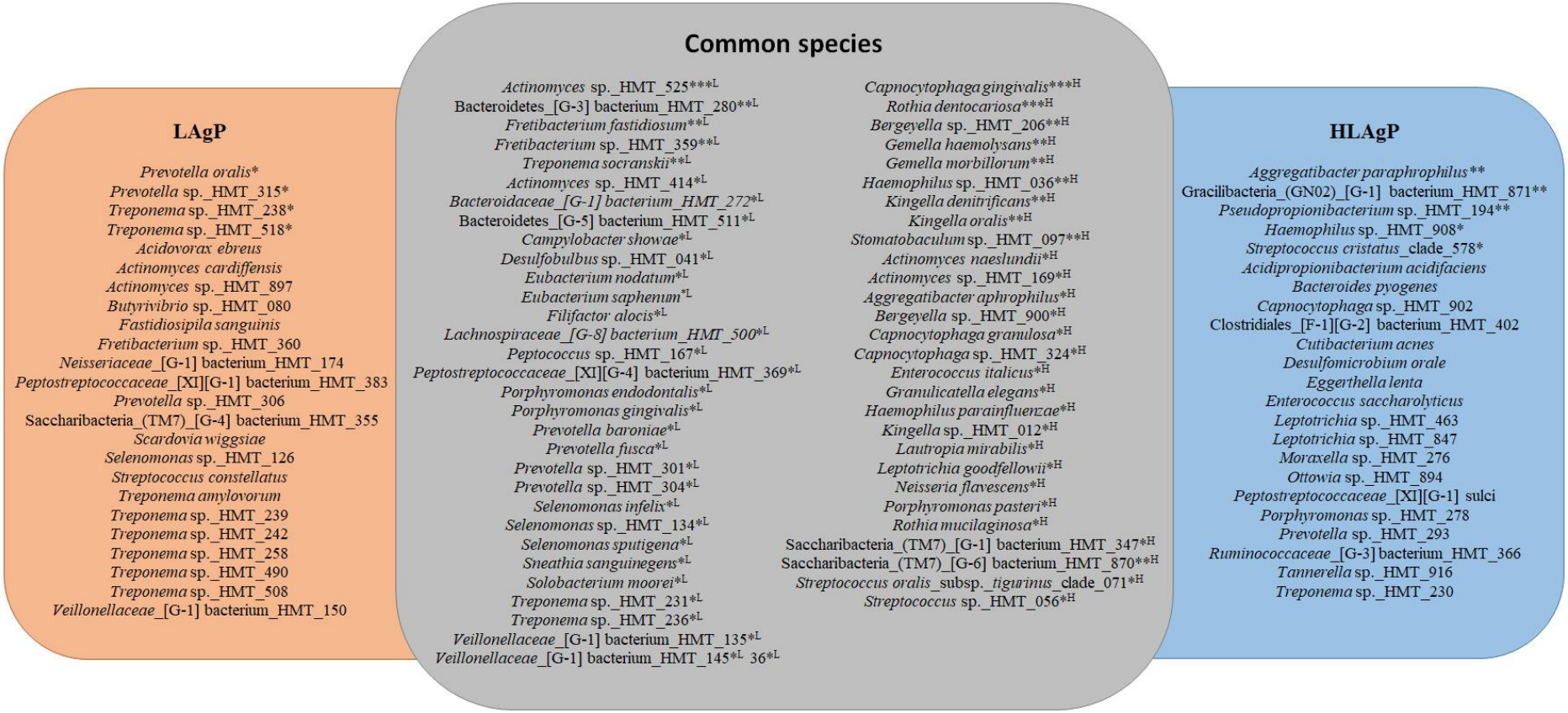

Figure 4. Core microbiome based on species present in oral biofilm of 50% of the subjects of LAgP, HLAgP and common species. ^{L} indicates species more abundant in LAgP and ^{H} in HLAgP. Asterisks indicate statistically significant difference in relative abundance between oral_LAgP and oral_HLAgP (*p<0.05; **p<0.01, ***p<0.001, nonparametric t test).

4.2.5 Firmicutes/Bacteroidetes ratio

Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated based on the total amount of Firmicutes and Bacteroidetes present in samples from each site (gut, SP, SH and MD) compared between LAgP and HLAgP (Figure 5). The mean of F/B ratio of each group and site was as follow: 1.03 for gut_LAgP, 1.00 for gut_HLAgP, 0.77 for oral_LAgP, 0.97 for oral_HLAgP, 0.81 for SP_LAgP, 0.96 SP_HLAgP, 0.81 for SH_LAgP, 0.97 for SH_HLAgP, 0.69 for MD_LAgP. F/B ratio of each site was compared between LAgP and HLAgP, as well as different sites of the same group. Despite the tendency towards the reduction of RA of Firmicutes and increase of Bacteroidetes in LAgP oral sites samples when compared to HLAgP, and the opposite observed in the gut microbiome, no statisticaly significant difference was observed ($p>0.05$, non-parametric Wilcoxon test).

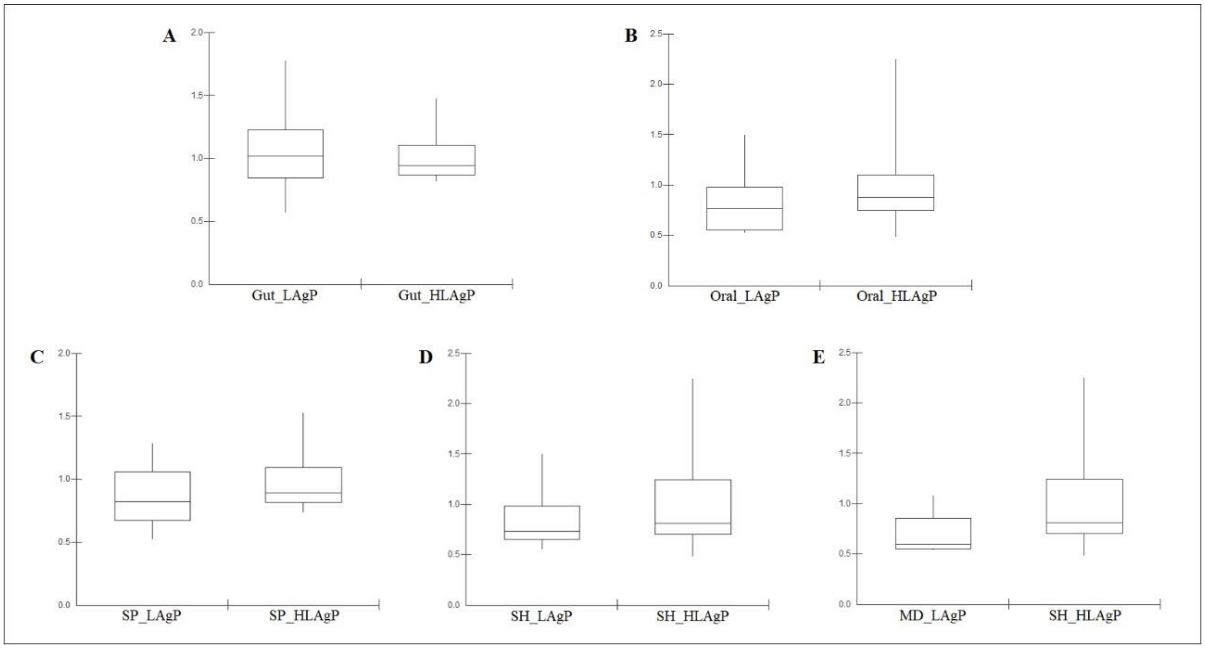

Figure 5. Box-plots of Firmicutes/Bacteroidetes ratios in each site: gut (A), oral (B), supragingival (C), shallow (D) and medium/deep vs. shallow (E). Horizontal lines represent the median. Boxes contain 50% of all values and whiskers represent the 25th and 75th percentiles. No statisticaly significant difference was observed ($p>0.05$, non-parametric Wilcoxon test).

4.2.6 Diversity analysis

Alpha diversity indexes analysis, a measure of the overall community structure, did not show significant differences in the oral and faecal samples between groups ($p>0.05$, Student *t* test). The alpha diversity results are provided in figure 6.

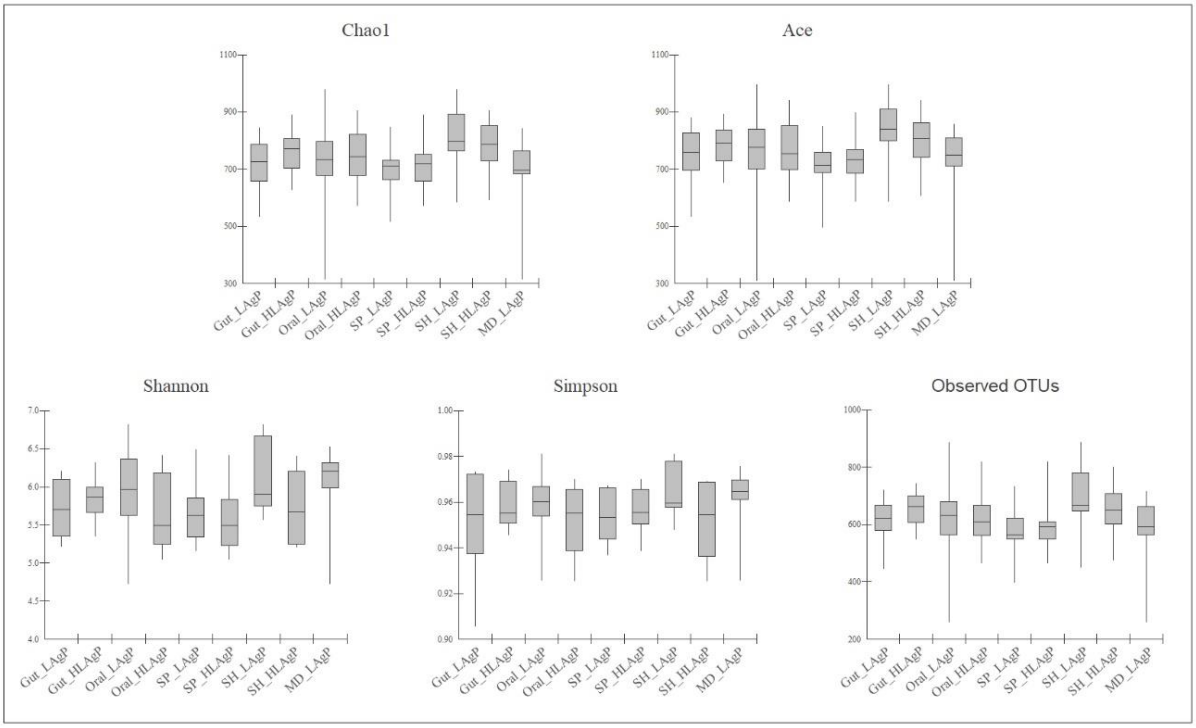

Figure 6. Box-plots of alpha diversity indexes calculated for each site. Boxes contain 50% of all values and whiskers represent the 25th and 75th percentiles. No statisticaly significant difference was observed ($p>0.05$, Student *t* test).

Beta diversity analysis was performed to evaluate the phylogenetic distance matrix based on the Weighted UniFrac distance measurement between groups of samples and visualized through PCoA (Figure 7). Percentage values at the axes indicate contribution of the principal components to the explanation of total variance in the dataset. The percentages of variation explained by PC1 and PC2 were, at least, 37.23% and 14.43%, respectively. Statistical analysis revealed that communities of oral_LAgP and MD_LAgP tended to cluster apart from the communities of oral_HLAgP and SH_HLAgP, respectively ($p<0.05$). Interestingly, one MD sample of subject part of LAgP group that was positive for *Aa* JP-like in qPCR cluster apart from the communities of oral samples and MDxSH (Figure 7B and 7E, respectively). The

sample JP2-like+ presented 6 times more reads for *A. actinomycetemcomitans* than LAgP oral samples altogether and 4.5 times more reads than all oral samples together.

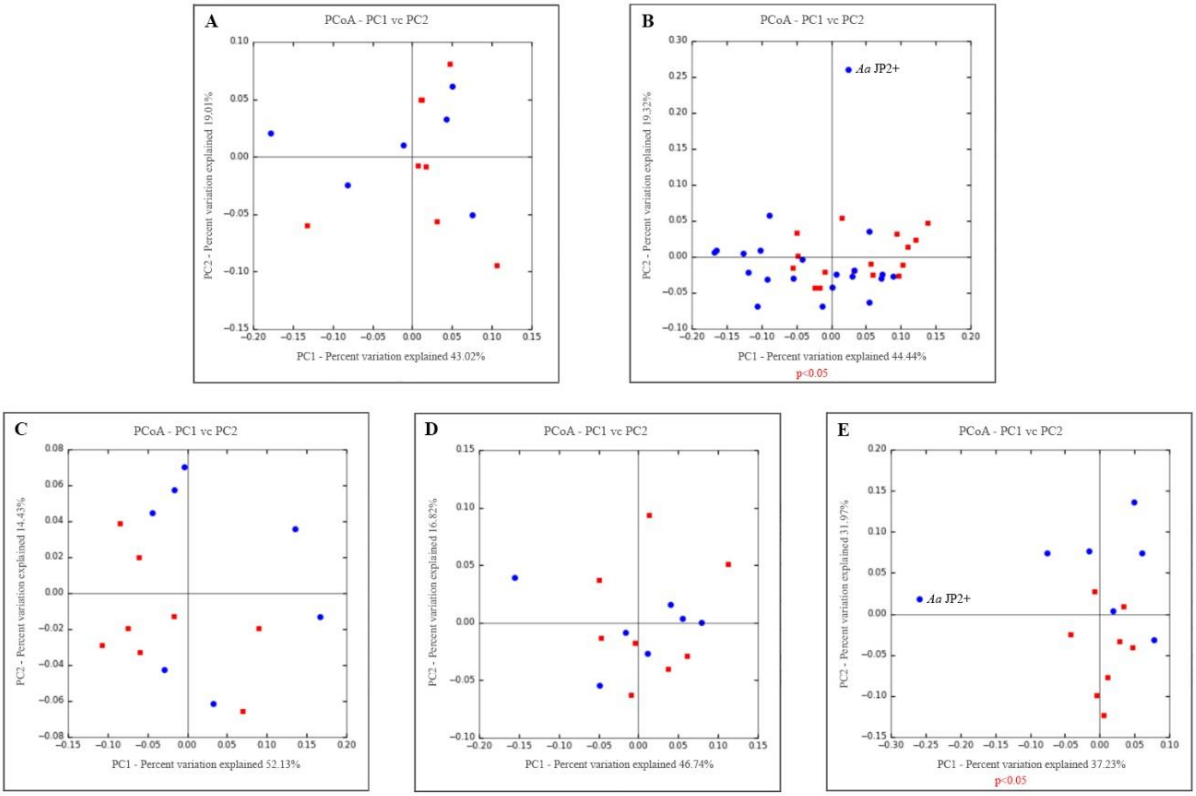

Figure 7. Principal Coordinates Analysis (PCoA) based on Weighted UniFrac distance matrix of the comparison between LAgP (blue dots) and HLAgP (red dots) and sites: gut (a), oral (b), supragingival (c), shallow (d) and shallow vs. medium/deep (e). Statisticaly significant difference $p<0.05$ PERMANOVA test (vegan::adonis).

4.2.7 Species correlation analysis

Spearman's rank correlation among oral biofilm species were calculated based on species which presented statisticaly significant differences in abundance between oral_LAgP and oral_HLAgP (the complete list is in Appendix 2). Only strong correlations were considered (positive correlation: $R \geq 0.97$, $p<0.001$; negative correlation: $R \leq -0.97$, $p<0.001$). Of 81 bacterial species, there were significant correlations in 30 in oral samples of the HLAgP (Figure 8A), and in 17 species in samples of the LAgP group (Figure 8B).

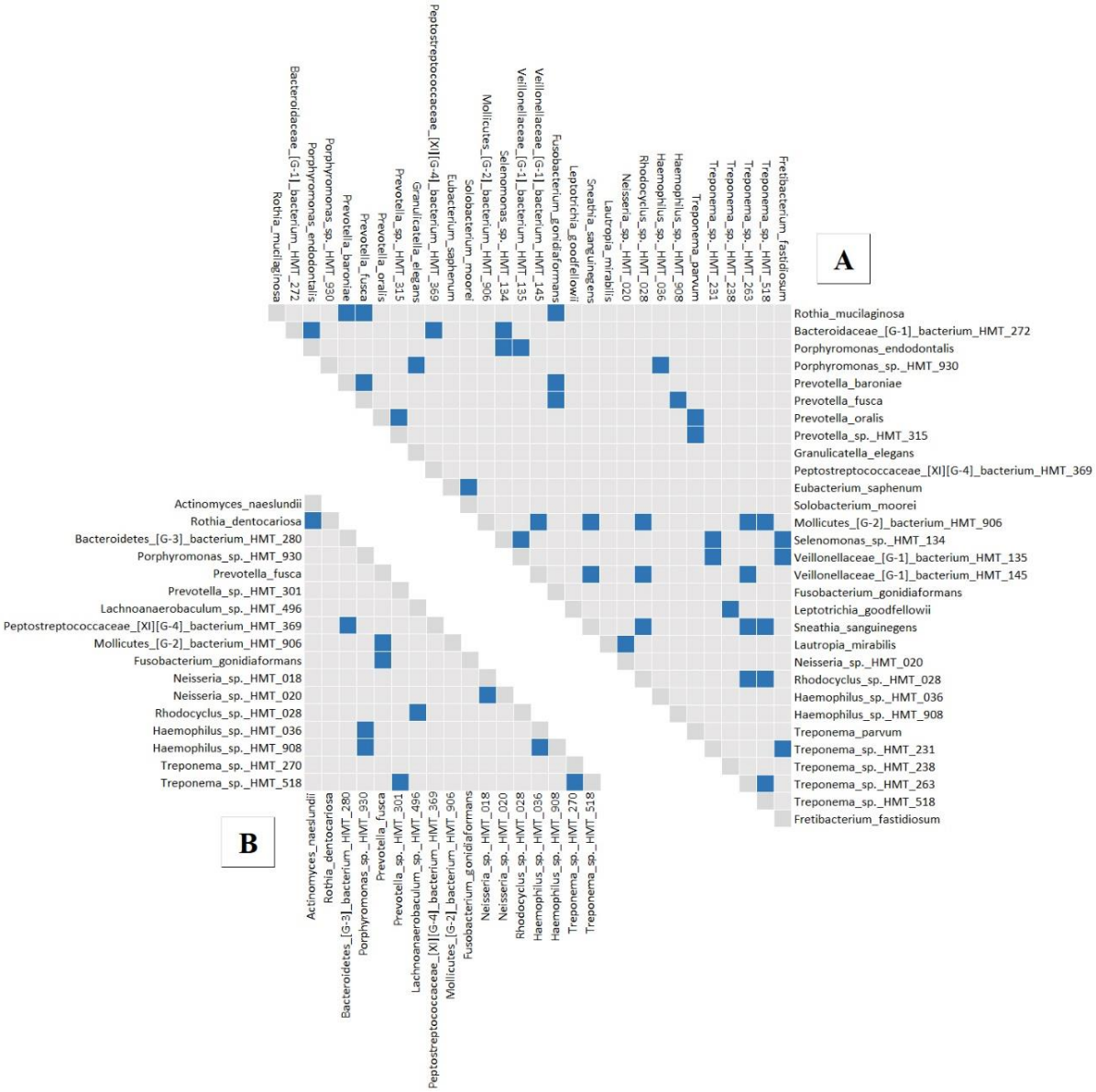

Figure 8. Spearman's rank correlation matrix of oral biofilm species of HLAgP (A) and LAgP (B). Blue squares indicate strong positive correlations ($R \geq 0.97$, $p < 0.001$).

4.3 Chemokines and cytokines profiles in whole saliva

Salivary levels data of 31 CM and 8 CT were analyzed in samples of ustimulated whole saliva of the LAgP and HLAgP groups. Our data indicated that several CT were detected in higher levels in saliva samples of LAgP than HLAgP, although these differences did not reach significance (Mann-Whitney, $p > 0.05$) (Appendix 5, Table 1). Furthermore, GM-CSF was undetectable in all saliva samples.

Data on CM revealed that the salivary levels of CTAK/CCL27, MCP-1/CCL2, TARC/CCL17, and TECK/CCL25 differed between LAgP and HLAgP (Appendix 5, Table 2). Mean levels of CTAK/CCL27 and TARC/CCL17 were significantly higher (2.2 and 2.9 times, respectively) in the saliva of LAgP, whereas levels of MCP-1/CCL2 and TECK/CCL25 were lower (1.9 and 1.5 times, respectively) ($p < 0.05$).

Disease severity in LAgP revealed a strong positive correlation with IL-4 salivary levels (CAL/IL-4: r=0.756, p=0.048; PD/IL-4: r=0.774, p=0.04). Furthermore, LAgP CAL showed a positive correlation with MDC/CCL22 (r=0.785, p=0.03), MIP-3β/CCL19 (r=0.821, p=0.023), MPIF-1/CCL23 (r=0.785, p=0.03), SDF1α+β/CXCL12 (r=0.792, p=0.033) and TARC/CCL17 (r=0.872, p=0.01), and LAgP PB showed a positive correlation with I309/CCL1 (r=0.785, p=0.03) and a negative correlation with MIF (r=-0.75, p=0.05).

Spearman's rank correlation was calculated for CT and CM detected in saliva, as well as the correlations between CT and CM and oral biofilm species. Only strong correlations were considered (positive correlation: $R \geq 0.97$, $p<0.001$; negative correlation: $R \leq -0.97$, $p<0.001$). Data on Spearman's rank correlation index of CT and CM levels in the saliva samples of healthy subjects and LAgP are shown in Figure 9.

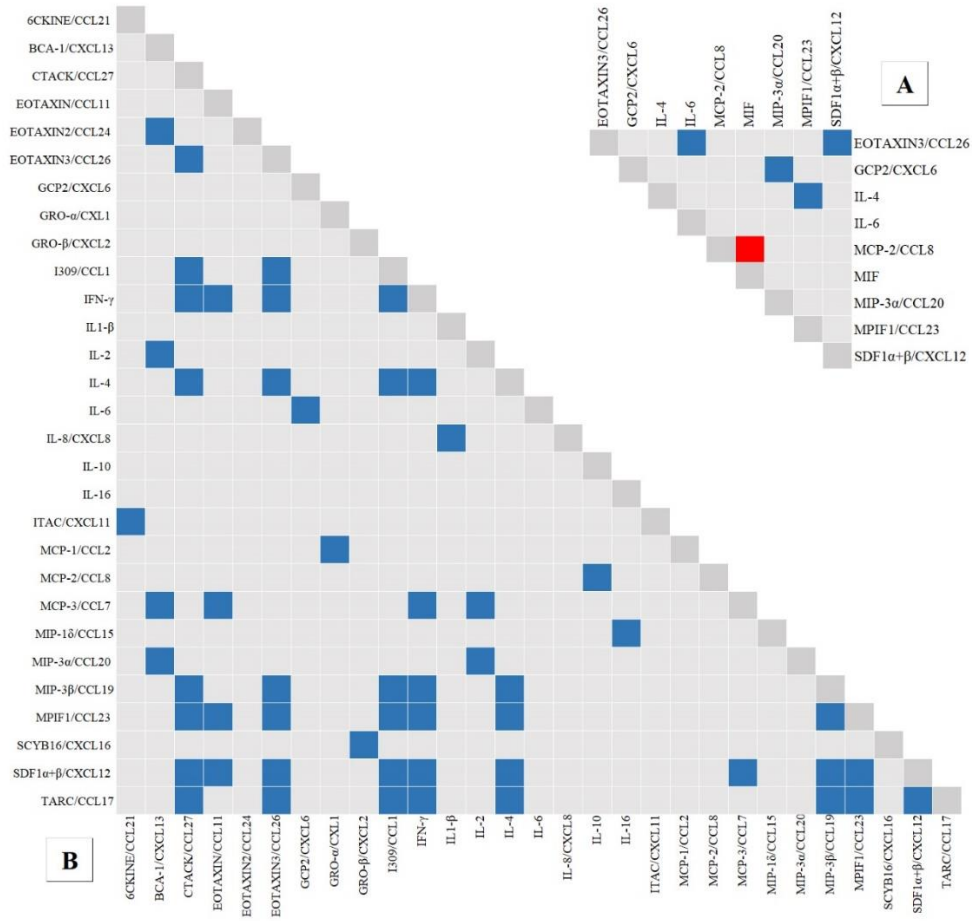

Figure 9. Spearman's rank correlation matrix of chemokines and cytokines profiles in whole saliva of HLAgP (A) and LAgP (B). Blue squares indicate strong positive correlations ($R \geq 0.97$, $p < 0.001$) and red square indicates strong negative correlation ($R \leq -0.97$, $p < 0.001$).

We have also calculated the Spearman's rank correlation between 512 oral biofilm species, 31 CM and 8 CT levels in saliva. Only strong correlations were considered ($R > 0.97$ or < -0.97 and $p < 0.01$) and data are shown in Figure 10.

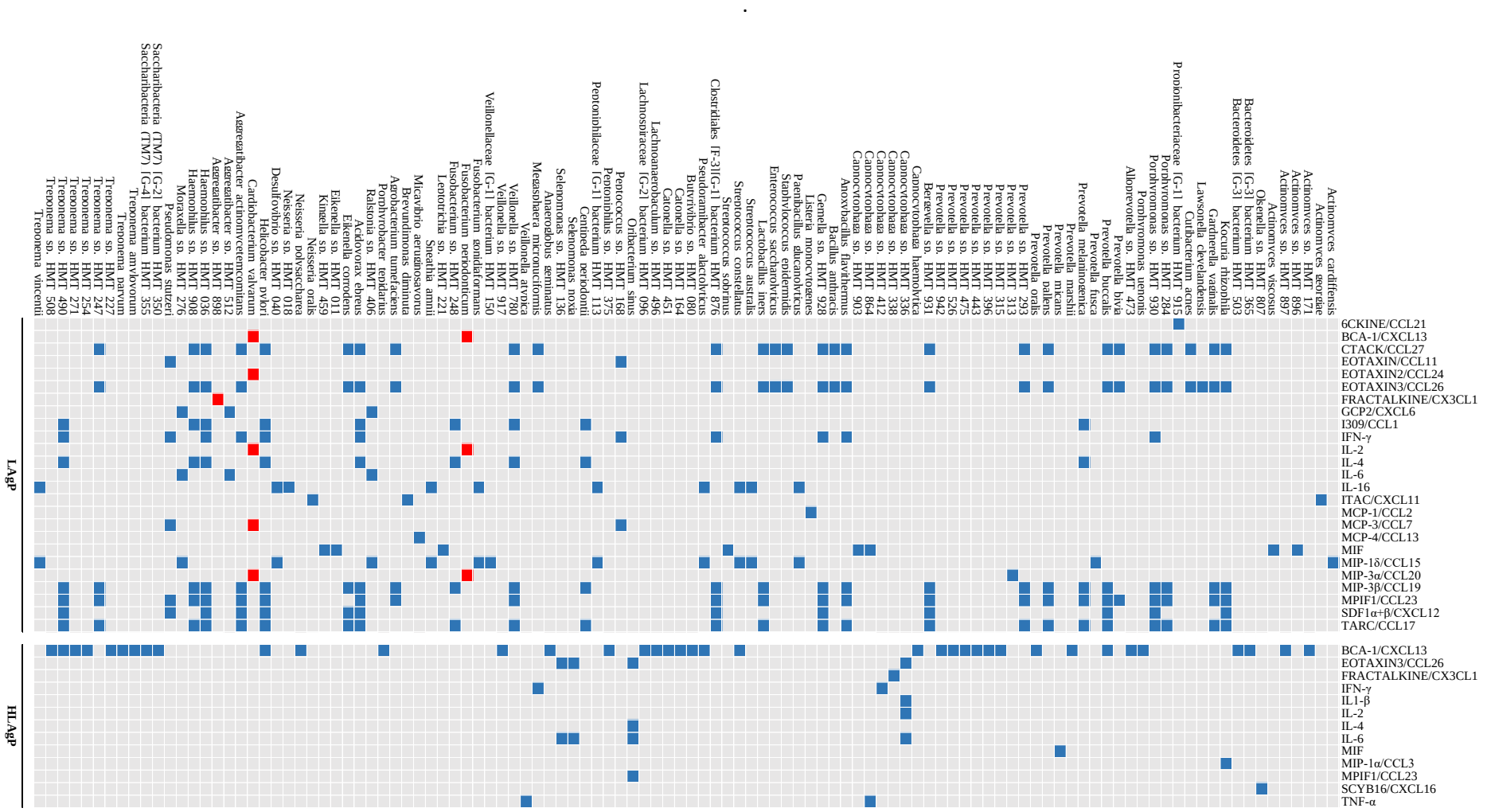

Figure 10. Spearman's rank correlation matrix of chemokines and cytokines profiles in whole saliva and species detected in oral biofilm of HLAgP (A) and LAgP (B). Blue squares indicate strong positive correlations ($R \geq 0.97$, $p < 0.001$) and red squares indicate strong negative correlation ($R \leq -0.97$, $p < 0.001$).

4.4 Evaluation of acquired enamel pellicle and whole saliva samples

A total of 41 samples were analyzed: 13 AEP, 14 stimulated, 14 unstimulated saliva samples. Initially, all these samples were individually evaluated for the amount of protein by BCA assay and results of the protein quantification are shown in Table 2.

Table 2. Total protein concentration of all groups (Mean ± SD). * indicates AEP samples of 6 individuals of the respective group.

Samples	Stimulated (µg/mL)	Unstimulated (µg/mL)	AEP (µg/0.1mL)
LAgP (n=7)	1075.5 ±416.9	1192 ±435.2	4 ±0.6
HLAgP (n=7)	874.9 ±196.4	988.7 ±240.1	6.2 ±1.7*

Aliquots (20µg of protein) of saliva of each individual were subjected to SDS-PAGE to check for the presence and quality of proteins in the samples (Appendix 6 and 7). AEP samples could not be tested in SDS-gel since the amount of proteins recovered from the collection paper was very low, being sufficient only for the proteomic analysis.

All samples showed good protein separation and quality, allowing their use in later experiments (Appendix 6). The pattern of salivary proteins between subjects of the same groups and their respective controls is similar, despite some variability, mainly due to differences in some proteins. The most abundant proteins in StS of LAgP and HLAgP correspond to MW between 50-70 kDa (Appendix 6, Figure A).

Important note: The results presented below will be used for patent applications, so they contain sensitive and confidential information.

4.5 Proteome analysis of acquired enamel pellicle and stimulated saliva samples

The proteomic analysis was performed at the School of Dentistry, University of Western Ontario, Canada, under the supervision of Professor Walter Siqueira, during a one-year internship (BEPE).

AEP samples of 7 LAgP and 6 HLAgP subjects, as well as samples of StS of 7 LAgP and 7 HLAgP subjects were prepared and the tryptic digested peptides were subjected to LC-ESI-MS/MS. All proteins were identified by at least two unique peptides, which ensured results with high confidence. Protein identification was made against a *Homo sapiens* database (Uniprot).

AEPs were collected as four strips from each subject, although they yielded a low amount of proteins. However, our data indicated a high diversity through the identification of a total of 361 unique proteins. The mean numbers of proteins identified in the AEP of HLAgP and LAgP groups were 43.7 (±42.1) and 46.7 (±30.1), respectively. Proteins lists of subjects of the same group were than combined and yielded a total of 222 proteins for AEP of LAgP and 201 of healthy subjects. Sixty-two proteins (17.2% of the total) were common to both groups (Figure 11A), comprising classical salivary proteins as serum albumin, annexin A1, lysozyme, statherin, cystatins, lactoferrin and mucins. Of the total of 361 proteins detected in AEP samples, 160 (44.3%) were exclusively detected in LAgP, while 139 (38.5%) were exclusively detected in HLAgP group (Figure 11A). Some important salivary proteins were detected only in one of the groups, e. g., his1 was found only in the AEP of LAgP group, whereas alpha-amylase 1 and small proline-rich protein 3 were found only in the AEP of healthy controls.

A total of 812 unique proteins were identified in StS samples of LAgP and HLAgP. The mean numbers of identified proteins in the StS of LAgP and LAgP groups were 121.3 (±74.9) and 121.9 (±39.7), respectively. Proteins lists of subjects of the same group were than combined and yielded a total of 518 proteins for LAgP and 498 for HLAgP. Among all proteins, 202 (24.9% of the total) for both healthy and LAgP subjects (Figure 11B). Two hundred and ninety-four (36.2%) proteins were exclusively detected in HLAgP, while 316 (38.9%) were exclusively detected in the LAgP group (Figure 11B).

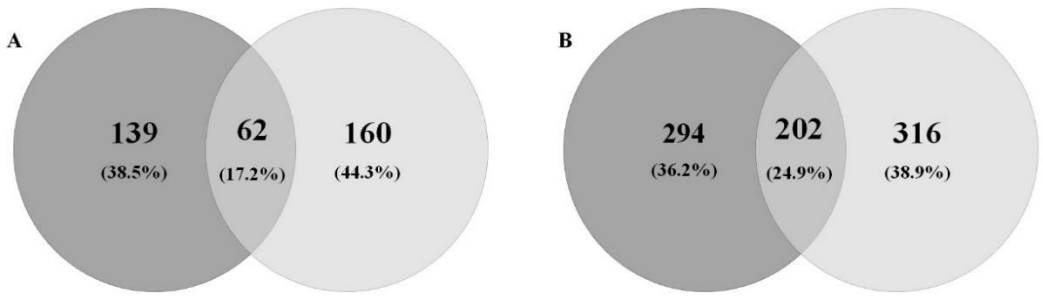

Figure 11. Venn diagram summarizing the absolute number and percentage of differential proteins and overlaps between HLAgP (dark grey) and the LAgP group (light grey) in *in vivo* AEP (A) and in stimulated saliva (B) by proteomic analysis.

Proteins identified in the AEP and StS samples of LAgP and HLAgP were classified by molecular function, biological process and protein class based on annotations in the PANTHER database (Figura 12).

It was possible to classify 189 of 201 (94%) AEP proteins of the HLAgP group, and 214 of 222 (96.4%) AEP proteins of the LAgP group, based on proteins/genes database. Most AEP proteins classified by molecular function are mainly involved in binding (n=55 for HLAgP and n=66 for LAgP) and catalytic activity (n=21 for HLAgP and n=32 for LAgP). AEP proteins were categorized by ther biological functions as: metabolic process (n=37 for HLAgP and n=47 for LAgP), biological regulation (n=33 for HLAgP and n=45 for LAgP), cellular processes (n=27 for HLAgP and n=37 for LAgP), cellular component organization or biogenesis (n=28 for HLAgP and n=30 for LAgP), and localization (n=26 for HLAgP and n=22 for LAgP). The most representative protein classes were nucleic acid binding proteins (n=16 for HLAgP and n=24 for LAgP) and enzyme modulators (n=10 for HLAgP and n=22 for LAgP). Subtle differences can be observed in the percentage of proteins of each category between the two studied groups. Interestingly, the amount of proteins classified in all categories cited above is slightly increased in the disease, except for those related to localization (biological process).

Most of proteins/genes of StS proteins could be classified as well: 453 of 498 (91%) and 467 of 518 (90.6%) of HLAgP and LAgP group, respectively. According to their molecular functions, most StS proteins are mainly involved in binding (n=148 for HLAgP and n=147 for LAgP) and catalytic activity (n=101 for HLAgP and n=102 for LAgP). StS proteins were classified in 4 categories according to their biological functions: cellular processes (n=168 for HLAgP and n=169 for LAgP), metabolic process (n=90 for HLAgP and n=95 for LAgP), biological regulation (n=73 for HLAgP and n=90 for LAgP) and localization (n=50 for HLAgP and n=54 for LAgP). The more representative proteins classes were enzyme modulators (n=36 for HLAgP and n=27 for LAgP), nucleic acid binding (n=32 for HLAgP and n=27 for LAgP), hydrolases (n=23 for HLAgP and n=29 for LAgP) and transcription factors (n=21 for HLAgP and n=28 for LAgP). Subtle differences could be observed in the percentage of proteins in each category between the two studied groups.

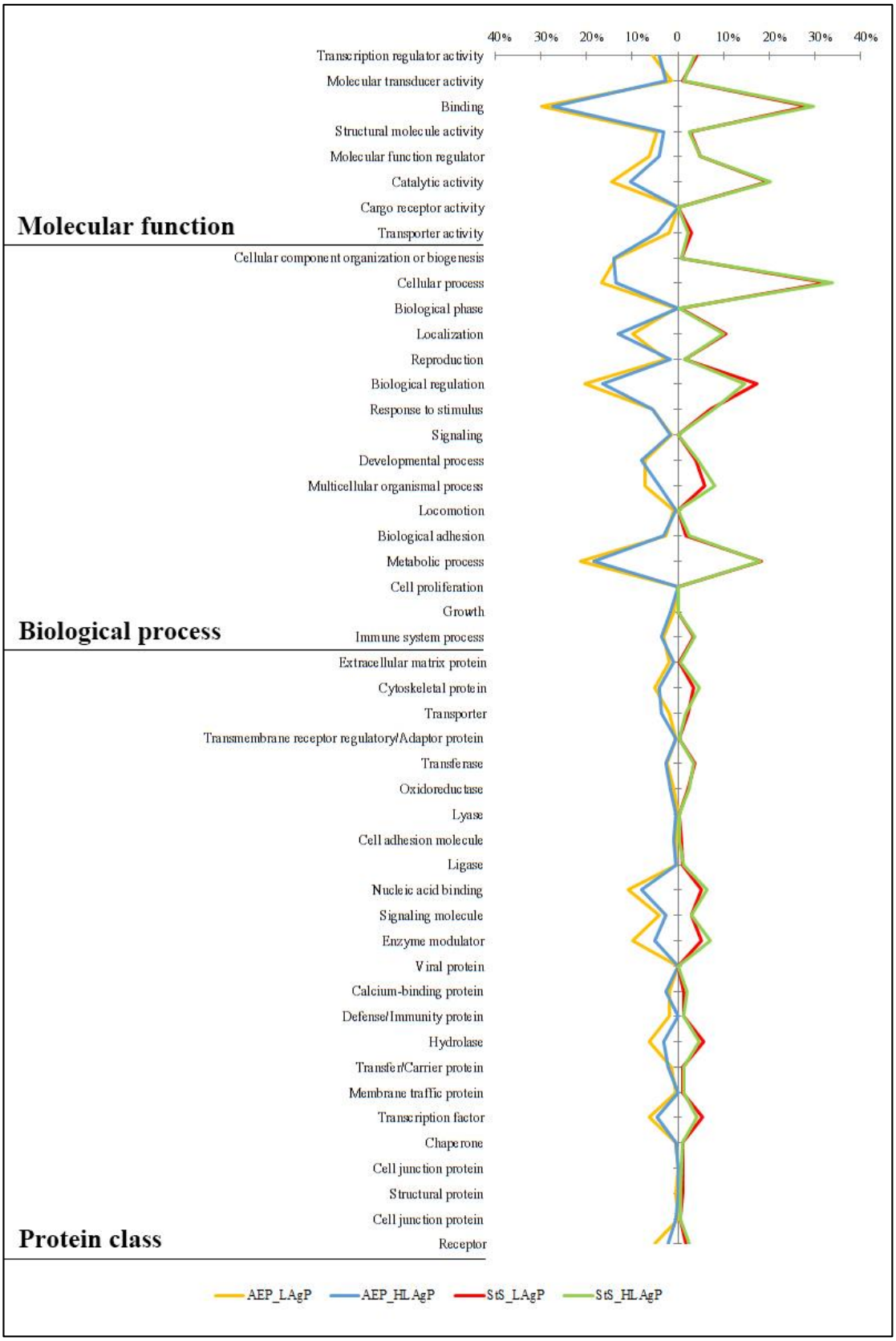

Figure 12. Classification of the identified proteins in Acquired Enamel Pellicle (AEP) and stimulated saliva (StS) by their molecular function, biological process and protein class. Percentage values represent the number of proteins/genes classified in each category by the total of proteins identified in each group (LAgP and HLAgP) by proteomic analysis. Proteins from multiple sources were counted multiple times.

Proteins were also classified according to important salivary processes, including processes relevant to AEP structure and function based on annotations in the Gene Ontology (GO) database (Figure 13). Differences between the amount of proteins identified in each category were not statiscaly significant ($p<0.05$, Student t test), however, there are differences in the protein composition associated to each process.

As shown in figure 13, the AEP and StS proteins were mainly involved in immune response processes. AEP proteins classified in this category corresponded to 24.1% (n=87) of the total of unique proteins (n=361). Twenty-seven proteins were common between both groups, whereas 34 were detected only in the AEP of healthy subjects and 26 were exclusive of LAgP subjects. Among the 87 proteins involved in immune response processes, 30 proteins were classified as having antimicrobial properties. Among the proteins with antimicrobial properties, 11 proteins were exclusively detected in the AEP of healthy subjects, while 8 proteins were detected only in LAgP. Some of the immune response proteins were also involved in inflammatory response, totaling 18 proteins. Among inflammatory response proteins, 6 were exclusively detected in the AEP of HLAgP and 5 in LAgP subjects.

Regarding the StS samples, 20.7% (n=168) of the total of unique proteins (n=812) were classified as being involved in immune response processes. Seventy-four proteins were common between both groups, whereas 44 were detected only in the StS of HLAgP and 50 were exclusive of LAgP subjects. Among the 168 proteins involved in immune response processes, 63 proteins were classified as having antimicrobial properties. Among the proteins with antimicrobial properties, 9 proteins were exclusively detected in the StS of HLAgP, while 22 proteins were detected only in LAgP. Of 168 immune response proteins, 36 were also involved in inflammatory response. Among inflammatory response proteins, 12 proteins were exclusively detected in HLAgP and 11 in LAgP subjects' StS samples.

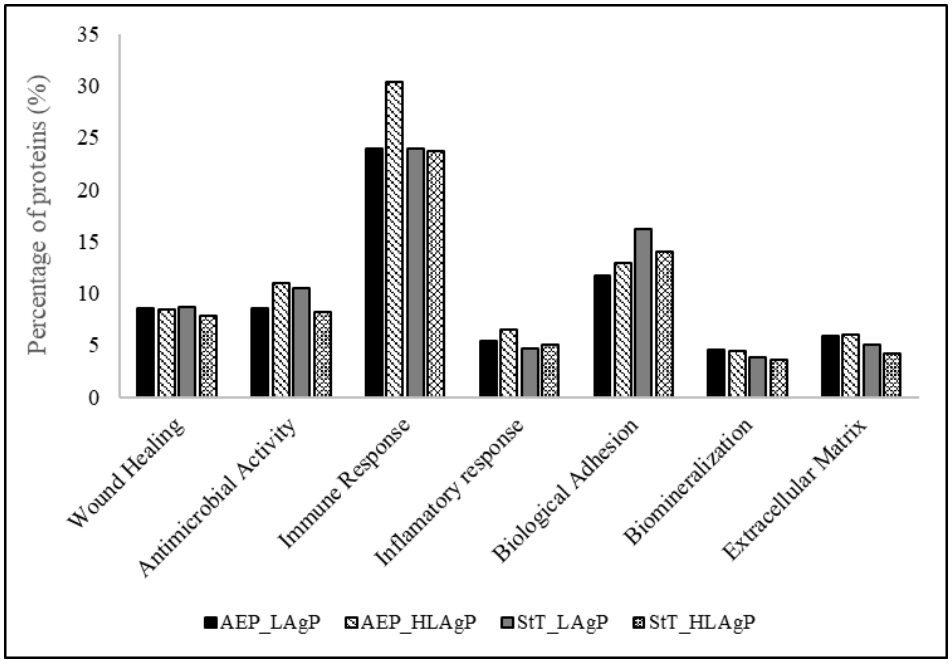

Figure 13. Percentage of proteins from LAgP and healthy controls (HAgP) in Acquired Enamel Pellicle (AEP) and stimulated saliva (StS) classified according to their salivary role. Percentage values represent the number of proteins/genes classified in each category by the total of proteins identified in each group (LAgP and HLAgP) by the proteomic analysis. Proteins with multiple roles were counted multiple times.

Proteins that are involved in these three processes (immune response, antimicrobial activity and inflammatory response) that were detected exclusively in AEP samples or in StS samples, and proteins that were common to AEP and StS are listed in table 3. Among the proteins common to both sampling sites (AEP and StS), some were not detected in LAgP and HLAgP subjects. Complement decay-accelerating factor and Ig lambda constant 1 were detected only in the AEP of the HLAgP group and only in the StS of LAgP. Ig heavy constant gamma 1 was detected only in the AEP of HLAgP and in the StS of both groups. Lysozyme C and myeloperoxidase were detected in the AEP of both groups, although lysozyme C was only detected in the StS of HLAgP, while myeloperoxidase was only detected in the StS of LAgP.

Table 3. Proteins involved in immune response, antimicrobial activity and inflammatory response that were detected exclusively in AEP samples, exclusively in StS samples and proteins that were common between AEP and StS samples. [L] indicates proteins that were detected exclusively in LAgG, and [H] indicates proteins that were detected exclusively in HLAgG, [C] indicates proteins that were common between both groups.

AEP	Common	StS
High mobility group protein B1[L]	Complement decay-accelerating factor[H L]	Complement C4-B[L]
Regenerating islet-derived protein 3-alpha[H]	Immunoglobulin heavy constant gamma 1[H C]	Proteinase-activated receptor 2[L]
	Immunoglobulin lambda constant 1[H L]	Peroxiredoxin-2[L]
	Immunoglobulin kappa constant[C]	Immunoglobulin kappa variable 3-20[L]
	Lysozyme C[C H]	Tumor necrosis factor alpha-induced protein 3[L]
	Myeloperoxidase[C L]	Clusterin[C]
	Protein S100-A9[C]	Immunoglobulin lambda constant 7[C]
		Interleukin-1 receptor antagonist protein[C]
		Complement C3[C]
		Haptoglobin[C]
		Glutathione S-transferase P[C]
		Immunoglobulin heavy constant gamma 4[H]
		NACHT, LRR and PYD domains-containing protein 6[H]
		Protein S100-A8[H]
		Stabilin-1[H]
		Prostaglandin E2 receptor EP1 subtype[H]

In order to determine the representativeness of some proteins in health and disease, AEP and StS proteins detected in at least 50% of subjects of the same group were listed in table 4.

In AEP samples, 57.6% proteins were originated in cells, 24.2% were serum proteins, and 18.2% derived from salivary glands. The proportions of proteins of these 3 origins were very similar in LAgP and heatlh (HLAgP). On the other hand, 50.5% of StS proteins were originated in cells, 31.9% were proteins in salivary glands, and 18.7% derived from serum.

All proteins listed in table 4 were common to both studied groups, except for cystatin-S and alstrom syndrome protein 1 that were found only in the AEP of HLAgP and LAgP subjects (respectively), and the protein dystonin that was detected only in StS samples of HLAgP ($p<0.05$, Student t test). Moreover, the detection of alpha-actinin-4 in, alpha-2-macroglobulin-like protein 1 and Ig heavy constant alpha 1 in more than 65% of the HLAgP subjects was statistically significant ($p<0.05$). On the other hand, the detection of alpha-enolase and profilin-1 in 100% of the LAgP subjects was statistically significant ($p<0.05$).

Table 4. Proteins identified in *in vivo* AEP and stimulated saliva of ≥50% of subjects of the same group (HLAgP and LAgP) by LC-ESI-MS/MS. AC corresponds to the UniProt accession number. Proteins in bold were exclusively detected in the specified group. *Statisticaly significant difference between groups ($p<0.05$, Student t test).

AEP samples

Percentage of individuals	HLAgP			LAgP		
	AC	**Protein name**	**Origin**	**AC**	**Protein name**	**Origin**
≥50%	P63261	Actin, cytoplasmic 2	Cellular	Q8TCU4	**Alstrom syndrome protein 1***	Cellular
	P04083	Annexin A1	Serum	P80723	Brain acid soluble protein 1	Cellular
	P08311	Cathepsin G	Cellular	Q9UBG3	Cornulin	Cellular
	P01036	**Cystatin-S***	Salivary gland	P04792	Heat shock protein beta-1	Cellular
	P61626	Lysozyme C	Serum	P12273	Prolactin-inducible protein	Salivary gland
	P08493	Matrix Gla protein	Cellular	P06702	Protein S100-A9	Serum
	Q8WXI7	Mucin-16	Cellular	P02814	Submaxillary gland androgen-regulated protein 3B	Salivary gland
	Q7Z5P9	Mucin-19	Salivary gland	Q8WZ42	Titin	Cellular
	Q8WZ42	Titin	Cellular			
>65%	P01040	Cystatin-A	Cellular	P63261	Actin, cytoplasmic 2	Cellular
	P04264	Keratin, type II cytoskeletal 1	Cellular	P20930	Filaggrin	Cellular
	P02814	Small proline-rich protein 3	Salivary gland	Q8WXI7	Mucin-16	Cellular
	P62328	Thymosin beta-4	Serum	Q7Z5P9	Mucin-19	Salivary gland
				P98088	Mucin-5AC	Cellular
				P62328	Thymosin beta-4	Serum
>80%	P20930	Filaggrin	Cellular	P04083	Annexin A1	Serum
	P01833	Polymeric immunoglobulin receptor	Cellular	P01040	Cystatin-A	Cellular
100%	P02768	Serum albumin	Serum	P02768	Serum albumin	Serum

Stimulated saliva samples

Percentage of individuals	HLAgP			LAgP		
	AC	**Protein name**	**Origin**	**AC**	**Protein name**	**Origin**
≥50%	Q9UQ26	Regulating synaptic membrane exocytosis protein 2	Cellular	P69905	Hemoglobin subunit alpha	Serum
	P52566	Rho GDP-dissociation inhibitor 2	Cellular	P06744	Glucose-6-phosphate isomerase	Cellular
	P01023	Alpha-2-macroglobulin	Serum	P27482	Calmodulin-like protein 3	Cellular
	P10909	Clusterin	Serum	P01877	Immunoglobulin heavy constant alpha 2	Serum
	P02647	Apolipoprotein A-I	Serum	P62937	Peptidyl-prolyl cis-trans isomerase A	Cellular
	P80303	Nucleobindin-2	Serum	P19013	Keratin, type II cytoskeletal 4	Cellular
	Q03001	**Dystonin***	Cellular	P20061	Transcobalamin-1	Salivary gland
	P06702	Protein S100-A9	Serum	P07237	Protein disulfide-isomerase	Cellular
				P29401	Transketolase	Cellular
				Q8IYB3	Serine/arginine repetitive matrix protein 1	Cellular
				P98088	Mucin-5AC	Cellular
				Q99102	Mucin-4	Salivary gland
				P60174	Triosephosphate isomerase	Cellular
>65%	P98088	Mucin-5AC	Cellular	Q8N4F0	BPI fold-containing family B member 2	Salivary gland
	Q8WZ42	Titin	Cellular	Q96DR5	BPI fold-containing family A member 2	Salivary gland
	Q8IYB3	Serine/arginine repetitive matrix protein 1	Cellular	P09211	Glutathione S-transferase P	Cellular
	P01834	Immunoglobulin kappa constant	Serum	P0DMV9	Heat shock 70 kDa protein 1B	Cellular
	O43707	Alpha-actinin-4*	Cellular	P68871	Hemoglobin subunit beta	Serum
	P19013	Keratin, type II cytoskeletal 4	Cellular	P25311	Zinc-alpha-2-glycoprotein	Salivary gland
	P02538	Keratin, type II cytoskeletal 6A	Cellular	Q7Z5P9	Mucin-19	Salivary gland
	P27482	Calmodulin-like protein 3	Cellular	Q6UWP8	Suprabasin	Cellular
	P30740	Leukocyte elastase inhibitor	Cellular			
	P60174	Triosephosphate isomerase	Cellular			
	P04406	Glyceraldehyde-3-phosphate dehydrogenase	Cellular			
	Q8WXI7	Mucin-16	Cellular			
	Q86UR5	Regulating synaptic membrane exocytosis protein 1	Cellular			
	Q8TDL5	BPI fold-containing family B member 1	Salivary gland			
>80%	P32926	Desmoglein-3	Cellular	P04264	Keratin, type II cytoskeletal 1	Cellular
	Q9UBC9	Small proline-rich protein 3	Salivary gland	P10599	Thioredoxin	Cellular
	P13796	Plastin-2	Cellular	P52566	Rho GDP-dissociation inhibitor 2	Cellular
	P02814	Submaxillary gland androgen-regulated protein 3B	Salivary gland	P04080	Cystatin-B	Salivary gland
	Q96DR5	BPI fold-containing family A member 2	Salivary gland	P02647	Apolipoprotein A-I	Serum
	P09211	Glutathione S-transferase P	Cellular	P06396	Gelsolin	Cellular
	P04264	Keratin, type II cytoskeletal 1	Cellular	P04406	Glyceraldehyde-3-phosphate dehydrogenase	Cellular
	P22079	Lactoperoxidase	Salivary gland	P02538	Keratin, type II cytoskeletal 6A	Cellular
	P20061	Transcobalamin-1	Salivary gland	P13796	Plastin-2	Cellular
				P06702	Protein S100-A9	Serum
100%	A8K2U0	Alpha-2-macroglobulin-like protein 1*	Cellular	P04745	Alpha-amylase 1	Salivary gland
	P04745	Alpha-amylase 1	Salivary gland	P06733	Alpha-enolase*	Cellular
	P04083	Annexin A1	Serum	P04083	Annexin A1	Serum
	Q8N4F0	BPI fold-containing family B member 2	Salivary gland	P23280	Carbonic anhydrase 6	Salivary gland
	P23280	Carbonic anhydrase 6	Salivary gland	P01036	Cystatin-S	Salivary gland
	P04080	Cystatin-B	Salivary gland	P22079	Lactoperoxidase	Salivary gland
	P01036	Cystatin-S	Salivary gland	Q8WXI7	Mucin-16	Cellular
	P01876	Immunoglobulin heavy constant alpha 1*	Serum	P01833	Polymeric immunoglobulin receptor	Cellular
	P01833	Polymeric immunoglobulin receptor	Cellular	P07737	Profilin-1*	Serum
	P12273	Prolactin-inducible protein	Salivary gland	P12273	Prolactin-inducible protein	Salivary gland
	P02768	Serum albumin	Serum	P02768	Serum albumin	Serum
	P10599	Thioredoxin	Cellular	Q9UBC9	Small proline-rich protein 3	Salivary gland
	Q96DA0	Zymogen granule protein 16 homolog B	Salivary gland	P02814	Submaxillary gland androgen-regulated protein 3B	Salivary gland
	P25311	Zinc-alpha-2-glycoprotein	Salivary gland	Q8WZ42	Titin	Cellular
				Q96DA0	Zymogen granule protein 16 homolog B	Salivary gland

4.6 Histatin 1 and 5 degradation assay

The assay evaluating the degradation rate of his1 and 5 in StS was also performed at Professor Siqueiras's laboratory during the internship in Canada.

In order to certify that histatins aliquots had good quality and that these proteins remained intact for a long period in the absence saliva, synthetic his1 (Appendix 7, Figure 1A) and his5 (Appendix 7, Figure 1B) were incubated in water bath at 37 °C for 48h. Cationic-PAGE results showed that both histatins were not degraded after this period and the prepared aliquots could be used in the degradation assay.

In order to confirm the absence or undetectable levels of histatins from StS in the Cationic gel, pooled samples diluted 1:10 in water were incubated at 37 °C for 48h (Appendix 7, figure 2). None of the three histatins (1, 3 and 5) could be visualized in the gel, indicating that the amount of these protein in diluted saliva would not interfere in the evaluation of synthetic histatins degradation.

Gel photographs of histatins 1 and 5 degradation and their products formation in the presence of DStSS of each individual after incubation for up tp 48h at 37 ºC are shown in Appendixes 8 and 9. The degradation at the different time points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours) was calculated considering the pixel intensity of the histatin band at t=0 as 100% and the results are presented in Table 5.

The degradation of histatin 5 occurred faster than the degradation of his1 in DStSS. The detection of degradation products (peptides), i. e., bands of MW lower than the intact protein, could be clearly visualized in the gel images (Appendix 9). Peptide bands derived from his5 could be detected right after the contact of this protein with the DStSS of most subjects, whereas his1 peptides started to appear between 0.5 and 1.5 h of incubation (Appendix 8).

Intact his1 and his5 were detected in all the samples after 1.5 hour of incubation. However, after 4h, DStSS of only one LAgP individual (14.3%) and none of the DStSS of HLAgP subjects had completely degraded his5. His5 was totally degraded in the saliva of 57.7% of LAgP subjects after 6h of incubation, whereas less than 15% of the saliva of healthy controls had completely degraded hist5. Intact his5 was detected in some subjects (42.8% of LAgP and 57% of HLAgP) after 8 of incubation. However, his5 was totally degraded in the saliva of all subjects after 24h of incubation. On the other hand, intact his1 was detected in 71.4% of DStSS of LAgP and HLAgP subjects even after 48h.

Table 5. Percentage of histatin 1 and 5 (mean and standard deviation) (band pixel intensity in relation to T0) after different incubation periods in the presence of DStSS of LAgP and HLAgP subjects. *Statisticaly significant difference between groups ($p<0.05$, ANOVA).

Time of incubation	Histatin 1		Histatin 5	
	LAgP (n=7)	HLAgP (n=7)	LAgP (n=7)	HLAgP (n=7)
0h	100 (±0.00)	100 (±0.00)	100 (±0.00)	100 (±0.00)
0.5h	103.43 (±4.11)	99.62 (±4.13)	86.45 (±9.63)	89.22 (±5.59)
1.5h	92.76 (±11.73)	94.78 (±6.36)	67.86 (±12.58)	78.75 (±9.59)
4h	76.74 (±18.63)	82.85 (±15.05)	30.67 (±20.96)*	51.92 (±16.97)
6h	67.57 (±17.57)	76.69 (±20.58)	14.54 (±18.50)*	34.88 (±22.90)
8h	60.47 (±17.26)	68.08 (±21.34)	8.95 (±11.29)	18.57 (±20.45)
24h	34.20 (±18.72)	48.91 (±27.38)	0	0
48h	22.30 (±17.43)	32.17 (±23.61)	0	0

Data on the average of degradation of his1 and his5 (Figure 14A and 14B, respectively) incubated in DStSS of subjects with LAgP and healthy controls demonstrated that the degradation occurs faster in the presence of periodontitis compared to the absence of disease.

In order to evaluate the influence of studied groups on histatins degradation upon different incubation periods, ANOVA with repeated measures was applied. For his1 and his5, a significant interaction effect was observed between group and time ($p<0.001$). A higher degradation rate of his5 was observed in comparison to his1, and the degradation rate of these histatins was greater in longer incubation periods. The degradation of his5 was more intense in subjects affected by periodontitis when compared to healthy subjects, especially after 4 and 6h in the DStSS of LAgP (Figure 14B). No statistical significant difference was observed in the rate of degradation os his1 between LAgP and HLAgP subjects (Figure 14A).

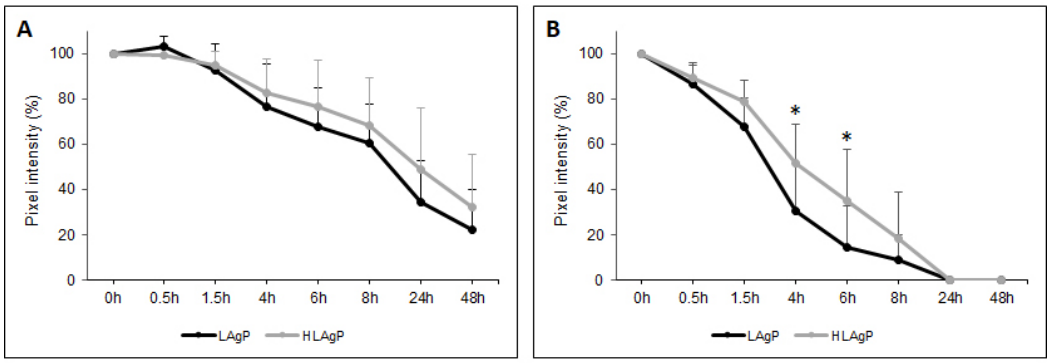

Figure 14. Effect of DStSS of LAgP and HLAgP subjects on the degradation of histatin 1 (A) and histatin 5 (B) at different time points (% calculated by the mean pixel intensity of the bands in relation to T0 in Cationic-PAGE gels). Bars represent standard deviation and asterisks indicate significant differences between groups (ANOVA, $p<0.05$).

In order to identify pairs of means that differed from each other, a multiple comparison of means with Bonferroni correction was performed. Figure 15 (A and B) shows the differences in pairs of means in his1 degradation. Figure 15 (C and D) shows the differences in pairs of means in his5 degradation.

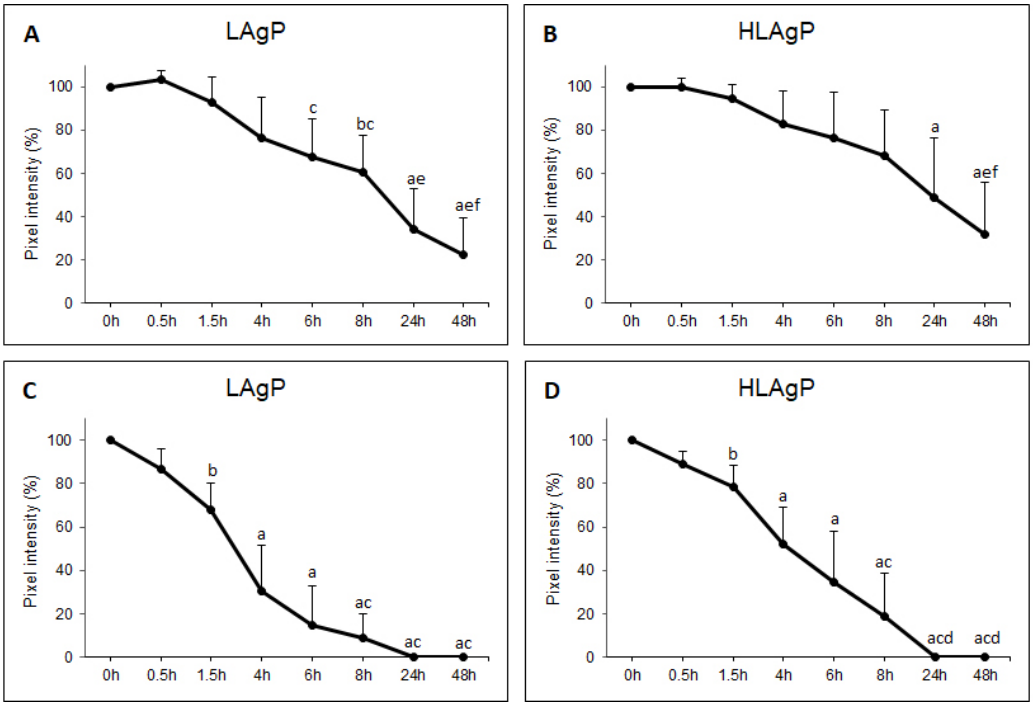

Figure 15. Curves of degradation of histatin 1 (A and B) and histatin 5 (C and D) incubated in DStSS of LAgP and HLAgP subjects calculated by the pixel intensity (%) of the bands in Cationic-PAGE throughout different time points. Bars represent standard deviation and different letters indicate significant differences in pairs of means. In figures A and B: a ≠ 0h, 0.5, 1.5 and 4h; b ≠ 0h; c ≠ 0.5h; e ≠6 h, f ≠ 8h. In figures C and D: a ≠ 0h, 0.5 and 1.5h; b ≠ 0h; c ≠ 4h; d ≠ 6h (Bonferroni, $p<0.05$).

In order to compare the amino acids sequences of degradation products of his1 and his5 submitted to DStSS from LAgP subjects and healthy controls, some his1 and 5 peptide bands were excised from the gel, and samples analysed by LS-ESI-MS/MS. Sequences are shown in Appendix 10.

5 DISCUSSION

5.1 Oral and gut microbiome

We reported the oral and fecal microbiome of LAgP and healthy controls, paired by age, gender, and race. Alterations observed in the microbial composition of the oral biofilm in LAgP periodontitis was previously reported using culture (ASIKAINEN et al., 1987; CHAHBOUN et al., 2015; DELANEY; KORNMAN, 1987; EISENMANN et al., 1983), quantitative PCR (OETTINGER-BARAK et al., 2014; TAKEUCHI et al., 2003) and hybridization based methods (FAVERI et al., 2009; FINE et al., 2013b; MOUTSOPOULOS et al., 2015; SHADDOX et al., 2012). To the best of our knowledge, this is the first study evaluating the oral and gut microbiome of LAgP using a NGS method. Furthermore, we have evaluated not only subgingival biofilm of affected periodontitis sites and compared to control sites of affected subjecs and control non affected subjects, but the composition of the supragingival biofilms of both groups was also evaluated. It should be noticed then, that there are no data in the literature about supragingival microbiota of LAgP so far.

Succession of microbial groupsin early biofilms of supra and subgingival sites seems not to differ between healthy and periodontitis subjects (TELES et al.; 2012) However, the gingival inflammation can alter the microbial composition of the supragingival dental biofilm (RÜDIGER et al., 2002). Moreover, the microbial composition of the subgingival biofilm is highly affected by the supragingival biofilm, since supra and subgingival dental biofilms share 65% of species (MASON et al., 2018).

Supra and subgingival microbiome analysis of LAgP and HLAgP revealed some aspects not yet described in the literature, and our data contribute to the understanding of the microbiota associated with the disease.

Although the SP biofilm in LAgP is less exuberant than that observed in ChP, microbial alterations in SP and subgingival biofilms were demonstrated, with the reduction of facultative

anaerobic organisms, biofilm initial colonizers towards a strict anaerobic microbiota. A higher abundance of facultative anaerobic, mostly strict anaerobic species, in the SP and healthy SH sites of LAgP was observed than in HLAgP subjects. On the other hand, facultative anaerobic species were more abundant in the same sites of HLAgP.

Johnsonella is a Gram negative, obligate anaerobic organism that was recently associated to subgingival plaque of periodontitis (CHEN et al., 2018) and *Johnsonella* sp._HMT_166 was previously associated to ChP (PÉREZ-CHAPARRO et al., 2018). The RA of *Johnsonella* sp._HMT_166 was higher in SP_LAgP (log_2FC of 4.7) compared to SP_HLAgP. It was also more abundant in SH and MD sites of LAgP compared to SH_HLAgP, but this difference was not statistically significant.

The RA of *Sneathia sanguinegens* and *Acidovorax ebreus* were also higher in SP_LAgP (log_2FC >4), and the abundance of *A. ebreus* was also increased in MD_LAgP sites compared to SH_HLAgP (log_2FC= 4.08). Although these species have not been associated to periodontitis so far, previous data reported that *S. sanguinegens* was associated to severe infections such as pelvic inflammatory disease (GOTTSCHICK et al., 2017; HAGGERTY et al., 2016; MUZNY et al., 2018), preterm prelabor rupture of membranes (KACEROVSKY et al., 2015), spontaneous abortion (SEO et al., 2017), and to a case of postpartum prosthetic valve infective endocarditis (KOTASKOVA et al., 2017).

A. ebreus, similar to other species in the *Acidovorax* genus, is a nitrate reducing bacteria (NRB), proficient at catalyzing the oxidation of ferrous iron [Fe(II)] coupled to nitrate (NO_3^-) reduction, often referred to as nitrate-dependent iron oxidation (NDFO) (CARLSON et al., 2013). Inorganic nitrate (NO_3^-) is mainly derived from green leafy vegetables, and is taken up by the intestinal mucosa, and achieves the oral cavity upon salivation (MA et al., 2018). Under NDFO phenomenon, redox transformations of NO_3^- can produce intermediates, such as nitric oxide (NO), which is able to bind to and react with heme cofactors (MCCLEVERTY, 2004) or Fe-S clusters (HARROP et al., 2008) or, in the presence of transition metals, nitrosates protein thiols to inhibit or alter protein activity (SPIRO, 2007). NO is a potent antimicrobial agent capable of inhibiting or killing a broad spectrum of microorganisms (FANG; VAZQUEZ-TORRES, 2002), which may confer a competitive advantage to *A. ebreus* and other NRB.

NO is endogenously produced in vascular endothelial cells, neurons and immune cells (MONCADA; PALMER; HIGGS, 1991), however, the reduction of salivary nitrate to nitrite is a reaction that mammalian cells are unable to perform during anaerobic respiration by nitrate reductases produced by facultative and obligate anaerobic commensal oral bacteria

(LUNDBERG et al., 2004). Oral NRB have been identified *in vitro* in human samples, including *Veillonella*, *Actinomyces*, *Rothia*, *Staphylococcus* and *Propionibacterium* (DOEL et al., 2005). Other NRB candidate species are *Neisseria*, *Haemophilus parainfluenzae*, *Prevotella* and *Granulicatella,* which are considered potent contributors to oral nitrite production (HYDE et al., 2014). NO and $ONOO^-$ (peroxynitrite, the reactive reaction product of NO and superoxide) participate in the pathogenesis of periodontitis (LOHINAI et al., 1998, 2001; Lohinai; Szabo, 1998) and oral bacteria appear to trigger the host inducible NO synthase (iNOS) up-regulation in periodontal tissues (LOHINAI et al., 1998, 2001; LOHINAI; SZABO, 1998). NO levels increase with the severity of periodontitis (REHER et al., 2007) and patients with ChP and GAgP present similar salivary levels of NO, higher than those found in healthy patients (SUNDAR et al., 2013).

In the present study, the saliva nitrite concentration was higher in LAgP subjects when compared to HLAgP, although this difference was not statisticaly significant. The suggestion of higher nitrite levels in LAgP may be due to endogenous production of NO by the inflamed periodontal tissues, but the abundance of NRB observed in the oral biofilm of LAgP subjects, including some species of *Veillonella*, *Actinomyces* and *Prevotella* as well as the previous described specie *A. ebreus,* may also play a role. On the other hand, the lack of difference in salivary nitrite levels in LAgP when compared to health, differing from more generalized disease, may rely on the few affected sites in the localized form of periodontitis.

Interestingly, NO production can occur in the presence of hydrogen sulphide (H_2S), since in a low pH environment, nitrite (NO_2^-) is reduced to HNO_2, which allows H_2S to form NO ($HNO_2 + H_2S \rightarrow HS\text{-}NO \rightarrow NO$) (GROSSI, 2009). In its turn, NO can release H_2S of Fe-S clusters of proteins through non-enzymatic reaction, as mentioned before (TRAN; WILLIARD; KIM, 2014), creating a synergistic production process that may have an important role in periodontal disease, since the excess of NO and H_2S can be extremely toxic for host cells (LINDEN, 2014; MURPHY, 1999).

H_2S production in the oral cavity occurs through the reduction of sulfates derived from the diet and from the degradation of proteoglycans from the conjunctive tissue. In periodontal pockets, there is a predominance of proteolytic and fermentative anaerobic bacteria (MOORE, 1987). Their metabolic end-products (organic compounds of low molecular weight and hydrogen) can be utilized as electron donors in the anaerobic respiration of sulfates by sulfate reducing bacteria (SRB). Thus, SRB must rely on a complex microbiota to obtain substrates necessary for their growth, which may explain the association of these bacteria with advanced cases of periodontitis (LANGENDIJK; HANSSEN; VAN DER HOEVEN, 2000). Our data

revealed a significant increase of Deltaproteobacteria class in subjects affected by periodontitis, being 10 times more abundant in oral_LAgP and 20 times in MD_LAgP when compared to oral and SH_HLAgP, respectively.

Desulfobulbus sp._HMT_041 and *Desulfovibrio* sp._HMT_040, members of Deltaproteobacteria class were more abundant in MD_ LAgP than in SH_HLAgP. *Desulfovibrio* sp._HMT_040 was detected only in MD_ LAgP. These organisms are Gram-negative anaerobic, sulfate reducers whose role in periodontitis has been suggested (CAMPBELL et al., 2013). *Desulfobulbus* sp._HMT_041 was previously identified in higher proportions in ChP and GAgP subgingival biofilms (OLIVEIRA et al., 2016), and the abundance of this phylotype was increased in subgingival sites of LAgP adolescents prior to bone loss (FINE et al., 2013b) and was associated to refractory periodontitis (COLOMBO et al., 2009). Furhtermore, an association between *Desulfobulbus* and the periodontitis severity was reported (CAMELO-CASTILLO et al., 2015a, 2015b).

Moreover, the overload of iron in biological fluids, including the GCF, has been associated with severe periodontitis (BOYER et al., 2018; MEURIC et al., 2017; MUKHERJEE, 1985). The genome-wide transcriptome analysis of the subgingival microbiome of periodontitis-affected subjects compared to healthy subjects showed that iron acquisition, lipopolysaccharide synthesis and flagellar synthesis were major activities defining the disease (DURAN-PINEDO et al., 2014). Since iron is essential for pathogens growth, alterations in iron homeostasis can lead to dysbiosis (BOYER et al., 2018). A study analyzing the microbiota of deepest periodontal pocket of affected subjects with increased transferrin saturation (TSAT), observed high abundance of Deltaproteobacteria and a significant increase of the genus *Desulfobulbus* and *Desulfobulbus* sp. when samples were compared to subjects with normal transferrin saturation (TSAT) levels (BOYER et al., 2018). Thus, the increased abundance of *Desulfobulbus* in oral sites of LAgP may be a result of increased iron availability in the periodontitis patients.

The release of H_2S can also occur via desulfhydration of cysteine or serum proteins by subgingival bacteria, such as *Peptosteptococcus anaerobius*, *Micros prevotii*, *Eubacterium limosum*, *Centipedia periodontii*, *Selenomonas artermidis*, *Prevotella intermedia*, *Prevotella loescheii*, and members of red complex according to the Socransky classification (SOCRANSKY et al., 1998): *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (LINDEN, 2014). It is hypothesized that *A. actinomycetemcomitans* can release H_2S through glutathione catabolism, however it is dependent of enzymes produced by other oral bacteria such as *Peptostreptococcus micros*, *Fusobacterium*, *P. gingivalis* and *T. forsythia*

(CHU et al., 2009). Our data indicated that *A. actinomycetemcomitans* was the species whose abundance increased the most in MD_LAgP (log_2FC of 5.65) compared to SH_HLAgP, and besides its other virulence properties, this organism could also contribute with the microbial community to H_2S release.

Solobacterium moorei can convert cysteine into H_2S, in a process dependent on external enzymes to produce volatile sulfur compounds, such as *P. gingivalis* gingipains (TANABE; GRENIER, 2012). *S. moorei* and *P. gingivalis* were more abundant in oral_LAgP than in oral_HLAgP. The RA of *C. periodontii* and *S. artermidis* was increased in SP_LAgP, while RA of *T. denticola* and *T. forsythia* was increased in MD_LAgP compared to SH_HLAgP. Moreover, *Helicobacter pylori* was one of the species whose abundance increased the most in MD_LAgP (log_2FC of 4.56) and this specie releases H_2S not only from cysteine but also from methionine desulfhydration (LEE et al., 2006). As a consequence, the accumulation of H_2S produced by SRB in periodontal pockets should play a relevant role in the destructive process (LOUBINOUX et al., 2002b).

Our findings suggest that the synergistic production of toxic compounds (NO and H_2S) by the microbial community present in periodontal affected sites may favor a shift from a balanced to a dysbiotic microbiota, favoring and being favored by the periodontal destruction observed in LAgP.

Pseudopropionibacterium propionicum and *Stenotrophomonas maltophilia* are species not previously associated with periodontitis. Both species were 10 times more abundant in supragingival biofilm of diseased (SP_LAgP) than in health (SP_HLAgP). *Propionibacterium* are gram-positive, pleomorphic, rod-shaped, non-spore forming, non-motile organism, which produce propionic acid and acetic acid from glucose and a *P. propionicum* closely related specie, *P. rubrum*, was recently reported in the gingival crevice (SAITO et al., 2018), whereas *P. propionicum* was associated to endodontic lesions and actinomycosis in humans (SIQUEIRA, 2003). *S. maltophilia* is a Gram-negative, Gammaproteobacteria, multi-drug-resistant global opportunistic nosocomial pathogen, which harbors several virulence factors (BROOKE et al., 2017). *S. maltophilia* is associated with various infections such as bacteremia, endocarditis, pneumonia, meningitis, ocular infections, urinary tract infection, enteritis, and skin/soft tissue infections (ABBOTT et al., 2011; SENOL, 2004).

When comparing subgingival healthy shallow sites of both groups, Absconditabacteria_(SR1)_[G-1] bacterium_HMT_875 was more abundant increased in SH_LAgP. Absconditabacteria (SR1) was recently found in increased levels in subjects with malodourous associated to H_2S production and other gases (NAKANO; SUZUKI; KUWATA,

2018). Species of *Selenomonas* (*S. flueggei*, *S. infelix*, *S.* sp._HMT_126 and *S.* sp._HMT_936) and *Actinomyces* (*A. cardiffensis*, *A.* sp._HMT_414 and *A.* sp._HMT_525) were also more abundant in SH_LAgP than in SH_HLAgP. Moreover, *Actinomyces* sp._HMT_525 was more abundant in all LAgP oral sites than in oral samples from healthy subjects. *Actinomyces* has been associated to healthy compared to periodontal-affected subjects (CHEN et al., 2018; FINE et al., 2013b), however this genus is highly heterogeneous, comprising species associated to health and disease (KÖNÖNEN; WADE, 2015).

Selenomonas is an anaerobic microorganism and was associated to necrotizing ulcerative periodontitis (GMÜR et al., 2004), periodontitis with rapid progression (KAMMA; NAKOU; MANTI, 1995) and active periodontal lesions (HAFFAJEE et al., 1984; TANNER et al., 1998). A previous study involving cloning and sequencing of *16S rRNA* had also shown a higher proportion of *Selenomonas* in GAgP, mainly *S. sputigena* (FAVERI et al., 2008). Gonçalves et al. (2012) analyzed the proportion of different *Selenomonas* species using specific oligonucleotide probes complementary to *16S rRNA* and confirmed the relation between the proportion of *Selenomonas* in subgingival sites of GAgP and disease severity. Our data indicated that *S. sputigena* and *S.* sp._HMT_134 were more abundant in MD_LAgP when compared to SH_HLAgP. *S. sputigena* is considered a periodontopathogen due to its virulence factors and its key role in coaggregation and maturation of the dental biofilm (HIRANMAYI et al., 2017). Its lipopolysaccharides induce the production of IL-6 and IL-1α in macrophages thereby provoking inflammation (HOFSTAD; NAESS; SKAUG, 1986; MCCARTHY; CARLSON, 1981). *S. sputigena* was suggested as a potential pathogen and diagnostic marker for active periodontal disease (HIRANMAYI et al., 2017). Moreover, Fine et al. (2013b) identified that *S sputigena* together with *A. actinomycetemcomitans*, *Filifactor alocis*, *Treponema socranskii*, *T. forsythia*, *P. gingivalis*, *Eubacterium nodatum*, *Eubacterium infirmum*, *Eubacterium brachy* and *Prevotella intermedia* were part of the prominent named species with increased prevalence in adolescents affected by LAgP prior to bone loss compared to healthy sites of healthy subjects. In accordance with this study, we have shown that all of these species were more abundant in MD_LAgP than in healthy SH_HLAgP, although the difference in the amount of *E. infirmum*, *E. brachy* and *P. intermedia* between these sites did not reach statistical significance, and *P. gingivalis* was more abundant in oral_LAgP than in oral_HLAgP.

Our data indicated an association between *F. alocis* and LAgP, as previously reported (FINE et al., 2013b; SHADDOX et al., 2012). *F. alocis* is the third most prevalent pathogen in GAgP (45%), second most prevalent in ChP (90%) and is less prevalent in periodontitis

resistant groups (HIRANMAYI et al., 2017). *F. alocis* possess unique virulence characteristics which could explain its high prevalence in periodontal diseases, such as oxidative stress resistance, induction of proinflammatory cytokines production, and involvement in biofilm formation that triggers host response by secretion of several proteases (ARUNI et al., 2015; ARUNI; ROY; FLETCHER, 2011; SCHLAFER et al., 2010). It was demonstrated that *F. alocis* is more resistant than *P. gingivalis* to hydrogen peroxide-induced oxidative stress, which favors its persistence in periodontal pockets (MOFFATT et al., 2011; WANG et al., 2014).

Previous studies indicated that periodontal disease is associated to the increase of anaerobic bacteria in deep periodontal pockets (SOCRANSKY et al., 1998; SOCRANSKY; HAFFAJEE, 2005). Other studies using target molecular methods indicated a higher proportion of strict anaerobic pathogens as *P. gingivalis, T. forsythia* and *Treponema denticola* in subgingival biofilm of patients with AgP compared to control subjects (FAVERI et al., 2009; SARAIVA et al., 2014), although this difference has been observed with more emphasis in patients with GAgP.

Our data revealed that *Porphyromonas gingivalis* abundance was increased in patients with MD_LAgP compared to SH_HLAgP, although this difference was not statistically significant, as described previously. *P. gingivalis* and *P. endodontalis* were part of the common oral core microbiome and were more abundant in disease ($p<0.05$) when comparing oral samples altogether of LAgP to HLAgP. *P. gingivalis* is considered a key stone pathogen in periodontitis since it can modulate the host immunological response, enhance its adaptation to the periodontal tissues, and promote environmental alteration which benefits the microbial community, leading to alteration in the amount and composition of the subgingival microbiota (HAJISHENGALLIS; LAMONT, 2012). *P. endodontalis* and *Porphyromonas* sp._HMT_285 were more abundant in MD_LAgP when compared to SH_HLAgP ($p<0.05$). Both were present in increased levels in subgingival sites of adolescents 6 months prior to bone loss (FINE et al., 2013b). *P. endodontalis* was also associated with severe periodontitis in a leukocyte adhesion deficiency 13 years -old patient (MOUTSOPOULOS et al., 2015). These findings suggest that *P. endodontalis* may have an important role in early-onset periodontitis.

We have performed qPCR analysis to evaluate *A. actinomycetemcomitans* prevalence in the biofilm of LAgP and HLAgP subjects, and this specie was detected only in subgingival samples of LAgP. However, sequencing analysis revealed that *A. actinomycetemcomitans* was present in low abundance in all samples of healthy controls. On the other hand, *A. actinomycetemcomitans* was almost 20 times more abundant in oral_LAgP than in oral_HLAgP samples. Despite the association of the JP2 clone with AgP in African

descendants (HAUBEK et al., 1995, 1997), this highly leukotoxic clone was detected in only one patient of the LAgP group subjects (biofilm of MD site) by qPCR, while the others harbored minimal leukotoxic *A.actinomycetemcomitans*, in agreement with studies in other populations (FINE et al., 2007).

Although only one patient harboring the JP2 clone was evaluated, the JP2-like+ subject presented 6 times more reads for *A. actinomycetemcomitans* than other LAgP patients and 4.5 times more reads than all oral samples together. Interestingly, the beta diversity analysis indicated the microbiome of SH and MD subgingival sites of the JP2+ subject was separated from all other oral, SH and MD samples of LAgP and HLAgP. Moreover, *A. actinomycetemcomitans* was 50 times more abundant in MD_LAgP than in SH_HLAgP.

Several studies have considered *A. actinomycetemcomitans* as the etiology agent of AgP (CASARIN et al., 2010; FINE et al., 2013b; KAPLAN et al., 2002; SCHACHER et al., 2007) and it has been detected among subjects of different ethnicities with periodontal lesions, mainly serotypes b and c (CHEN; WANG; CHEN, 2010; CORTELLI et al., 2005; SAKELLARI et al., 2011; TEIXEIRA et al., 2006; THIHA et al., 2007). However, *A. actinomycetemcomitans* was also previously detected in healthy subjects (FINE et al., 2013b; SHADDOX et al., 2012).

In contrast to what we found using a NGS method, Faveri et al. (2009), applying a target-directed method (DNA-DNA hybridization) in subgingival biofilm samples, observed that the proportion of *A. actinomycetemcomitans* was significantly higher in SH and intermediate periodontal pockets in subjects with LAgP, but not in patients with GAgP or ChP. Furthermore, *A. actinomycetemcomitans* was not associated with deep pockets, but recognized pathogens such as *P. gingivalis* and *T. denticola*, suggesting that *A. actinomycetemcomitans* is associated with the onset of the disease, being replaced by species of the red group with the progression of disease. On the other hand, Shaddox et al. (2012) observed that *A. actinomycetemcomitans* was also more abundant in diseased sites in LAgP when compared to healthy sites in both LAgP and healthy children using *16S rRNA*-based microarrays.

Previous data revealed that 70% of healthy adolescents harboring *A. actinomycetemcomitans* in their oral cavities did not present LAgP, whereas 25% of those initially *A. actinomycetemcomitans* positive developed LAgP over time (FINE et al., 2007). Lateron, the same group (FINE et al., 2013b) reported that AgP disease progression was associated to a consortium formed by *A. actinomycetemcomitans*, *Streptococcus parasanguinis*, and *F. alocis* and suggested that *A. actinomycetemcomitans* is necessary but sufficient to the development of LAgP (FINE et al., 2013b).

S. parasanguinis was not detected in any of the samples in the present study, although both *A. actinomycetemcomitans* and *F. alocis* were in increased abundance in MD of LAgP. *S. parasanguinis* and other oral streptococci, such as *S. sanguinis* and *S. gordonii*, produce peroxide, and *A. actinomycetemcomitans* produces catalase which protects *P. gingivalis* from the oxidative stress in a multi-species biofilm model, suggesting that *A. actinomycetemcomitans* may support the colonization of pathogens (ZHU et al., 2019)

We had also observed an increased abundance of Spirochaetae in MD_LAgP, due to increased abundance of the genus *Treponema*. Eleven *Treponema* species were more abundant in MD_LAgP including putative pathogens such as *T. denticola*, *T. maltophilum*, *T. parvum* and *T. socranskii*, some associated with LAgP (CAMPISCIANO et al., 2017; FAVERI et al., 2009; TAKEUCHI et al., 2003). *Treponema* sp._HMT_238 and _HMT_518 were part of the core microbiome of LAgP (increased in oral and MD_LAgP) with higher RA than in HLAgP.

Members of Synergistetes such as *Fretibacterium* (*Synergistaceae*) were more abundant in MD sites of LAgP than in health. *F. fastidiosum*, *F.* sp._HMT_358, _359 and _361 were detected in higher RA in LAgP. Synergistetes cluster A, which includes *F. fastidiosum*, was significantly in higher levels in the saliva of subjects affected by ChP and GAgP compared to healthy subjects (BELIBASAKIS et al., 2013). Other data indicated that *F. fastidiosum* should be considered a putative periodontal pathogen both in ChP and GAgP (DENG et al., 2017; OLIVEIRA et al., 2016).

Organisms that were detected in higher abundance in SP_LAgP such as *Centipeda periodontii*, and *Selenomonas artemidis*, (MOORE; JOHNSON; MOORE, 1987; RAMS et al., 2015), as well as species of *Treponema*, *Leptotrichia* and *Prevotella* were previously detected in subgingival sites of periodontitis-affected subjects, although the last two genera were associated with both disease and health, suggesting a variability of pathogenic potentials within the same genus (CHEN et al., 2018). *Prevotella oralis*, *P.* sp._HMT_304 and _315 were more abundant in MD_LAgP than in SH_HLAgP. Species of *Prevotella*, such as *P. intermedia* and *P. nigrescens* were detected in high prevalence in patients with LAgP, GAgP and ChP (CHAHBOUN et al., 2015; FAVERI et al., 2009; TOPCUOGLU; KULEKCI, 2015).

Another interesting aspect observed in the present study refers to the reduction of health-associated species in LAgP samples. Even is SP sites, it was possible to see features of a dysbiotic microbiota in LAgP, characterized by a reduction in organisms commonly associated to health (AAS et al., 2005; CHEN et al., 2018; COLOMBO et al., 2009; FINE et al., 2013b; KUMAR et al., 2006; SHADDOX et al., 2012). *Streptococcus* sp._HMT_056, *Capnocytophaga gingivalis*, *Aggregatibacter paraphrophilus*, *Leptotrichia* sp._HMT_879 and

Gemella haemolysans were less abundant in SP_LAgP compared to SP_HLAgP. The RA of *Actinomyces* sp._HMT_169 was decreased in SH_LAgP compared to SH_HLAgP, while the RA of *Rothia dentocariosa* and *Kingella oralis* was decreased in SH and MD_LAgP compared to SH_HLAgP. A total of 29 different species were less abundant in affected sites, including species of *Bergeyella*, *Capnocytophaga*, *Gemella*, *Prevotella*, *Streptococcus*, as well as *Granulicatella adiacens* and *Haemophilus parainfluenzae*.

These data are in agreement with previous studies where species of *Rothia* genus were described as members of the oral microbiota associated to health (AAS et al., 2005; COLOMBO et al., 2009; MOUTSOPOULOS et al., 2015) or were predominants in healthy subjects (COLOMBO et al., 2009). However, the association of the specie *R. dentocariosa* to disease or health is controversial. Despite its high abundance in both healthy and periodontitis samples (ABUSLEME et al., 2013; AI et al., 2017), this specie was also detected in higher abundance in periodontitis (KUMAR et al., 2003), or shown to be associated to health (GRIFFEN et al., 2012; PÉREZ-CHAPARRO et al., 2018). Furthermore, species of *Kingella* and *Haemophilus* were previously associated to health when the microbiota of Afro-descendants infants with LAgP and healthy controls was compared (SHADDOX et al., 2012).

The detection of microorganisms associated to periodontal disease in SP and SH sites of LAgP suggests that the change on the microbial community is not only associated to the to the presence of deep sites with low oxygen tension, and indicates that these sites in subjects with periodontitis can represent an intermediary stage on the disease development (GRIFFEN et al., 2012). *Porphyromonas*, *Tannerella* and *Treponema* were more abundant in SP and SH of LAgP when compared to the same sites of HLAgP, and their role in disease is reinforced by their highest abundance in MD sites of LAgP. Longitudinal studies in humans demonstrated that level of *P. gingivalis, T. forsythia* and *T. denticola* are correlated with periodontal loss (BYRNE et al., 2009), and interventional studies showed a relation between the presence of this pathogens in treated sites and disease progression (SOCRANSKY; HAFFAJEE, 2002).

Correlation analyzes was performed among species whose relative abundances differ between LAgP and health. In LAgP, the increased abundance of several strict anaerobes were correlated including members of the genus *Treponema*, *Prevotella*, *Peptostreptococcaceae Lachnoanaerobaculum*, and *Mollicutes* and *Bacteroidetes*.

In healthy subjects, abundance of several strict anaerobes correlated such as *Selenomonas*, *Fretibacterium*, *Eubacterium*, *Solobacterium*, and *Sneathia*. Moreover, both in LAgP and HLAgP, species that were associated to health strongly positively correlated. In HLAgP, the higher abundance of *Rothia mucilaginosa*, *Granulicatella elegans*, *Lautropia*

mirabilis and *Haemophilus* strongly positively correlated to *Neisseria,* but also to *Fusobacterium gonidiaformans,* and *Porphyromonas* sp. In LAgP, the low abundant members of the genus *Actinomyces, Neisseria* and *Rothia dentocariosa* were correlated. Moreover, in LAgP, the abundance of *Porphyromonas* sp._HMT_930 was correlated to the abundance of certain *Haemophilus* sp. These findings corroborate with the idea that the lack of beneficial organisms in the oral biofilm may be just as important as the presence of pathogens in the contribution to disease (BEREZOW; DARVEAU, 2011). The increase of obligate anaerobes and the reduction of health-associated species that correlated to each other suggest that the development of oral dysbiosis is likely to occur with changes in the symbiotic relationship between host and microbe's complex to a pathogenic one.

The gut microbiome of healthy subjects is dominated by Bacteroidetes and Firmicutes (HUMAN MICROBIOME PROJECT CONSORTIUM, 2012; QIN et al., 2010). Both HLAgP and LAgP gut microbiome were dominated by these phyla and feces analysis revealed that there were no significant differences in the microbiome between the groups at a high taxonomic level, except for the association of Deltaproteobacteria to disease, particularly *Desulfovibrio* (*Desulfovibrionaceae*). Interestingly, *Desulfovibrio* was more abundant in affected oral sites of LAgP as well, as described above. *Desulfovibrio* accounts for 66% and *Desulfobulbus* accounts for 16% of SRB in the human colon (LINDEN, 2014). Our data have also revealed that *Bilophila,* another SRB, was also more abundant in gut microbiome of LAgP subjects compared to HLAgP, but this difference was not statistically significant. In the intestinal tract, SBR convert sulphate into H_2S, which is toxic to epithelial cells, inhibits butyrate and other short chain fatty acids oxidation preventing their use by colonocytes, reducing the nutritionalsupport for these cells and leading to the destruction of intestinal barrier function (PITCHER; CUMMINGS, 1996; ROEDIGER; MOORE; BABIDGE, 1997). Exogenous H_2S is also genotoxic at levels commonly found in the colon, and a higher level of H_2S in feces was associated to colorectal cancer (CRC) (O'KEEFE et al., 2015). SRB are also associated to inflammatory bowel disease (GIBSON; CUMMINGS; MACFARLANE, 1991; LOUBINOUX et al., 2002a; PITCHER; CUMMINGS, 1996). An association between periodontitis and inflammatory bowel disease has been suggested, since the pathogenesis of both diseases involves a complex interplay between the immune inflammatory response and the dysbiotic microbiota, under the influence of environmental and genetic factors (LIRA-JUNIOR; FIGUEREDO, 2016).

Species of *Desulfovibrio* are commonly find in the environment and intestinal tract (JOUSIMIES-SOMER et al., 2003). However, *Desulfovibrio fairfieldensis* was associated to

compromised gastrointestinal tract, such as liver abscesses, choledocholithiasis, perforated appendage and rectorragia (PIMENTEL; CHAN, 2007). Moreover, greater RA of *Desulfovibrio* in detriment of health-associated microorganisms was reported in the intestinal microbiota of patients with fungicide-associated colon intoxication (JIN et al., 2016).

It is interesting to notice that race is strongly associated with the higher abundance of sulfidogenic bacteria in African-americans (AAs) compared to non-Hispanic whites, whereas diet had relatively small effects (YAZICI et al., 2017). However, this variable imposed no effect in the studied population, since all studied subjects both of the LAgP and control groups were of African descendancy. On the other hand, in AAs, a non significant increase in the RA of *Bilophila* and *Desulfovibrio* was demonstrated when CRC fecal samples were compared to healthy, suggesting the role of sulfidogenic bacteria as a risk factor contributing to CRC development in AAs.

Shuttleworthia (phylum Firmicutes) was only detected in LAgP feces than in HAgP. *Shuttleworthia satelles* was previously detected in periodontal pockets of subjects with refractory periodontitis (COLOMBO et al., 2009), and levels of this genus were increased significantly in cavitated dentin lesions (JIANG et al., 2014). The pathogenic potential of this genus in the gut has not been established, except for a report that cerebral ischemic stroke was associated with increased gut barrier permeability triggered by gut microbial disturbances which included incresead levels of *Shuttleworthia* (CHEN et al., 2019).

In sum, data obtained from the study of the oral microbiome associated with LAgP have shown that the microbial imbalance in disease is not restricted to subgingival affected sites, but also to supragingival and subgingival biofilm in still healthy periodontal sites. The imbalance is characterized by reduction of organisms considered beneficial such as *Kingella* and *Streptococcus*, and increased abundance of pathogens such as *A. actinomycetemcomitans*, *Porphyromonas, Tannerela* and *Treponema*. Our data suggested that treatment strategies should aim not only the reduction of pathogens, but favour the beneficial bacteria both at the supra and subgingival sites. On the other hand, we have shown an association of LAgP with organisms such as *Johnsonella, Acidovorax, Desulfobulbus* and other not well known SRB, and suggested a synergistic relation between NO and H_2S production as an important phenomenon of periodontal destruction. These observations should be investigated in future studies. Furthermore, our data revealed that dysbiosis of oral microbial in LAgP is accompanied by a certain imbalance of the gut microbiota, since LAgP patients presented an increased abundance of SRB of the genus *Desulfovibrio* in feces, an organism associated with gut inflammatory

conditions. Moreover, the association between LAgP in African descendants and enhance of sulfidogenic bacteria in the gut should be also investigated.

5.2 Cytokines and chemokines profile in LAgP and healthy subjects

AgP has been previously associated to several gene polymorphisms, suggesting that host related factors mediated by genetics may play a relevant role in AgP (LOOS et al., 2015).

In the present study, the salivary cytokines and chemokines profiles of LAgP and HLAgP were analyzed and compared. A strong positive correlation between disease severity (PD and CAL) in LAgP and IL-4 salivary levels was demonstrated. IL-4-expressing cells were previously reported in the periodontal tissues of AgP when compared to ChP patients. (LAPPIN et al., 2011).

Our main findings revealed differences in chemokines levels between LAgP and HLAgP, but not in cytokines levels. The salivary concentrations of CTAK/CCL27 and TARC/CCL17 were significantly higher in the saliva of LAgP patients compared with the healthy control group, whereas the levels of MCP-1/CCL2 and TECK/CCL25 were reduced in LAgP. Strong positive correlations were observed between TARC/CCL17, CTACK/CCL27, and EOTAXIN3/CCL26, I309/CCL1, IFN-γ, IL-4, MIP-3β/CCL19, MPIF1/CCL23, SDF1α+β/CXCL12. All of them were increased in disease, although only TARC/CCL17 and CTACK/CCL27 levels differed between LAgP and HLAgP ($p<0.05$).

Furthermore, the increase of TARC/CCL17 and CTACK/CCL27 in LAgP were strongly positively correlated to the increase of *A. ebreus*, *H. pylori* and *A. actinomycetemcomitans*, whereas TARC/CCL17 was also positively correlated to *Treponema* sp._HMT_490 as well. Moreover, our data indicated that *A. actinomycetemcomitans*, the specie that increased the most in MD_LAgP, was positively correlated not only to CTACK/CCL27 and TARC/CCL17 salivary levels, but also to EOTAXIN3/CCL26, IFN-γ, MIP-3β/CCL19, MPIF1/CCL23, SDF1α+β/CXCL12 salivary levels.

It is known that periodontopathogenic bacteria induce the expression of chemokines, which trigger chemotaxis and activation of various leukocytes (MURDOCH; FINN, 2000). The lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, stimulates innate immune cells via Toll-like receptor 4, triggering the inflammatory response (PÅLSSON-MCDERMOTT; O'NEILL, 2004). It has been reported that the activation of T-lymphocyte by *A. actinomycetemcomitans* serotype a stimulated a higher expression CCL17 ($p>0.05$)

(ALVAREZ et al., 2015), suggesting a role of this pathogen in the altered chemokines profile of LAgP.

TARC/CCL17 is a ligand for the receptor CCR4 (IMAI et al., 1999; YOSHIDA et al., 1998), which is predominantly expressed by Th2-polarized cells, but also in skin-homing of T cells, regulatory T cells (Treg) and Th17 cells (YOSHIE; MATSUSHIMA, 2015), TARC/CCL17 expression is upregulated during Chron´s disease (JUDGE; LICHTENSTEIN, 2001), whereas the absence of TARC/CCL17 results in increased induction of regulatory T cells (Tregs), reduction of Th17 and lower levels of proinflammatory cytokines in an experimental colitis model (HEISEKE et al., 2012). In the oral cavity, TARC/CCL17 is produced by dendritic cells and gingival fibroblasts of diseased periodontal tissues (HOSOKAWA et al., 2008), driven by macrophage colony–stimulation factor (M-CSF) (ACHUTHAN et al., 2016). Data on gingival biopsies of ChP patients indicated increased expression of CCL17 and its receptor CCR4, as well as Tregs in the inflammatory infiltrate (CARDOSO et al., 2008). Thus, although TARC/CCL17 involvement in periodontitis should still be elucidated, the increased salivary levels of TARC/CCL17 in LAgP may indicate a possible role of this chemokine in the disease.

CTAK/CCL27 and its receptor CCR10 are involved in immunity of epithelium (XIONG et al., 2012). CTAK/CCL27 is supposed to be involved in T cell-mediated inflammation (HOMEY et al., 2002), and its expression is induced by TNF-α and downregulated by INF-γ and IL-17 (KANDA; KOIKE; WATANABE, 2005). In animal models, a reduction in CTAK/CCL27 expression was observed when *Streptococcus mutans* growth increased in oral cavity, leading to alveolar bone loss, and caries under a sucrose rich diet (TAMASAS; COX, 2017). Despite its protective role, higher levels of CTAK/CCL27 in serum were associated with not only with atopic dermatitis (REISS et al., 2001), but also with psoriasis and eczema (GARZORZ; EYERICH, 2015), and multiple sclerosis (KHAIBOULLINA et al., 2015).

Our data have also indicated significantly lower levels of MCP-1/CCL2 and TECK/CCL25 in the saliva of patients with LAgP than in their controls. Higher MCP-1/CCL2 levels were reported in saliva, GCF and serum of ChP (GUPTA; CHATURVEDI; JAIN, 2013), and in GCF of GAgP patients when compared to healthy controls (EMINGIL; ATILLA; HÜSEYINOV, 2004), although these levels could not differentiate ChP and GAgP (KURTIŞ et al., 2005). On the other hand, previous data on AgP patients reported that GCF of periodontitis sites (GUNPINAR; ALPTEKIN; DUNDAR, 2017) and the plasma (ZEIN ELABDEEN et al., 2017) yielded lower concentrations of MCP-1 than their healthy controls. Furthermore, lower MCP-1/CCL2 levels were demonstrated in the GCF of diseased sites of

LAgP when compared to healthy sites (SHADDOX et al., 2011), which would result in a lower content of MCP-1/CCL2 in the saliva of LAgP when compared to controls, in accordance with our observation.

MCP-1/CCL2 is involved in the recruitment of inflammatory monocytes to mucosa surfaces (TSUI et al., 2007; YU; GRAVES, 1995), since its receptor, CCR-2, is only expressed by these cells (GEISSMANN; JUNG; LITTMAN, 2003; PALFRAMAN et al., 2001). Moreover, MCP-1/CCL2 and may regulate the angiogenic process by affecting the expression of vascular endothelial growth factor A (STAMATOVIC et al., 2006), which is highly expressed by inflammatory monocytes (QIAN et al., 2011), and although MCP-1/CCL2 is also associated with osteoclast chemotaxis and differentiation in periodontitis (KIM et al., 2006), and is produced in inflamed gingival tissues (YU; GRAVES, 1995), the reduced levels of MCP-1/CCL2 in the saliva of LAgP patients when compared to controls may indicate reduced defenses at the affected gingival sites.

Our data have also indicated reduced salivary levels CCL25 or thymus-expressed chemokine (TECK) in LAgP. This chemokine also mediates inflammatory monocytes and T cells recruitment to the infection sites (LINTON et al., 2012), and it leads to resistance to induced colitis in experimental models of the small intestine by participating in the regulatory mechanisms for immune tolerance (MIZUNO et al., 2012). The role of TECK/CCL25 in tolerance was further demonstrated by data indicating that ligation of the receptor CCR9 to TECK/CCL25 inhibits Treg cell differentiation (EVANS-MARIN et al., 2015).

Tissue destruction is mediated by several mediators, and differing from most studies which evaluated a limited number of potential biomarkers, we reported data on 40 mediators in saliva. Thus, despite the limited number of subjects involved in this study, our data indicated that a combination of chemokines may be useful as biomarkers for AgP, since LAgP showed a profile of decreased salivary levels of MCP-1/CCL2 and TECK/CCL25, and increased salivary levels of TARC/CCL17 and CTAK/CCL27. The altered chemokines profile in LAgP may shape the recruitment of inflammatory monocytes and the traffic and differentiation of regulatory T cells to the periodontal area, thus altering the homeostasis of the gingival tissues to commensal bacteria.

Further data with higher number of patients and other populations should still be obtained. The analysis of expression and polymorphism of genes encoding these chemokines will help to elucidate the genetic background of LAgP and may allow improvement in diagnosis and better treatment options for this disease.

5.3 Acquired enamel pellicle and stimulated saliva proteome

In recent years, it became evident that the salivary composition can reflect alterations in response to certain disease states. Salivary biomarkers are not only arising in correlation with oral disorders, but also to distal pathologies affecting other tissues and organs, suggesting that saliva may represent a key reservoir of molecular and microbial features capable of transmiting valuable information on the onset or presence of diseases throughout the body (SHAH, 2018). In the present study, we aimed to evaluate the proteome profile of the acquired enamel pellicle and stimulated saliva in order to find biomarkers that could be associated to the periodontal status observed in LAgP-affected subjets and determine differences on the proteome between LAgP and health.

The oral biofilm formation starts with the recognition of salivary proteins adsorbed to the enamel surface by initial colonizers such as *Actinomyces* and *Streptococcus* species, which subsequently promotes co-aggregation of microorganisms, and is of utmost importance to determine if the microbial succession will happen towards health or to a dysbiotic environment (LI et al., 2004a).

A total of 361 unique proteins from *in vivo* AEP was identified, a greater amount than previously reported (SIQUEIRA et al., 2007; ZIMMERMAN et al., 2013). This achievement might be due to two aspects: (1) AEP elution from collection strips: protocol adaptations were made with sequential centrifugations to improve the removal of debris from the collection strips, facilitating the protein purification and elution afterwards; (2) AEP samples: AEP was collected from each individual and samples were singly submitted to LC-ESI-MS/MS, differing from other studies that used pooled samples (DELECRODE et al., 2015; LEE et al., 2013; ZIMMERMAN et al., 2013). A high variability in the amount of proteins among subjects was observed. Despite the improvements made to the collection protocol and protein recovery procedures, our data are in accordance to others who had shown that proteins can vary between subjects (DELIUS et al., 2017).

A total of 812 unique proteins were identified in StS samples of LAgP and HLAgP. More than 1,000 proteins were already identified in the proteome of human saliva, including data obtained from periodontitis patients and with other oral diseases such as oral cancer (BOSTANCI; BAO, 2017). The number of proteins detected in periodontitis can vary depending on the applied proteomic method, the saliva sample (stimulated or unstimulated) and sample size (BALIBAN et al., 2012; GONÇALVES et al., 2010; HAIGH et al., 2010; WU et

al., 2009). Despite the considerable effort and the application of the state-of-the art MS methods, the salivary proteome of periodontitis is not yet fully mapped (BOSTANCI; BAO, 2017).

The microbial composition of the biofilm differs between healthy and LAgP, as we and others have shown (FAVERI et al., 2009; FINE et al., 2013a), with higher colonizing levels of *A. actinomycetemcomitans* and lower levels *Actinomyces* spp., *S. gordonii* and *S. oralis* in LAgP. *A. naeslundii* type 1 fimbriae and adhesins of oral streptococci bind peptide motifs of proline-rich proteins (PRPs) (GIBBONS et al., 1990; LI et al., 1999) and these interactions contribute to their attachment to the AEP (RUHL; SANDBERG; CISAR, 2004). In the present study, two PRPs were detected in the AEP samples: small PPR 3 (SPRR3) and submaxillary gland androgen-regulated protein 3B (SMR3B), also known as proline-rich protein 3. Although both PPRs were detected in the StS of all LAgP and in more than 80% of the HLAgP subjects, SPRR3 was detected in the AEP of more than 65% of the HLAgP subjects, while SMR3B was detected in more than 50% of the LAgP group.

SPRR3 is the most abundant type of PPR in oral and esophageal epithelia, although two isoforms could be detected in fetal parotid, submandibular gland and saliva of human preterm newborns (MANCONI et al., 2010). On the other hand, SMR3B can bind to bacteria and bacterial LPS, the endotoxin of gram negative bacteria which triggers tissue destructive inflammatory reactions (CHOI et al., 2011). Although SMR3B was more frequently detected in the AEP of LAgP (50% of LAgP), it was present in all StS studied samples in this study. A possible explanation for this phenomena should be the adsortion of LPS to the AEP first, which should be more abundant in saliva of LAgP than HLAgP, followed by the ligation of SMR3B to the already adsorbed LPS. Our suggestion is reinforced by observations that saliva present high level of endotoxin (LEENSTRA et al., 1996) and induces macrophages to M1, similar to LPS (POURGONABADI et al., 2017). However, this hypothesis still needs to be confirmed, since these studies did not evaluate healthy and LAgP subjects.

Both SPRR3 and alpha-amylase 1 were found only in the AEP of healthy subjects, although they were part of the StS proteome of most subjects. *S. gordonii* and *S. mitis* bind to alpha-amylase, while other streptococci (*S. sanguinis*, *S. oralis*, *S. vestibularis*, and *S. mutans*) lack alpha-amylase-binding capacity (KILIAN; NYVAD, 1990). Alpha-amylase 1, also known as alpha-amylase, is one the major constituents of saliva and is widely-known for its endoglicosidase activity. However, it plays an important antimicrobial role in the oral cavity, inhibiting the growth of certain bacteria, bacterial pili and LPS (FÁBIÁN et al., 2012). Moreover, salivary alpha-amylase plays a significant role in inhibiting bacteria growth

including of *P. gingivalis* species, and interfere on the adherence and biofilm formation of *A. actinomycemcomitans*, indicating that alpha-amylase may have an effective role in preventing periodontal diseases (BAIK et al., 2013; OCHIAI et al., 2014).

Cystatin-S was detected in 50% of HLAgP subjects, but in none of the AEP of LAgP subjects ($p<0.05$), although it was detected in the StS of all studied the subjects. This protein also binds to bacteria as well as bacterial LPS (CHOI et al., 2011). Cystatin-S and some of its peptides can also lead to growth inhibition of *P. gingivalis* (BLANKENVOORDE et al., 1996, 1998), and partially inhibited its proteolytic activity (cysteine proteinase) (BLANKENVOORDE et al., 1996), suggesting that cystatin C may modulate colonization by this key stone pathogen in periodontitis (HAJISHENGALLIS; LAMONT, 2012).

Cystatins act as inhibitors of cysteine proteinases and could play a protective and regulatory role under inflammatory conditions (HENSKENS et al., 1994). Total cystatin activity of WS is increased in periodontitis-affected patients (HENSKENS et al., 1993). Previous data reported the detection of cystatin-S in WS of healthy subjects, whereas cystatin-C was barely detectable. In contrast, both cystatin-C and -S levels were in higher levels in WS of gingivitis and periodontitis-affected patients (HENSKENS et al., 1994). Total cystatin activity was about five times higher in submandibular than in parotid saliva (HENSKENS et al., 1994). In submandibular and sublingual saliva, both cystatin-S and -C were observed. In contrast, in parotid saliva samples, only cystatin-C was detectable. Moreover, increased cystatin activity was shown in WS of subjects with inflammatory periodontal disease (HENSKENS et al., 1993).

In this present study, cystatin-C was detected in only one StS sample of HLAgP and in none LAgP StS samples or AEP samples of both groups. Cystatin-A was only detected in AEP samples, while cystatin-B was detected in both AEP and StS samples. Cystatin-D and –SN were only detected in StS saliva samples. In general, cystatins were detected more frequently in LAgP subjects than in HLAgP, despite the association of cystatin-S to healthy AEP samples. The lack of quantitative data makes inadequate the comparison between these findings to the previously described studies, however our data suggest that the presence of cystatin-S in the AEP may contribute to the maintanence of a healthy dental biofilm.

A total of 11 mucins were detected in the AEP in the present study (mucin -4, -5AC, -5B, -6, -7, -12, -16, -17, -19, -20, -22). Mucins are large highly glycosylated salivary proteins mainly involved in lubrication and hydration of teeth surface and oral mucosa, confering a protective barrier to the epithelial cells as well as against bacterial proteases in the AEP (AMERONGEN; BOLSCHER; VEERMAN, 1995; FRENKEL; RIBBECK, 2015; TABAK,

1995). Moreover, mucins can present an indirect antimicrobial activity by aggregating oral microorganisms and other proteins such as IgA, lactoferrin and lysozyme, altering their capacity to modulate the microbial colonization in the oral cavity (AL-HASHIMI; LEVINE, 1989; BRUNO et al., 2005; FRENKEL; RIBBECK, 2017; IONTCHEVA; OPPENHEIM; TROXLER, 1997; TABAK, 1995; WICKSTRÖM et al., 2000). Mucins containing oligosaccharide side-chains with a terminal sialic acid can be detected in epithelial cells and on the enamel surface (GIBBONS et al., 1990). Species such as *A. naeslundii* produce neuraminidase, which cleaves sialic acid and exposes a hidden receptor in mucins, the second last galactosyl sugar residue. This receptor will serve as binding sites to organisms which synthetize galactosyl-binding lectins, such as *A. naeslundii*, *Leptotrichia buccalis*, *F. nucleatum*, *Eikenella corrodens* and *Prevotella intermedia* (MARSH et al., 2016).

Although mucin-4 was detected in more than 50% of StS samples of LAgP subjects, it was not detected in the AEP of these patients. Mucin-4 was detected in the StS and AEP of two healthy individuals. Mucin-4 is secreted by the submandibular gland and its reduced level was appointed as a saliva biomarker for periodontitis, since it is detected in lower levels in the saliva and gingival crevicular fluid of periodontitis subjects compared to healthy controls (LUNDMARK et al., 2017). It was suggested that mucin-4 may be degraded by bacterial or host derived proteolytic proteins and that lower levels of mucin-4 in saliva may impact its capability to agglutinate and promote the clearance of oral pathogens, triggering the inflammatory response by the accumulation of oral biofilm in periodontitis affected subjects (LUNDMARK et al. 2017).

In the present study, 87 AEP proteins and 168 StS proteins were involved in immune response processes, which corresponded to the most abundant group of proteins involved in biological processes. Proteins involved in the innate and acquired immune response are compositional of the AEP (SIQUEIRA et al., 2007; ZIMMERMAN et al., 2013) and are mostly multifunctional (FÁBIÁN et al., 2012). Among the proteins involved in immune response, we have identified 30 distinct proteins in the AEP with antimicrobial properties and 63 in StS samples. Antimicrobial proteins also play an important role in the maintenance of the biofilm composition. These saliva components synergistically co-interact and can have direct or indirect antimicrobial activities (MARSH et al., 2016). Both studied groups shared some well-known salivary proteins with antimicrobial activity in the AEP such as lactotransferrin, lysozyme C, mucin-7, myeloperoxidase, cystatins and statherin. On the other hand, the antimicrobial arsenal of each group also contained exclusive contributors that are associated to other important processes to the maintenance of a homeostatic environment.

Despite the higher number of proteins with antimicrobial properties in the StS of LAgP compared to the HLAgP group, this difference was not reflected in the AEP composition. The number of AEP proteins with antimicrobial properties detected in LAgP samples was lower than in HLAgP. A higher number of immunoglobulin allotypes was observed in the AEP of healthy subjects compared to LAgP patients, including two involved in antimicrobial activity and inflammatory response: Ig heavy constant gamma 1 (IGHG1) and Ig lambda constant 1 (IGLC1).

Immunoglobulins are fundamental components of the adaptive immune response in the oral cavity, excluding antigens in saliva, on the mucus layer and on the epithelia, as well as in the AEP (BRANDTZAEG, 2007; FÁBIÁN et al., 2012). We have also detected peptides of the polymeric immunoglobulin receptor (pIgR) in the AEP of 80% healthy subjects and in less than 50% of the LAgP subjects, although pIgR was detected in all of StS samples. pIgR may be associated to the higher amount of immunoglobulins allotypes in the AEP of HLAgP, since its secretory component (SC) presents strong affinity for the J chain of dimeric and polymeric IgA and pentameric IgM (BRANDTZAEG, 2007). pIgR is responsible for the molecular transport of these antibodies into the glandular lumen, whereas SC remains incorporated into SIgA and SIgM permanently, conferring resistance to these secretory immunoglobulins against the proteolytic degradation (BRANDTZAEG, 2007).

Wu et al. (2009) using two dimensional gel electrophoresis had shown that the levels of Ig allotypes were increased in the UtS of GAgP-affected individuals compared to healthy controls. The reduction in the number of Ig on the dental surface of affected individuals may be influenced by the oral microbiota. It is well known that the periodontopathogen *P. gingivalis* produces many cysteine proteases such as gingipains, periodontain, PrtT protease, and Tpr protease (POTEMPA et al., 2003). Gingipains are the most important proteases produced by this bacterium and can cleave several host components, such as the extracellular matrix, cytokines, immunoglobulins, and complement factors through trypsin-like activity (GUO; NGUYEN; POTEMPA, 2010; IMAMURA; TRAVIS; POTEMPA, 2003; POTEMPA et al., 2003; POTEMPA; BANBULA; TRAVIS, 2000). Gingipain K (Kgp) cleaves IgG1 and IgG3 at the hinge region, leading to the separation of the antigen binding Fab fragment from the effector Fc fragment of immunoglobulins, which was demonstrated not only *in vitro*, but also in the gingival crevicular fluid *in vivo* (GUENTSCH et al., 2013; VINCENTS et al., 2011), one of the main sources of AEP proteins (SIQUEIRA et al., 2007). These findings indicate that microbial proteases may have an important role in the modulation of immunological components on the tooth surface.

Histatins are multifunctional histidine-rich proteins mainly acting in the modulation of mineral formation, buffering process, wound healing and can present antifungal and antibacterial properties (EDGERTON; KOSHLUKOVA, 2000; OPPENHEIM et al., 2007; RAJ; EDGERTON; LEVINE, 1990; reviewed in TORRES et al., 2018). Histatins 1 and 3 were detected in the StS samples of LAgP and HLAgP groups, however, his1 was detected only in the AEP of two subjects with LAgP and in none of healthy controls. His1 plays an important role in the enamel maintenance by adsorbing to hydroxyapatite, and it seems to play a role in re-epithelialization of the oral mucosa and angiogenic responses by cell migration mechanisms (TORRES et al., 2018). His1 may also be relevant to homeostasis by its wound healing and antimicrobial properties, especially due to LPS neutralization (OUDHOFF et al., 2008; SUGIYAMA, 1993). The absence of detection of histatins in the AEP of LAgP subjects and control group may be due time of collection (after 2h of formation). Lee et al. (2013), analyzing *in vivo* AEP formation by proteomics at different time points, observed that histatins 1 and 3 abundance drastically decreased after 60 and 120 minutes of pellicle formation compared to the first 5 minutes. Despite of this method limitation, it was previously detected through slot blot quantification a higher concentration of his1 in WS of subjects with ChP compared to healthy controls (TRINDADE et al., 2015).

Some groups of proteins also involved in immune response processes were only detected in the AEP of healthy subjects, such as ATP-binding cassete subfamily a and b, E3 ubiquitin-protein ligases and histone-lysine N-methyltransferases. With the exception of ATP-binding cassete proteins, proteins of the other two groups were detected also in the StS of LAgP group. Such proteins are involved in several activities, including phagocytosis, DNA repair, neutrophil degranulation, protein-protein binding, negative regulation of cytokine production involved in inflammatory response, negative regulation of reactive oxygen species biosynthetic process, activation of MAPK activity, between others (Uniprot annotations). PMNs of LAgP and GAgP patients present alterations, such as reduced migration and affected antibacterial functions (GENCO et al., 1980), and the manipulation of PMNs survival and functions by microbial factors such as gingipains, serine proteases, lipid phosphatases, or fimbriae produced by *P. gingivalis* (SOCHALSKA; POTEMPA, 2017).

In response to pathogenic microorganisms, several immune-inflammatory mediators are released to the periodontal pocket (DI BENEDETTO et al., 2013; KINANE, 2001; KORNMAN, 2008). Cumulative proteolytic activities executed by host derived molecules (e.g. collagenases, matrix metalloproteinases, hyaluronidase) complemented by bacterial proteases (e.g. gingipain R and K produced by *Porphyromonas gingivalis*) lead to the breakdown of

collagen and other extracellular matrix derived proteins (ECM) at both soft and hard tissue level, endorsing the destruction of the tooth support apparatus (DI BENEDETTO et al., 2013; INTO et al., 2006; KINANE, 2001; RUGGIERO et al., 2013; YASUHARA et al., 2009). In the AEP samples, we detected a total of 4 proteins with proteolytic activity according to GO annotations (GO:0006508). Cathepsin G and PIP were common between LAgP and healthy subjects, whereas the metalloprotease ADAMTS9 (a disintegrin and metalloproteinase with thrombospondin motifs 9) and low-density lipoprotein receptor-related protein 8 (also known as apolipoprotein E receptor 2 or ApoER2), both cell-derived proteins, were detected only in the AEP of LAgP, while ADAMTS9 was also detected in StS samples of these patients.

ADAMTS9 is a zinc metalloendopeptidase, whose substrates are components of ECM (MEAD; APTE, 2018; SOMERVILLE et al., 2003). ADAMTS-like proteins lack a metalloprotease domain, are ECM constituents and present regulatory roles, such as papilin (KRAMEROVA et al., 2000), which present serine-type endopeptidase inhibitor activity (GO:0004867). Papilin was also detected only in the AEP of LAgP.

On the other hand, ApoER2 is a cellular receptor involved in apolipoprotein E-containing lipoproteins, originally identified in neurons (KIM et al., 1996), although it is expressed on platelets (thrombocytes) (PENNINGS et al., 2007), endothelial cells (KORSCHINECK et al., 2001) and monocytes/macrophages (CHEN et al., 2012; YANG et al., 2009). Possibly, it was detected in saliva due to extracellular cleavages to release its soluble forms (KOCH et al., 2002). Gao et al. (2015) suggested that polymorphisms in gene encoding the low density lipoprotein receptor (LRP5) and apolipoprotein E may lead to dyslipidemia and are associated with subjects affected by generalized aggressive periodontitis. Moreover, they suggested that dyslipidemia may be a risk indicator for GAgP in the Chinese population. However, we could not find any association between ApoER2 and periodontitis in the literature.

Protein S100-A9, a calcium- or zinc-binding protein, involved in the regulation of pro-inflammatory processes and immune response (MARENHOLZ; HEIZMANN; FRITZ, 2004), was detected in the AEP of more than 50% of the subjects and in the StS of more than 80% of the LAgP subjects, and in the AEP of two HLAgP subjects and in the StS of more than 50% of the HLAgP subjects. This protein was recently pointed as a candidate biomarker for periodontitis (SHIN et al., 2018). Patients affected by periodontitis and gingivitis presented increased levels of S100A9/S100A8 etherodimer (calprotectin) in the gingival crevicular fluid (KIDO et al., 2012; KOJIMA et al., 2000). Moreover, calprotectin concentration was correlated with periodontal markers of inflammation such as pocket probing depth or gingival bleeding (ANDERSEN et al., 2010; ELLIAS et al., 2012). S100A9 also promotes apoptosis and

modulate the inflammatory response in periodontal ligament cells, so its down-regulation could suggest a suppression of inflammation (ELLIAS et al., 2012; ZHENG et al., 2014). A recent study using shotgun proteomics confirmed by ELISA showed that salivary levels of S100A8 and S100A9 were higher in subjects with periodontitis compared to healthy controls and pointed these proteins as candidate biomarkers for periodontitis (SHIN et al., 2018).

Alstrom syndrome protein 1 was the only protein exclusively detected in the AEP of patients with LAgP that was present in more than 50% of subjects ($p<0.05$). It is an ubiquitous protein that localizes to centrosomes and basal bodies of ciliated cells (ANDERSEN et al., 2003; HEARN et al., 2005; LI et al., 2007). Mutation in its encoding gene (ALMS1) is associated to Alström syndrome (HEARN et al., 2002; MARSHALL et al., 2007), a condition associated with retinal degeneration, hearing loss, cardiomyopathy, obesity and diabetes mellitus (COLLIN et al., 2002), with severe early-onset insulin resistance (MARSHALL et al., 2005, 2011; MINTON et al., 2006). ALMS1 has not been associated to periodontal disease before, however ALMS1 has a lot of splice variants which are not totally understood (COLLIN et al., 2002).

Regarding the significant differences between the StS protein profile of LAgP and HLAgP, dystonin was detected only on HLAgP samples, present in more than 50% of these subjects. Dystonin, known as bullous pemphigoid antigen 1 (BPAG1 or BP230) is a member of the plakin protein family of cytolinkers that is also present in the gingival basement membrane protein. This protein is the target of antibodies detected in subtypes of autoimmune bullous diseases (SCHMIDT; ZILLIKENS, 2013), manifested as blisters and erosions which affect the oral mucosa, referred to as desquamative gingivitis (GAGARI; DAMOULIS, 2011). Association between periodontitis and bullous diseases has been reported (AKMAN et al., 2008; THORAT; RAJU; PRADEEP, 2010; TRICAMO et al., 2006). Peng, Nisengard and Levine (1986) demonstrated that gingival biopsy specimens of individuals with periodontitis appear to be affected by the sera of an individual with bullous pemphigoid, whereas a clinically normal gingivae specimen remained intact, suggesting that basement membrane changes may be related to the pathogenesis of periodontitis. One of the hypotheses raised in a systematic review is that not only clinical characteristics overlap, but also pathophysiologic similarity or interference may relate in both diseases (JASCHOLT et al., 2017), since periodontitis is an inflammatory disease where leukocyte infiltration and persistent activation in response to the oral biofilm results in periodontal tissue destruction. Periodontitis is associated to the release of proinflammatory cytokines such as IL-6, IL-8 and matrix metalloproteinases (e. g., MMP9) (YUCEL-LINDBERG; BÅGE, 2013). Similar inflammatoty mechanisms are involved in the

pathogenesis of bullous pemphigoid diseases, including antihemidesmosomal autoantibody-induced cytokine production (e.g., IL-6 and IL-8), complement-mediated recruitment of matrix metalloproteinase (e. g., MMP9) and reactive oxygen species–releasing leukocytes that lead to the degradation of the basal membrane zone components resulting in dermoepidermal splitting (JASCHOLT et al., 2017; SCHMIDT; ZILLIKENS, 2013).

Alpha-2-macroglobulin-like protein 1 (A2ML1), a protein that was also more prevalent in the StS of HLAgP than in LAgP subjects ($p<0.05$), is a broad range protease inhibitor, acting *in vitro* against chymotrypsin, papain, thermolysin, and subtilisin A (GALLIANO et al., 2006). However, it is a unique target for Paraneoplastic pemphigus, a multiorgan autoimmune syndrome with intractable stomatitis and polymorphous cutaneous lesions (ANHALT et al., 1990; SCHEPENS et al., 2010). Antibodies in the sera of individuals afflicted by Paraneoplastic pemphigus bind to plakin family proteins, including dystonin (BP230) (KIYOKAWA et al., 1998). Increased expression levels of A2ML1 is associated to keratinocyte differentiation (GALLIANO et al., 2006; SCHEPENS et al., 2010). Moreover, A2ML1 is similar to alpha-2-macroglobulin, and appears to participate in defense mechanisms, maintenance of epidermal homeostasis, and regulation of cytokines and growth factors release (GALLIANO et al., 2006).

Alpha-actinin 4 was also more prevalent in the StS of HLAgP than in LAgP subjects ($p<0.05$). Alpha-actinins are cytoskeletal proteins which cross-link filamentous actin to establish cytoskeletal structure, protecting cells from mechanical stress and controlling cell movement (SJÖBLOM; SALMAZO; DJINOVIĆ-CARUGO, 2008). Unlike other ACTNs, alpha-actinin 4 displays unique features in signaling transduction, nuclear translocation, and gene expression regulation (HSU; KAO, 2013). Overexpression of actinin-4 has been detected in several invasive cancers, including salivary gland carcinoma (WATABE et al., 2014), oral squamous cell carcinoma (YAMADA et al., 2010) and tongue cancer (KAKUYA et al., 2017). On the other hand, deficiencies in this protein appear to contribute to kidneys's cells defect in multiple human glomerulopathies, including sporadic focal segmental glomerulosclerosis, minimal change disease, and IgA nephropathy (LIU et al., 2011). Kidney epithelial cells depleted of α-actinin-4 or expressing a disruptive mutant appear to make more massive stress fibers that are less dynamic than those in wildtype cells, leading to defects in cell motility and wound healing (KEMP; BRIEHER, 2018).

Immunoglobulin heavy constant alpha 1 (IGHA1) was detected in the StS of all HLAgP individuals and in less than 50% of LAgP, indicating a possible reduction of these molecules in the saliva of affected individuals. IGHA1 is the constant region of Ig heavy chains (SCHROEDER; CAVACINI, 2010), associated to immune response and antimicrobial activity.

Uniprot annotations show that IGHA1 is part of SIgA. In healthy individuals, secretory IgA (SIgA) is the most abundant antibody fulfilling the function of microbial exclusion on the lumen of mucosal surfaces (MACPHERSON et al., 2008). In the oral cavity, SIgA is one of the key molecules on effective antimicrobial activity (PEDERSEN; BELSTRØM, 2019). The combination of innate and inducible cellular and molecular mechanisms ensures mucosa protection against colonization or invasion of microrganisms (BRANDTZAEG, 2009). Thus, our observations should indicate more SIgA in the saliva of healthy subjects than in LAgP, which deserves further investigations.

Two proteins were more prevalent in the StS of LAgP than in health, present in the saliva of all LAgP subjects and in less than 50% of healthy controls: alpha-enolase and profilin-1.

Alpha-enolase, also known as enolase 1 (ENO1), is a glycolytic enzyme expressed in most tissues, and is a highly conserved protein pointed as possible candidate for molecular mimicry between bacterial and human proteins (ALBERT; INMAN, 1999). Human ENO1 presents homology and cross-reactivity with enolase of *P. gingivalis* (KINLOCH et al., 2011; LUNDBERG et al., 2008). A correlation between antibody titers to *P. gingivalis* and anti-ENO1 and the severity of periodontitis and rheumatoid arthritis (RAR) was reported (LEE et al., 2015). Studies have shown an increased frequency of periodontitis in RAR-affected individuals compared to individuals without RAR (CHEN et al., 2013; DISSICK et al., 2010; TORKZABAN et al., 2012). *P. gingivalis* can mediate citrullination of host peptides through peptidylarginine deiminase (PAD) expression, leading to the generation of systemic immunogens that contain epitopes against which anti-citrullinated protein antibodies could be produced, such as the anti-citrullinated α-enolase peptide-1, a RA-associated autoantibody (MCGRAW et al., 1999; ROSENSTEIN et al., 2004). Furthermore, the leukotoxin of *A. actinomycetemcomitans* can induce endogenous peptidylarginases by neutrophils which citrulinate endogenous proteins, and was associated to RAR (KONIG et al., 2016). Since ENO1 appears to be more prevalent in StS samples of LAgP subjects and its peptides can be modified by PADs from *P. gingivalis* or from neutrophils submitted to *A. actinoycetemcomitans* leukotoxin, this protein may contribute to the increased inflammatory response observed in LAgP.

Profilins constitute a group of conserved small actin-binding proteins with regulatory roles in several cellular activities, including actin-proliferation and motility (DING; BAE; ROY, 2012). Profilin-1 is an actin-depolymerizing factor that appears to be involved in advantageous processes such as remodeling of the cytoskeleton and stress response in inflamed

gingival tissue (TOMASELLO et al., 2017). Thus, the high prevalence of profilin-1 in StS samples of LAgP may be related to active remodeling and inflammation of periodontitis affected sites.

We have only evaluated the proteome of AEP and StS qualitatively, although these data shed light on differences between LAgP and health. The abundance of common salivary proteins in the AEP and in StS may vary depending on the status of healthy or disease, however in the absence of a quantitative data, alterations in the abundance of these common proteins that could direct influence in the oral biofilm composition could not be evaluated in this present study.

Several proteins involved in relevant processes to AEP were more frequently detected in healthy than in the LAgP subjects, despite the absence of statistical significance, suggesting that undetectable levels or the lack of some proteins involved in immune response processes, antimicrobial activity and anti-inflammatory molecules could have a negative impact in the maintenance of the homeostatic environment on the tooth surface. The WS protein profile appear not to reflect the AEP composition in LAgP, since the number of proteins involved in immune response, antimicrobial activity and inflammatory response were higher in the StS, but were decreased in the AEP of these subjects. Non-detectable levels or the lack of proteins such as cystatin-S, alpha-amylase and some mucins in the AEP of subjects affected by LAgP may have an impact on the adhesion of initial colonizers (beneficial bacteria), as well as in the inhibition of pathogens colonization on the dental surface, leading to the dysbiotic environment observed in this disease. On the other hand, the lack or low frequency of several proteins such as cystatin S, alpha-amylase and SPRR3 in the AEP of a certain group, healthy or diseased, but their presence in StS saliva samples of both groups may indicate saturation of ligation with planktonic bacteria in saliva. Thus, their antimicrobial activity or aggregation roles would be performed in saliva, leading to clearance of specific bacterial groups from the oral cavity. Alternatively, these proteins may have been degraded in saliva during the AEP formation. Due to their importance in adhesion or as inhibitors of bacterial growth, this hypothesis should be elucidated in *in vitro* models.

Moreover, our data suggests that the AEP of subjects affected by LAgP may influence the colonization of beneficial bacteria (initial colonizers) and other organisms leading to the dysbiotic environment observed in this disease. These data are in accordance to the oral microbiome results, where healthy subjects harbored more benefical organisms than LAgP-subjects. The dysbiosis observed in disease may be correlated to reduced numbers of immunoglobulins and other immune-related molecules in the AEP of LAgP subjects.

Furthermore, the impact of the absence or decreased prevalence of proteins such dystonin, A2ML1 and alpha-actinin-4 in WS of the LAgP group should be investigated.

Our data also support the hypothesis of a genetic background determinant for the establishment of LAgP. The observation of correlation between chemokines and certain organisms, especially *A. actinomycetemcomitans* suggested that the microbial community modulate host response, or alternatively, host response variation in LAgP may have allowed conditions for the dysbiosis of the microbial community. The latter hypothesis is reinforced by the demonstration of a dysbiotic microbiota not only in the oral cavity, but also in the gut of LAgP patients.

Lastly, our data indicated that the detection of proteins in AEP and StS samples, in a multianalyte evaluation system, including the detection of Alstrom syndrome protein 1 and absence of Cystatin-S in AEP samples in AEP samples, as well as the detection of alpha-enolase and profilin-1 and absence dystonin, A2ML1, alpha-actinin-4 and IGHA1 in StS samples, as well as certain salivary chemokines should be tested as biomarkers for AgP in more diverse larger population.

5.4 Saliva proteolytic activity (histatin 1 and 5 degradation in stimulated saliva)

Histatins plays an important role in tooth enamel protection, therefore their degradation is an unfavorable event (MCDONALD et al., 2011). Studies evaluating this event were carried out in WS of healthy subjects (CAMPESE et al., 2009; HELMERHORST et al., 2006; MCDONALD et al., 2011; SIQUEIRA et al., 2012b; SUN et al., 2009; THOMADAKI et al., 2011), however there is a lack of information regarding histatins degradation in the saliva of periodontitis patients (THOMADAKI et al., 2013). Proteolytic events may still occur within the salivary glands during posttranslational proteolytic processes (HARDT et al., 2005; HAY et al., 1988; JENSEN et al., 1991; OPPENHEIM; HAY; FRANZBLAU, 1971; SABATINI; AZEN, 1989; TROXLER et al., 1990), and are triggered by host and bacterial enzymes in the oral cavity (HELMERHORST et al., 2006). In WS, proteolysis events occur extremely rapid (BAUM et al., 1976; PAYNE et al., 1991). Although histatins can be degraded when in free-form or attached, the proteolysis of the his1 bound to hydroxyapatite is five times slower than of free his1 (MCDONALD et al., 2011).

In order to evaluate if the periodontal condition can influence the rate and pattern of degradation, stimulated saliva of LAgP and healthy subjects was incubated with synthetic his1 and his5.

Once histatins degradation is really rapid in WS (BAUM et al., 1976; PAYNE et al., 1991), we decided not to perform this assay with undiluted SS samples. The magnitude of degradation in WS is such that it can impair the detection of some native salivary protein by electrophoretic, chromatographic or immuno-quantitation methods (JENSEN et al., 1991; KOUSVELARI et al., 1980; LI et al., 2004b; OPPENHEIM, 1970). Fractions of 1:10 diluted WS do not contain detectable levels of cationic proteins (HELMERHORST et al., 2006), which makes possible the evaluation of degradation of synthetic histatins and their degradation peptides.

In this present study, the degradation of his5 in saliva occurred faster than the degradation of his1. Even after 48 hours of incubation, it was still possible to detect intact his1, whereas his5 was totally degraded in the DStSS of all subjects after 24h. Similar results were observed in a study developed in Professor Siqueira's lab using exactly the same protocol and comparing samples of patients with ChP, Down Syndrome (DS), ChP+DS and healthy subjects (data not yet published). These findings also corroborate to a previous study which observed that his5 is more susceptible to proteolytic events than his1 in WS (SUN et al., 2009). Sun et al., 2009 suggested that human proteases have a high affinity to HRGY↓ and RGYR↓ regions, present in histatin 3 and 5, but not in his1.

McDonald et al. (2011) incubated his1 in 1:10 WS supernatant samples of health subjects and revealed by HPLC analysis that degradation peptides can be detected after 0.5 h of incubation and they tend to increase in the range of 1-2 h. However, the intact protein can resist to proteolysis until 40 h of incubation (SUN et al., 2009). Our findings in Cationic-PAGE, a less sensitive method, show his1 peptides starting to appear after 0.5 h of incubation in the DStSS of patients affected by periodontitis and after 1.5 h in healthy subjects' samples. Sun et al., 2009 observed by HPLC a loss of his1 around 50% after 1.5 h of incubation and its complete absence after 40 h, applying the same concentration of his1 in the DStSS of healthy donors. In this present study, even with a less sensitive method, more than 82% of intact his1 was detected after 1.5 h in all samples, and after 48 h, it was detected in 71.4% of healthy subjects anddiseased patients' samples, indicating a variability between subjects, especially in the presence of periodontitis.

In contrast, the rate of degradation of his5 was higher than of his1 in the same condition. Right after adding synthetic his5 to DStSS, the aliquot of this mixture corresponding to time 0

(t=0) was boiled to stop proteolytic activity. Despite this procedure was completed in a short interval (maximum of 1 min), degradation bands were detected at t=0 in both groups, indicating that, even in ten times diluted saliva, his5 degradation occurs extremely fast. After 24 h of incubation, we could not detect intact his5 in any of the samples. According to Helmerhorst et al. (2006), the HPLC analysis showed that the first degradation fragments were detectable after 4 h in 1:10 saliva samples of healthy subjects with 4 times more concentrated his5 than we used (400µg/mL). However, they still could detect intact his5 peak after 24 h. Previous studies observed a reduction of 75% in his5 concentration after 1.5 h of incubation (SUN et al., 2009; THOMADAKI et al., 2011) and a total loss of this protein after 8 h of incubation (THOMADAKI et al., 2011). We observed that saliva of one LAgP individual (14.3%) had completely degraded his5 after 4 h of incubation, whereas the saliva of healthy subjects had not completely degraded his5 after this period.

Proteolysis has been recognized as an important virulence determinant in periodontitis progression (ARMITAGE, 2004; KADOWAKI et al., 2000). Bretz and Loesche (1987) demonstrated that the rate of proteolysis in dental biofilm is indicative of the periodontal disease status and the degradation event was believed to be associated to proteolytic enzymes produced by bacteria associated to the development of periodontal disease. A study analyzing the microbial metatranscriptome showed that proteolysis is one of the events that are upregulated during periodontal disease progression, not only in members of the red complex (*P. gingivalis*, *T. denticola*, and *T. forsythia*) but also in members of the orange complex (*P. intermedia, P. nigrescens, P. micra, F. nucleatum, F. periodonticum, C. gracilis, C. rectus, S. constellatus, E. nodatum*, and *C. showae*) (YOST et al., 2015).

Helmerhorst et al., 2006 observed a high consistency in the early peptides generated after his5 degradation by WS from healthy subjects, suggesting that one or a set of proteases with similar specificities may be involved in this process. The consistency observed in the pattern of degradation in WS indicates that this process involves similar proteolytic features among healthy subjects. Studies demonstrated that subjects with high incidence of caries and caries-free subjects present differences in whole and glandular saliva protein composition (AYAD et al., 2000), as well as in their ability to serve as substrates to support the growth of *S. mutans* (COWMAN et al., 1979), suggesting that the degradation and utilization of certain salivary proteins by microorganisms may be associated to a particular oral disease. The WS proteolytic features shared among subjects with similar disease and differences in clinical phenotypes clearly open avenues toward of exploiting this saliva property for diagnostic purposes (HELMERHORST, 2007).

In view of the interesting results obtained in this present study, the evaluation of the rate of histatins degradation in diluted WS of affected subjects could be suggested as a future simple and complementary diagnostic method, since samples can be obtained in a painless and non-invasive manner and showed to have a discriminatory proteolytic activity in a short time period between healthy subjects and those affected by periodontitis. Furthermore, histatins degradation should be tested in order to monitor the development and progression of the disease, particularly in those families/populations with a higher prevalence of periodontitis.

In vitro studies indicated that his5 can inhibit both host metalloproteases (MMP-2 and MMP-9) (GUSMAN et al., 2001b) and bacterial-derived proteases such as clostripain (cysteine proteinase derived from the pathogen *Clostridium histolyticum*) (GUSMAN et al., 2001a) and Arg-gingipaine and Lys-gingipaine from *P. gingivalis* (GUSMAN et al., 2001b). In periodontal disease, his5 possess properties that are considered protective, acting in the inhibition of inflammatory cytokine expression by human gingival fibroblasts, induced by *P. gingivalis* (IMATANI et al., 2000) and neutralizes the leukotoxin produced by *A. actinomycetemcomitans* (MURAKAMI et al., 2002).

In this present study, we observed LAgP seems to have an impact on the speed of degradation of his5. WS of subjects affected by periodontitis presents a high proteolytic activity when compared to healthy subjects (THOMADAKI et al., 2013). Trindade et al. (2015) observed through inhibition assays that proteases with a higher activity in WS of ChP-affected subjects, and with higher activity towards gelatin-like substrates, are sorted in ascending order as: aspartic proteases < serine proteases < metalloproteinases.

His5 inhibitory mechanisms are possibly partially suppressed in the saliva of diseased patients by its extremely rapid degradation, since proteolysis associated with whole salivary enzymes does not instantly abolish all functional activity, and some initial peptides of his5 degradation still have antimicrobial activity (HELMERHORST et al., 2006). On the other hand, his1 and his5 end fragments appear to lose the zinc-binding motifs (HEXXH) which can chelate the zinc ion from the catalytic sites present in host proteases such as MMP-2 and MMP-9 (MELINO et al., 1999; SUN et al., 2009). The levels of these metalloproteases are elevated in the saliva of patients affected by periodontal disease (SORSA; TJÄDERHANE; SALO, 2004). These findings suggest that the degradation of these proteins in saliva of disease subjects may have a critical impact in the inhibition of MMP in the oral cavity (SUN et al., 2009).

The implementation of evaluation of MMP-protease activities in the clinic has been proposed (SORSA et al., 2011) and these activities would represent one of the best diagnostic markers for active periodontal disease (THOMADAKI et al., 2013). As observed in this present

study, the degradation of his5 could be suggested as a potent complementary diagnostic test for periodontitis as well. In association to other disease-associated markers, such as the detection of periodontopathogens, WS proteolytic activities could increase the discriminating power of such diagnostic assays for periodontal disease and its progression (THOMADAKI et al., 2013). Moreover, the elucidation of salivary protein degradation mechanisms in WS will not only contribute to the knowledge of how this process happens in the oral cavity, but also facilitate the selection of peptides for further functional analysis and enable the design of variants that are more resistant to proteases, enhancing its retention time and activity in the oral cavity (SUN et al., 2009).

6 CONCLUSION

Supra and subgingival microbiome analysis of LAgP and HLAgP revealed some aspects not yet described in the literature, and our data contribute to the understanding of the microbiota associated with the disease.

Oral microbiome analysis confirms the microbial dysbiosys in LAgP, with the reduction of abundance of organisms considered beneficial such as species of *Bergeyella*, *Capnocytophaga*, *Gemella*, *Streptococcus*, *Granulicatella*, *Haemophilus* and *Kingella* and increased abundance of pathogenic and putative organisms such as *A. actinomycetemcomitans*, *Porphyromonas*, *Tannerela* and *Treponema*.

The association of organisms such as *Johnsonella, Acidovorax, Desulfobulbus* and other not well known sulfate reducing bacteria with LAgP, as well as the synergistic relation between NO and H_2S producers as an important phenomenon of periodontal destruction should be investigated in future studies.

Dysbiosis of oral microbial community in LAgP may be accompanied by a certain imbalance of the intestinal microbiota, since LAgP patients presented an increased abundance of sulfate reducing bacteria of the genus *Desulfovibrio* in feces, an organism associated with gut inflammatory conditions. Moreover, the association between LAgP in African descendants and enhanced levels of sulfidogenic bacteria in the gut should be also investigated.

Despite the limited number of subjects involved in this study, our data indicated that a combination of chemokines may be useful as biomarkers for AgP, since LAgP showed a profile of decreased salivary levels of MCP-1/CCL2 and TECK/CCL25, and increased salivary levels

of TARC/CCL17 and CTAK/CCL27. The altered chemokines profile in LAgP may shape the recruitment of inflammatory monocytes and the traffic and differentiation of regulatory T cells to the periodontal area, thus altering the homeostasis of the gingival tissues to commensal bacteria.

The salivary levels of certain chemokines profile correlated the abundance of oral organisms. Our data suggested that oral colonization may be modultated by host factors such as chemokines, or alternatively, certain organisms such as *A. actinomycetemcomitans* may impair host defenses by altering chemokines production in the oral cavity.

The dysbiosys of the oral microbiota may be influenced by AEP and saliva composition in LAgP subjects, who presented undetectable levels or lacked some proteins involved in immune response processes, antimicrobial activity and anti-inflammatory molecules.

The salivary proteolytic activity in LAgP-affected subjects was higher compared to healthy controls, which may impact the salivary and AEP protein profile. Our findings suggest that the alterations observed in the StS and AEP protein composition in affected individuals may occur not only due to host proteolysis events, but also due to the increased abundance of known proteolytic bacteria in the oral microbiome, such as the periodonthopathogens *P. gingivalis, T. denticola*, and *T. forsythia*, and other protease producers such as *A. actinomycetemcomitans*, species of *Prevotella, F. nucleatum, E. nodatum*, among others. Moreover, WS proteolytic activities should be tested as diagnostic tool for periodontitis and its progression.

The detection of proteins in AEP and WS samples, in a multianalyte evaluation system should be tested as biomarkers for LAgP, including the detection of Alstrom syndrome protein 1 and absence of Cystatin-S in AEP samples in AEP samples, as well as the detection of alpha-enolase and profilin-1 and absence dystonin, A2ML1, alpha-actinin-4 and IGHA1 in WS samples.

Taken all toghether, our findings enlightened new aspects of AgP and collaborated to the understanding of mechanisms that may be involved in the development and progression of the disease, and open avenues to new innovational treatment strategies, aiming not only the reduction of pathogens, but favoring the colonization of beneficial bacteria, as well as in the use of antimicrobial designed peptides based on salivary proteins in order to enhance its retention time and activity in the oral cavity.

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Appendix 1. List of subjects' samples that were collected and used in each experiment. SP= supragingival, SH= shallow, MD= medium/deep, AEP= acquired enamel pellicle, CT= cytokines, CM= chemokines.

Subjects	Microbiome				AEP proteomics	Saliva		
	SP	SH	MD	Gut		CT and CM	Saliva proteomics	Proteolytic activity
LAgP 1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LAgP 2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LAgP 3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LAgP 4	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LAgP 5	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
LAgP 6	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
LAgP 7	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HLAgP 1	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 2	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 3	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 4	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 5	Yes	Yes	No	Yes	No	No	No	No
HLAgP 6	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 7	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
HLAgP 8	Yes	Yes	No	Yes	No	Yes	Yes	Yes

Appendix 2 – Species present in oral sites with statistical significant difference (nonparatric t test, $p<0.05$) between LAgP and HLAgP in each compared site: oral, supragingival (SP), shallow (SH) and medium/deep x shallow (MDxSH). Red squares indicate species more abundant in LAgP and blue squares indicate species more abundant in HLAgP. Asterisks indicate species present only in the specified group.

Phylum	Family	Genus and specie	Site
Absconditabacteria_(SR1)	Absconditabacteria_(SR1)_[F-1]	Absconditabacteria_(SR1)_[G-1] bacterium_HMT_875	SH
Actinobacteria	*Actinomycetaceae*	*Actinomyces cardiffensis*	SH
Actinobacteria	*Actinomycetaceae*	*Actinomyces naeslundii*	Oral, MDxSH
Actinobacteria	*Actinomycetaceae*	*Actinomyces* sp._HMT_169	Oral, SH
Actinobacteria	*Actinomycetaceae*	*Actinomyces* sp._HMT_414	Oral, SH, MDxSH
Actinobacteria	*Actinomycetaceae*	*Actinomyces* sp._HMT_525	Oral, SP, SH, MDxSH
Actinobacteria	*Atopobiaceae*	*Olsenella* sp._HMT_807	SP
Actinobacteria	*Atopobiaceae*	*Olsenella uli*	MDxSH
Actinobacteria	*Eggerthellaceae*	*Eggerthella lenta*	MDxSH
Actinobacteria	*Micrococcaceae*	*Rothia dentocariosa*	Oral, SH, MDxSH
Actinobacteria	*Micrococcaceae*	*Rothia mucilaginosa*	Oral
Actinobacteria	*Propionibacteriaceae*	*Pseudopropionibacterium propionicum*	SP
Actinobacteria	*Propionibacteriaceae*	*Pseudopropionibacterium* sp._HMT_194	Oral
Bacteroidetes	*Bacteroidaceae*	*Bacteroidaceae* _[G-1] bacterium_HMT_272	Oral, MDxSH
Bacteroidetes	*Bacteroidaceae*	*Bacteroides heparinolyticus*	MDxSH
Bacteroidetes	Bacteroidetes_[F-1]	Bacteroidetes_[G-3] bacterium_HMT_280	Oral, MDxSH
Bacteroidetes	Bacteroidetes_[F-1]	Bacteroidetes_[G-5] bacterium_HMT_511	Oral, MDxSH
Bacteroidetes	Bacteroidetes_[F-1]	Bacteroidetes_[G-7] bacterium_HMT_911	MDxSH
Bacteroidetes	*Flavobacteriaceae*	*Bergeyella* sp._HMT_206	Oral
Bacteroidetes	*Flavobacteriaceae*	*Bergeyella* sp._HMT_322	MDxSH
Bacteroidetes	*Flavobacteriaceae*	*Bergeyella* sp._HMT_422	MDxSH
Bacteroidetes	*Flavobacteriaceae*	*Bergeyella* sp._HMT_900	Oral
Bacteroidetes	*Flavobacteriaceae*	*Capnocytophaga gingivalis*	Oral, SP, MDxSH
Bacteroidetes	*Flavobacteriaceae*	*Capnocytophaga granulosa*	Oral
Bacteroidetes	*Flavobacteriaceae*	*Capnocytophaga* sp._HMT_324	Oral
Bacteroidetes	*Flavobacteriaceae*	*Capnocytophaga sputigena*	MDxSH
Bacteroidetes	*Porphyromonadaceae*	*Porphyromonas endodontalis*	Oral, MDxSH
Bacteroidetes	*Porphyromonadaceae*	*Porphyromonas gingivalis*	Oral
Bacteroidetes	*Porphyromonadaceae*	*Porphyromonas pasteri*	Oral
Bacteroidetes	*Porphyromonadaceae*	*Porphyromonas* sp._HMT_285	MDxSH
Bacteroidetes	*Porphyromonadaceae*	*Porphyromonas* sp._HMT_930	Oral
Bacteroidetes	*Porphyromonadaceae*	*Tannerella forsythia*	MDxSH
Bacteroidetes	*Porphyromonadaceae*	*Tannerella* sp._HMT_808	MDxSH
Bacteroidetes	*Prevotellaceae*	*Alloprevotella* sp._HMT_914	MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella baroniae*	Oral
Bacteroidetes	*Prevotellaceae*	*Prevotella fusca*	Oral
Bacteroidetes	*Prevotellaceae*	*Prevotella histicola*	MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella nanceiensis*	MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella oralis*	Oral, MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella scopos*	MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_301	Oral
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_304	Oral, MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_313	SP
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_315	Oral, MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_376	MDxSH
Bacteroidetes	*Prevotellaceae*	*Prevotella* sp._HMT_472	MDxSH
Firmicutes	*Carnobacteriaceae*	*Granulicatella adiacens*	MDxSH
Firmicutes	*Carnobacteriaceae*	*Granulicatella elegans*	Oral
Firmicutes	Clostridiales_[F-1]	Clostridiales_[F-1][G-2] bacterium_HMT_402	MDxSH
Firmicutes	*Enterococcaceae*	*Enterococcus italicus*	Oral
Firmicutes	*Erysipelotrichaceae*	*Bulleidia extructa*	MDxSH
Firmicutes	*Erysipelotrichaceae*	*Solobacterium moorei*	Oral
Firmicutes	*Gemellaceae*	*Gemella haemolysans*	Oral, SP
Firmicutes	*Gemellaceae*	*Gemella morbillorum*	Oral, MDxSH
Firmicutes	*Gemellaceae*	*Gemella sanguinis*	MDxSH
Firmicutes	*Lachnospiraceae* _[XIV]	*Johnsonella* sp._HMT_166	SP
Firmicutes	*Lachnospiraceae* _[XIV]	*Lachnoanaerobaculum* sp._HMT_496	Oral, MDxSH
Firmicutes	*Lachnospiraceae* _[XIV]	*Lachnospiraceae* _[G-3] bacterium_HMT_100	SH
Firmicutes	*Lachnospiraceae* _[XIV]	*Lachnospiraceae* _[G-8] bacterium_HMT_500	Oral, MDxSH
Firmicutes	*Lachnospiraceae* _[XIV]	*Stomatobaculum* sp._HMT_097	Oral, MDxSH
Firmicutes	*Lachnospiraceae* _[XIV]	*Stomatobaculum* sp._HMT_373	MDxSH
Firmicutes	Mollicutes_[F-2]	Mollicutes_[G-2] bacterium_HMT_906	Oral
Firmicutes	*Peptococcaceae*	*Peptococcus* sp._HMT_167	Oral, MDxSH
Firmicutes	*Peptostreptococcaceae* _[XI]	*Filifactor alocis*	Oral, MDxSH
Firmicutes	*Peptostreptococcaceae* _[XI]	*Peptostreptococcaceae* _[XI][G-3] bacterium_HMT_950	MDxSH
Firmicutes	*Peptostreptococcaceae* _[XI]	*Peptostreptococcaceae* _[XI][G-4] bacterium_HMT_369	Oral, MDxSH
Firmicutes	*Peptostreptococcaceae* _[XI]	*Peptostreptococcaceae* _[XI][G-5] bacterium_HMT_493	SH*, MDxSH*
Firmicutes	*Peptostreptococcaceae* _[XI]	*Eubacterium saphenum*	Oral, MDxSH
Firmicutes	*Peptostreptococcaceae* _[XI]	*Eubacterium nodatum*	Oral, MDxSH

Appendix 2. (continued)

Phylum	Family	Genus and specie		Site
Firmicutes	*Selenomonadaceae*	*Centipeda periodontii*		SP*
Firmicutes	*Selenomonadaceae*	*Mitsuokella* sp._HMT_131		MDxSH
Firmicutes	*Selenomonadaceae*	*Selenomonas artemidis*		SP
Firmicutes	*Selenomonadaceae*	*Selenomonas flueggei*		SH
Firmicutes	*Selenomonadaceae*	*Selenomonas infelix*		Oral, SH
Firmicutes	*Selenomonadaceae*	*Selenomonas* sp._HMT_126		SH
Firmicutes	*Selenomonadaceae*	*Selenomonas* sp._HMT_134		Oral, MDxSH
Firmicutes	*Selenomonadaceae*	*Selenomonas* sp._HMT_936		SH
Firmicutes	*Selenomonadaceae*	*Selenomonas sputigena*		Oral, MDxSH
Firmicutes	*Streptococcaceae*	*Streptococcus cristatus* _clade_578		Oral
Firmicutes	*Streptococcaceae*	*Streptococcus gordonii*		MDxSH
Firmicutes	*Streptococcaceae*	*Streptococcus oralis* _subsp._*tigurinus* _clade_071		Oral, MDxSH
Firmicutes	*Streptococcaceae*	*Streptococcus* sp._HMT_056		Oral, SP
Firmicutes	*Veillonellaceae*	*Dialister* sp._HMT_119		MDxSH
Firmicutes	*Veillonellaceae*	*Veillonella atypica*		MDxSH
Firmicutes	*Veillonellaceae*	*Veillonella* sp._HMT_917		Oral
Firmicutes	*Veillonellaceae*	*Veillonellaceae* _[G-1] bacterium_HMT_135		Oral, SP, SH, MDxSH
Firmicutes	*Veillonellaceae*	*Veillonellaceae* _[G-1] bacterium_HMT_145		Oral, MDxSH
Fusobacteria	*Fusobacteriaceae*	*Fusobacterium gonidiaformans*		Oral
Fusobacteria	*Leptotrichiaceae*	*Leptotrichia goodfellowii*		Oral
Fusobacteria	*Leptotrichiaceae*	*Leptotrichia* sp._HMT_212		SP
Fusobacteria	*Leptotrichiaceae*	*Leptotrichia* sp._HMT_215		SP
Fusobacteria	*Leptotrichiaceae*	*Leptotrichia* sp._HMT_392		MDxSH*
Fusobacteria	*Leptotrichiaceae*	*Leptotrichia* sp._HMT_879		SP
Fusobacteria	*Leptotrichiaceae*	*Sneathia sanguinegens*		Oral, SP
Gracilibacteria_(GN02)	Gracilibacteria_(GN02)_[F-1]	Gracilibacteria_(GN02)_[G-1] bacterium_HMT_871		Oral, MDxSH*
Proteobacteria	*Burkholderiaceae*	*Lautropia mirabilis*		Oral
Proteobacteria	*Campylobacteraceae*	*Campylobacter showae*		Oral
Proteobacteria	*Campylobacteraceae*	*Campylobacter sputorum*		Oral
Proteobacteria	*Cardiobacteriaceae*	*Cardiobacterium hominis*		MDxSH
Proteobacteria	*Commamonadaceae*	*Acidovorax ebreus*		SP, MDxSH
Proteobacteria	*Desulfobulbaceae*	*Desulfobulbus* sp._HMT_041		Oral, MDxSH
Proteobacteria	*Desulfovibrionaceae*	*Desulfovibrio* sp._HMT_040		MDxSH*
Proteobacteria	*Helicobacteraceae*	*Helicobacter pylori*		MDxSH
Proteobacteria	*Neisseriaceae*	*Kingella denitrificans*		Oral
Proteobacteria	*Neisseriaceae*	*Kingella oralis*		Oral, SH, MDxSH
Proteobacteria	*Neisseriaceae*	*Kingella* sp._HMT_012		Oral
Proteobacteria	*Neisseriaceae*	*Neisseria flavescens*		Oral
Proteobacteria	*Neisseriaceae*	*Neisseria* sp._HMT_018		Oral
Proteobacteria	*Neisseriaceae*	*Neisseria* sp._HMT_020		Oral
Proteobacteria	*Pasteurellaceae*	*Aggregatibacter actinomycetemcomitans*		MDxSH
Proteobacteria	*Pasteurellaceae*	*Aggregatibacter aphrophilus*		Oral
Proteobacteria	*Pasteurellaceae*	*Aggregatibacter paraphrophilus*		Oral, SP
Proteobacteria	*Pasteurellaceae*	*Aggregatibacter* sp._HMT_458		Oral
Proteobacteria	*Pasteurellaceae*	*Haemophilus parahaemolyticus*		Oral
Proteobacteria	*Pasteurellaceae*	*Haemophilus parainfluenzae*		MDxSH
Proteobacteria	*Pasteurellaceae*	*Haemophilus* sp._HMT_036		Oral
Proteobacteria	*Pasteurellaceae*	*Haemophilus* sp._HMT_908		Oral
Proteobacteria	*Rhodocyclaceae*	*Rhodocyclus* sp._HMT_028		Oral, SP*
Proteobacteria	*Xanthomonadaceae*	*Stenotrophomonas maltophilia*		SP
Saccharibacteria_(TM7)	Saccharibacteria_(TM7)_[F-1]	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_347		Oral
Saccharibacteria_(TM7)	Saccharibacteria_(TM7)_[F-1]	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_349		SP
Saccharibacteria_(TM7)	Saccharibacteria_(TM7)_[F-1]	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_952		SH
Saccharibacteria_(TM7)	Saccharibacteria_(TM7)_[F-1]	Saccharibacteria_(TM7)_[G-6] bacterium_HMT_870		Oral
Spirochaetes	*Spirochaetaceae*	*Treponema denticola*		MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema maltophilum*		MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema parvum*		Oral, MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema socranskii*		Oral, MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_227		SH*, MDxSH*
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_231		Oral, MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_234		MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_238		Oral, MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_249		MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_263		Oral
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_270		Oral
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_490		MDxSH
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_508		SP
Spirochaetes	*Spirochaetaceae*	*Treponema* sp._HMT_518		Oral, MDxSH
Synergistetes	*Synergistaceae*	*Fretibacterium fastidiosum*		Oral, MDxSH
Synergistetes	*Synergistaceae*	*Fretibacterium* sp._HMT_358		Oral, MDxSH
Synergistetes	*Synergistaceae*	*Fretibacterium* sp._HMT_359		Oral, MDxSH
Synergistetes	*Synergistaceae*	*Fretibacterium* sp._HMT_361		MDxSH

Appendix 3. Core microbiome based on species present in oral biofilm of 50% of the subjects of LAgP, HLAgP and common species. L indicates species more abundant in LAgP and H in HLAgP. Asterisks indicate statistically significant difference in relative abundance between oral_LAgP and oral_HLAgP (*p<0.05, **p<0.01, ***p<0.001, nonparametric t test)

LAgP	Common		HLAgP
Acidovorax ebreus	*Abiotrophia defectiva*	*Mycoplasma salivarium*	*Acidipropionibacterium acidifaciens*
Actinomyces cardiffensis	Absconditabacteria_(SR1)_[G-1] bacterium_HMT_345	*Neisseria bacilliformis*	*Aggregatibacter paraphrophilus* **
Actinomyces sp._HMT_897	*Actinomyces gerencseriae*	*Neisseria elongata*	*Bacteroides pyogenes*
Butyrivibrio sp._HMT_080	*Actinomyces israelii*	*Neisseria flavescens* *^{H}	*Capnocytophaga* sp._HMT_902
Fastidiosipila sanguinis	*Actinomyces massiliensis*	*Neisseria mucosa*	Clostridiales_[F-1][G-2] bacterium_HMT_402
Fretibacterium sp._HMT_360	*Actinomyces meyeri*	*Neisseria oralis*	*Cutibacterium acnes*
Neisseriaceae _[G-1] bacterium_HMT_174	*Actinomyces naeslundii* *^{H}	*Olsenella* sp._HMT_807	*Desulfomicrobium orale*
Peptostreptococcaceae _[XI][G-1] bacterium_HMT_383	*Actinomyces* sp._HMT_169*^{H}	*Olsenella uli*	*Eggerthella lenta*
Prevotella oralis *	*Actinomyces* sp._HMT_170	*Oribacterium sinus*	*Enterococcus saccharolyticus*
Prevotella sp._HMT_306	*Actinomyces* sp._HMT_414*^{L}	*Oribacterium* sp._HMT_078	Gracilibacteria_(GN02)_[G-1] bacterium_HMT_871**
Prevotella sp._HMT_315*	*Actinomyces* sp._HMT_448	*Parvimonas* sp._HMT_110	*Haemophilus* sp._HMT_908*
Saccharibacteria_(TM7)_[G-4] bacterium_HMT_355	*Actinomyces* sp._HMT_525***^{L}	*Peptidiphaga gingivicola*	*Leptotrichia* sp._HMT_463
Scardovia wiggsiae	*Actinomyces timonensis*	*Peptidiphaga* sp._HMT_183	*Leptotrichia* sp._HMT_847
Selenomonas sp._HMT_126	*Aggregatibacter actinomycetemcomitans*	*Peptococcus* sp._HMT_167*^{L}	*Moraxella* sp._HMT_276
Streptococcus constellatus	*Aggregatibacter aphrophilus** ^{H}	*Peptococcus* sp._HMT_168	*Ottowia* sp._HMT_894
Treponema amylovorum	*Aggregatibacter* sp._HMT_458	*Peptoniphilaceae* _[G-1] bacterium_HMT_113	*Peptostreptococcaceae* _[XI][G-1] *sulci*
Treponema sp._HMT_238*	*Aggregatibacter* sp._HMT_512	*Peptostreptococcaceae* _[XI][G-2] bacterium_HMT_091	*Porphyromonas* sp._HMT_278
Treponema sp._HMT_239	*Aggregatibacter* sp._HMT_898	*Peptostreptococcaceae* _[XI][G-3] bacterium_HMT_495	*Prevotella* sp._HMT_293
Treponema sp._HMT_242	*Aggregatibacter* sp._HMT_949	*Peptostreptococcaceae* _[XI][G-4] bacterium_HMT_369*^{L}	*Pseudopropionibacterium* sp._HMT_194**
Treponema sp._HMT_258	*Agrobacterium tumefaciens*	*Peptostreptococcaceae* _[XI][G-7] bacterium_HMT_081	*Ruminococcaceae* _[G-3] bacterium_HMT_366
Treponema sp._HMT_490	*Alloprevotella rava*	*Porphyromonas catoniae*	*Streptococcus cristatus* _clade_578*
Treponema sp._HMT_508	*Alloprevotella* sp._HMT_473	*Porphyromonas endodontalis* *^{L}	*Tannerella* sp._HMT_916
Treponema sp._HMT_518*	*Alloprevotella* sp._HMT_912	*Porphyromonas gingivalis* *^{L}	*Treponema* sp._HMT_230
Veillonellaceae _[G-1] bacterium_HMT_150	*Alloprevotella* sp._HMT_914	*Porphyromonas pasteri* *^{H}	
	Alloprevotella tannerae	*Porphyromonas* sp._HMT_275	
	Anaeroglobus geminatus	*Porphyromonas* sp._HMT_277	
	Anaerolineae _[G-1] bacterium_HMT_439	*Porphyromonas* sp._HMT_284	
	Atopobium parvulum	*Porphyromonas* sp._HMT_285	
	Bacillus anthracis	*Prevotella baroniae* *^{L}	
	Bacteroidaceae _[G-1] bacterium_HMT_272*^{L}	*Prevotella buccae*	
	Bacteroidales_[G-2] bacterium_HMT_274	*Prevotella dentalis*	
	Bacteroides heparinolyticus	*Prevotella denticola*	
	Bacteroides zoogleoformans	*Prevotella enoeca*	
	Bacteroidetes_[G-3] bacterium_HMT_280**^{L}	*Prevotella fusca* *^{L}	
	Bacteroidetes_[G-3] bacterium_HMT_281	*Prevotella histicola*	
	Bacteroidetes_[G-3] bacterium_HMT_899	*Prevotella intermedia*	
	Bacteroidetes_[G-5] bacterium_HMT_505	*Prevotella maculosa*	
	Bacteroidetes_[G-5] bacterium_HMT_507	*Prevotella marshii*	
	Bacteroidetes_[G-5] bacterium_HMT_511*^{L}	*Prevotella melaninogenica*	
	Bacteroidetes_[G-7] bacterium_HMT_911	*Prevotella micans*	
	Bergeyella sp._HMT_206**^{H}	*Prevotella nanceiensis*	
	Bergeyella sp._HMT_322	*Prevotella nigrescens*	
	Bergeyella sp._HMT_422	*Prevotella oris*	
	Bergeyella sp._HMT_900*^{H}	*Prevotella oulorum*	
	Bergeyella sp._HMT_907	*Prevotella pallens*	
	Bifidobacterium dentium	*Prevotella pleuritidis*	
	Bulleidia extructa	*Prevotella saccharolytica*	
	Burkholderia cepacia	*Prevotella salivae*	
	Butyrivibrio sp._HMT_090	*Prevotella scopos*	
	Campylobacter concisus	*Prevotella shahii*	
	Campylobacter gracilis	*Prevotella* sp._HMT_292	
	Campylobacter showae *^{L}	*Prevotella* sp._HMT_301*^{L}	
	Campylobacter sp._HMT_044	*Prevotella* sp._HMT_304*^{L}	
	Capnocytophaga ginqivalis ***^{H}	*Prevotella* sp._HMT_309	
	Capnocytophaga granulosa *^{H}	*Prevotella* sp._HMT_313	
	Capnocytophaga leadbetteri	*Prevotella* sp._HMT_314	
	Capnocytophaga sp._HMT_323	*Prevotella* sp._HMT_317	
	Capnocytophaga sp._HMT_324*^{H}	*Prevotella* sp._HMT_376	
	Capnocytophaga sp._HMT_326	*Prevotella* sp._HMT_396	
	Capnocytophaga sp._HMT_332	*Prevotella* sp._HMT_443	
	Capnocytophaga sp._HMT_336	*Prevotella* sp._HMT_472	
	Capnocytophaga sp._HMT_338	*Prevotella* sp._HMT_526	
	Capnocytophaga sp._HMT_412	*Prevotella veroralis*	
	Capnocytophaga sp._HMT_863	*Pseudomonas stutzeri*	
	Capnocytophaga sp._HMT_864	*Pseudopropionibacterium propionicum*	
	Capnocytophaga sputigena	*Pyramidobacter piscolens*	
	Cardiobacterium hominis	*Rothia aeria*	
	Cardiobacterium valvarum	*Rothia dentocariosa* ***^{H}	
	Catonella sp._HMT_164	*Rothia mucilaginosa* *^{H}	
	Catonella sp._HMT_451	*Ruminococcaceae* _[G-1] bacterium_HMT_075	
	Clostridiales_[F-1][G-1] bacterium_HMT_093	*Ruminococcaceae* _[G-2] bacterium_HMT_085	
	Corynebacterium durum	*Ruminococcaceae* _[G-3] bacterium_HMT_381	
	Corynebacterium matruchotii	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_346	
	Desulfobulbus sp._HMT_041*^{L}	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_347*^{H}	
	Desulfovibrio fairfieldensis	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_348	
	Dialister invisus	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_349	
	Dialister pneumosintes	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_352	
	Dialister sp._HMT_119	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_488	
	Eggerthia catenaformis	Saccharibacteria_(TM7)_[G-1] bacterium_HMT_952	
	Eikenella corrodens	Saccharibacteria_(TM7)_[G-2] bacterium_HMT_350	
	Enterococcus italicus *^{H}	Saccharibacteria_(TM7)_[G-3] bacterium_HMT_351	
	Erysipelotrichaceae _[G-1] bacterium_HMT_905	Saccharibacteria_(TM7)_[G-5] bacterium_HMT_356	
	Escherichia coli	Saccharibacteria_(TM7)_[G-6] bacterium_HMT_870**^{H}	
	Eubacterium brachy	*Saccharibacteria_(TM7)_[G-8] bacterium_HMT_955*	
	Eubacterium infirmum	*Schlegelella thermodepolymerans*	
	Eubacterium nodatum *^{L}	*Selenomonas artemidis*	
	Eubacterium saphenum *^{L}	*Selenomonas dianae*	
	Eubacterium yurii _subspp._*yurii* _&_*margaretiae*	*Selenomonas flueggei*	
	Filifactor alocis *^{L}	*Selenomonas infelix* *^{L}	
	Fretibacterium fastidiosum **^{L}	*Selenomonas noxia*	
	Fretibacterium sp._HMT_359**^{L}	*Selenomonas* sp._HMT_134*^{L}	
	Fusobacterium naviforme	*Selenomonas* sp._HMT_136	
	Fusobacterium nucleatum _subsp._*polymorphum*	*Selenomonas* sp._HMT_137	
	Fusobacterium nucleatum _subsp._*vincentii*	*Selenomonas* sp._HMT_442	
	Fusobacterium periodonticum	*Selenomonas* sp._HMT_501	
	Fusobacterium sp._HMT_248	*Selenomonas* sp._HMT_892	
	Fusobacterium sp._HMT_370	*Selenomonas* sp._HMT_936	

Appendix 3. (continued)

LAgP	Common		HLAgP
	Gemella haemolysans **[H]	*Selenomonas* sp._HMT_937	
	Gemella morbillorum **[H]	*Selenomonas sputigena* *[L]	
	Gemella sanguinis	*Shuttleworthia satelles*	
	Gemella sp._HMT_928	*Slackia exigua*	
	Gracilibacteria_(GN02)_[G-1] bacterium_HMT_872	*Sneathia sanguinegens* *[L]	
	Granulicatella adiacens	*Solobacterium moorei* *[L]	
	Granulicatella elegans *[H]	*Staphylococcus epidermidis*	
	Haemophilus parahaemolyticus	*Stomatobaculum longum*	
	Haemophilus parainfluenzae *[H]	*Stomatobaculum* sp._HMT_097**[H]	
	Haemophilus sp._HMT_036**[H]	*Stomatobaculum* sp._HMT_373	
	Johnsonella ignava	*Streptococcus anginosus*	
	Johnsonella sp._HMT_166	*Streptococcus gordonii*	
	Kingella denitrificans **[H]	*Streptococcus intermedius*	
	Kingella oralis **[H]	*Streptococcus mutans*	
	Kingella sp._HMT_012*[H]	*Streptococcus oralis* _subsp._*tigurinus* _clade_071*[H]	
	Klebsiella pneumoniae	*Streptococcus sanguinis*	
	Lachnoanaerobaculum saburreum	*Streptococcus sinensis*	
	Lachnoanaerobaculum sp._HMT_083	*Streptococcus* sp._HMT_056*[H]	
	Lachnoanaerobaculum sp._HMT_089	*Streptococcus vestibularis*	
	Lachnoanaerobaculum umeaense	*Tannerella forsythia*	
	Lachnospiraceae _[G-10] bacterium_HMT_094	*Tannerella* sp._HMT_286	
	Lachnospiraceae _[G-2] bacterium_HMT_088	*Tannerella* sp._HMT_808	
	Lachnospiraceae _[G-2] bacterium_HMT_096	*Treponema denticola*	
	Lachnospiraceae _[G-3] bacterium_HMT_100	*Treponema lecithinolyticum*	
	Lachnospiraceae _[G-7] bacterium_HMT_086	*Treponema maltophilum*	
	Lachnospiraceae _[G-8] bacterium_HMT_500*[L]	*Treponema pectinovorum*	
	Lactobacillus pentosus	*Treponema socranskii* **[L]	
	Lautropia mirabilis *[H]	*Treponema* sp._HMT_226	
	Leptotrichia buccalis	*Treponema* sp._HMT_231*[L]	
	Leptotrichia goodfellowii *[H]	*Treponema* sp._HMT_234	
	Leptotrichia hofstadii	*Treponema* sp._HMT_236*[L]	
	Leptotrichia hongkongensis	*Treponema* sp._HMT_237	
	Leptotrichia shahii	*Treponema* sp._HMT_247	
	Leptotrichia sp._HMT_212	*Treponema* sp._HMT_251	
	Leptotrichia sp._HMT_219	*Treponema* sp._HMT_254	
	Leptotrichia sp._HMT_221	*Treponema* sp._HMT_257	
	Leptotrichia sp._HMT_223	*Treponema* sp._HMT_262	
	Leptotrichia sp._HMT_225	*Treponema* sp._HMT_927	
	Leptotrichia sp._HMT_417	*Veillonella atypica*	
	Leptotrichia sp._HMT_498	*Veillonella denticariosi*	
	Leptotrichia sp._HMT_909	*Veillonella dispar*	
	Leptotrichia wadei	*Veillonella parvula*	
	Megasphaera micronuciformis	*Veillonella rogosae*	
	Megasphaera sp._HMT_841	*Veillonella* sp._HMT_780	
	Mitsuokella multacida	*Veillonellaceae* _[G-1] bacterium_HMT_129	
	Mitsuokella sp._HMT_131	*Veillonellaceae* _[G-1] bacterium_HMT_132	
	Mogibacterium neglectum	*Veillonellaceae* _[G-1] bacterium_HMT_135*[L]	
	Mogibacterium timidum	*Veillonellaceae* _[G-1] bacterium_HMT_145*[L]	
	Mycoplasma faucium		

Appendix 4. Core microbiome based on species present in the gut of 50% of the subjects of LAgP, HLAgP and common species. [L] indicates species more abundant in LAgP and [H] in HLAgP. Asterisks indicate statistically significant difference in relative abundance between gut_LAgP and gut_HLAgP (*p<0.05, **p<0.01, nonparametric t test)

LAgP	Common		HLAgP
[Bacteroides] pectinophilus group	*[Clostridium] innocuum* group	*Kingella*	*[Eubacterium] saphenum group*
[Eubacterium] yurii group	*[Eubacterium] brachy* group	*Lachnoanaerobaculum*	*Agathobacter*
Cloacibacillus	*[Eubacterium] coprostanoligenes* group	*Lachnoclostridium*	*Christensenella*
Cutibacterium	*[Eubacterium] eligens* group	*Lachnospira*	*Erysipelotrichaceae* UCG-004
Lachnoclostridium 5	*[Eubacterium] hallii* group	*Lachnospiraceae* CAG-56*[H]	*Gardnerella*
Lactobacillus	*[Eubacterium] nodatum* group	*Lachnospiraceae* FCS020 group	*Howardella*
Negativibacillus	*[Eubacterium] oxidoreducens* group	*Lachnospiraceae* GCA-900066575	*Intestinimonas*
Olsenella	*[Eubacterium] ruminantium* group	*Lachnospiraceae* NC2004 group	*Lachnospiraceae* UCG-003
Sanguibacteroides	*[Eubacterium] ventriosum* group	*Lachnospiraceae* NK4A136 group	*Libanicoccus*
Shuttleworthia **	*[Eubacterium] xylanophilum* group	*Lachnospiraceae* NK4B4 group	*Marvinbryantia*
Stomatobaculum	*[Ruminococcus] torques* group	*Lachnospiraceae* UCG-001	*Megasphaera*
Tyzzerella 4	*Actinomyces*	*Lachnospiraceae* UCG-004	*Parvimonas*
uncultured *Thermoanaerobacterales*	*Actinomycetaceae* F0332	*Lachnospiraceae* UCG-008	*Peptostreptococcus*
	Aggregatibacter	*Lachnospiraceae* UCG-010	*Pseudopropionibacterium*
	Akkermansia	*Lautropia*	*Rhizobium*
	Alistipes	*Leptotrichia*	*Scardovia*
	Allisonella	*Mitsuokella*	*Turicibacter*
	Alloprevotella	*Moryella*	
	Anaerofilum	*Mycoplasma*	
	Anaeroglobus	*Nasturtium officinale*	
	Anaerostipes	*Neisseria*	
	Atopobium	*Odoribacter*	
	Bacteroides	*Oribacterium*	
	Barnesiella	*Oscillibacter*	
	Bergeyella	*Oscillospira*	
	Bifidobacterium	*Oxalobacter*	
	Bilophila	*Paludibacteraceae* F0058	
	Blautia	*Parabacteroides*	
	Blvii28 wastewater-sludge group	*Paraprevotella*	
	Butyricicoccus	*Parasutterella*	
	Butyricimonas	*Peptoanaerobacter*	
	Butyrivibrio	*Peptococcus*	
	Campylobacter	*Phascolarctobacterium*	
	candidate division SR1 bacterium taxon 345	*Phocaeicola*	
	Candidatus Saccharibacteria bacterium UB2523	*Porphyromonas*	
	Candidatus Saccharimonas	*Prevotella*	
	Candidatus Soleaferrea	*Prevotella* 2	
	Capnocytophaga	*Prevotella* 6	
	Cardiobacterium	*Prevotella* 7	
	Catenibacterium	*Prevotella* 9	
	Catonella	*Propionibacterium*	
	Cedrus deodara (deodar cedar)	*Propionivibrio*	
	Christensenellaceae R-7 group	*Pseudomonas*	
	Clostridiales bacterium feline oral taxon 148	*Rikenellaceae* RC9 gut group	
	Clostridiales Family XIII UCG-001	*Roseburia*	
	Clostridium sensu stricto 1	*Rothia*	
	Collinsella	*Ruminiclostridium* 5	
	Coprococcus 1	*Ruminiclostridium* 6	
	Coprococcus 2	*Ruminiclostridium* 9	
	Coprococcus 3	*Ruminococcaceae* DTU089	
	Corynebacterium	*Ruminococcaceae* NK4A214 group	
	Defluviitaleaceae UCG-011	*Ruminococcaceae* UBA1819	
	Desulfobulbus	*Ruminococcaceae* UCG-002	
	Desulfovibrio *[L]	*Ruminococcaceae* UCG-003	
	Dialister	*Ruminococcaceae* UCG-004	
	Dorea	*Ruminococcaceae* UCG-005	
	Eggerthia	*Ruminococcaceae* UCG-009	
	Eikenella	*Ruminococcaceae* UCG-010	
	Eisenbergiella	*Ruminococcaceae* UCG-013	
	Enterobacter	*Ruminococcaceae* UCG-014	
	Enterorhabdus	*Ruminococcus* 1	
	Erysipelotrichaceae UCG-003	*Ruminococcus* 2	
	Erysipelotrichaceae UCG-006	*Selenomonas*	
	Escherichia-Shigella	*Selenomonas* 3	
	Ezakiella	*Selenomonas* 4	
	Faecalibacterium	*Senegalimassilia*	
	Filifactor	*Slackia*	
	Flavonifractor	*Sneathia*	
	Flexilinea	*Solobacterium*	
	Fournierella	*Streptococcus*	
	Fretibacterium	*Subdoligranulum*	
	Fusobacterium	*Succinivibrio*	
	Gemella	*Sutterella*	
	Granulicatella	*Tannerella*	
	Haemophilus	*Treponema* 2	
	Harryflintia	uncultured *Candidatus* Saccharibacteria bacterium	
	Holdemanella	uncultured eubacterium E1-K9	
	Holdemania	*Veillonella*	
	Hungatella	*Victivallis*	
	Johnsonella	*Weissella*	

Appendix 5. Cytokines and chemokines levels in whole saliva of LAgP and HLAgP

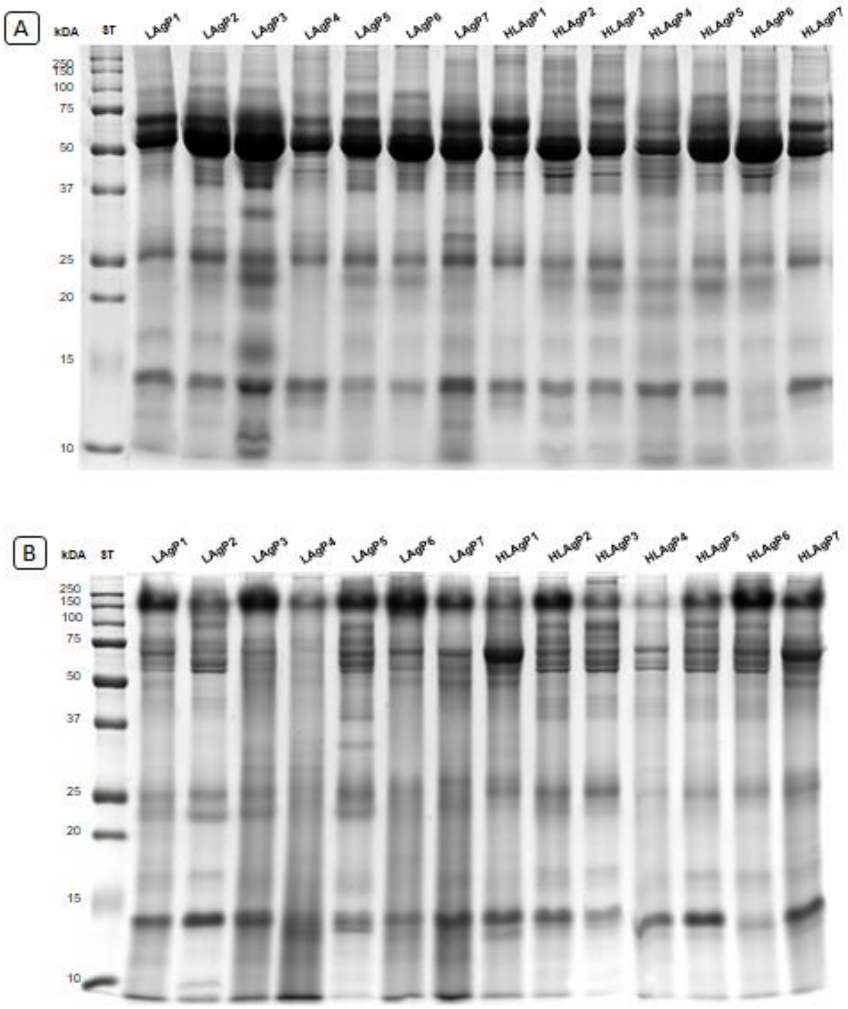
Table 1. Cytokines levels in unstimulated saliva of LAgP and HLAgP (mean pg/mL ± SD). No differences were found between the groups (p > 0.05, Mann-Whitney test).

Cytokine	Median (interquartile range)		Effect	Reference
	LAgP (n=7)	HLAgP (n=7)		
IL1-β *	397.61 (64.66-92.23)	145.26 (51.24-146.35)	Pro-inflammatory	(Evans-Marin et al., 2015; Shaddox et al., 2011)
TNF-α *	8.96 (7.67-11.79)	5.88 (4.81-6.05)	Pro-inflammatory	(Evans-Marin et al., 2015; Sakai, Ohshima, Sugano, Otsuka, & Ito, 2006; Shaddox et al., 2011)
IFN-γ *	6.6 (5.218.495)	6.6 (5.84-6.68)	Pro-inflammatory	(Shaddox et al., 2011)
IL-2 *	1.25 (1.1-1.71)	1.54 (1.28-1.6)	Pro-inflammatory	(Shaddox et al., 2011; Sokol & Luster, 2015)
IL-6 *	1.25 (0.88-1.4)	1.32 (1.11-1.67)	Pro-inflammatory	(Evans-Marin et al., 2015; Sakai et al., 2006)
IL-16 *	165.5 (35.22-412.645)	120.04 (97.84-151.11)	Pro-inflammatory	(Evans-Marin et al., 2015; Saraiva et al., 2014)
IL-10 *	3.12 (2.66-3.13)	2.63 (2.61-3.02)	Regulatory	(Shaddox et al., 2011)
IL-4 *	2.45 (2.38-2.67)	2.45 (2.38-2.45)	Regulatory	(Lappin, Murad, Sherrabeh, & Ramage, 2011)

Table 2. Chemokines levels in unstimulated saliva of LAgP and HLAgP (mean pg/mL ± SD). *Statistical significant difference between groups (p > 0.05, Mann-Whitney test).

Chemokine	Median (interquartile range)		Effect	Receptor
	LAgP (n=7)	HLAgP (n=7)	(Palomino & Marti, 2015; Sokol & Luster, 2015)	(Palomino & Marti, 2015; Sokol & Luster, 2015)
6CKINE/CCL21	41.41 (26.61-47.42)	30.85 (26.8-51.49)	Homeostatic	CXCR5
BCA-1/CXCL13	0.85 (0.72-1.045)	1.12 (0.87-1.28)	Homeostatic	CCR10
CTACK/CCL27	1.2 (1.081.255) *	0.98 (0.87-0.99)	Inflammatory	CXCR2
ENA-78/CXCL5	485.36 (398.38-2789.43)	2265.75 (860.09-3836.05)	Inflammatory	CCR3
EOTAXIN/CCL11	4.04 (3.23-4.67)	3.98 (3.43-3.99)	Inflammatory	CCR3
EOTAXIN2/CCL24	6.97 (6.397.98)	8.91 (7.35-9.05)	Inflammatory	CCR3
EOTAXIN3/CCL26	0 (0-0.02)	0 (0-0)	Inflammatory	CX3CR2
FRACTALKINE/CX3CL1	1341.87 (525.98-1576.22)	1008.01 (877.26-1061.17)	Inflammatory	CXCR2
GCP2/CXCL6	25.86 (7.21-27.74)	12.79 (7.14-20.19)	Inflammatory	CXCR2
GRO-α/CXCL1	1023.99 (357.15-1340.47)	644.93 (504.19-907.78)	Inflammatory	CXCR2
GRO-β/CXCL2	100.05 (42.64-191.45)	112.25 (67.71-188.65)	Dual function	CCR8
I309/CCL1	9.23 (8.15-10.08)	8.58 (8.27-9.09)	Dual function	CXCR3
IP-10/CXCL10	148.73 (38.33-152.8)	89.6 (48.91-124.17)	Dual function	CXCR3
ITAC/CXCL11	2.71 (1.68-2.92)	3.35 (1.47-6.23)	Inflammatory	CCR2
MCP-1/CCL2	59.24 (24.96-72.91)	137.49 (77.725139.635)*	Inflammatory	CCR5
MCP-2/CCL8	0.78 (0.59-0.89)	0.8 (0.58-0.94)	Inflammatory	CCR3
MCP-3/CCL7	12.23 (9.99-19.24)	18.18 (14.21-18.31)	Inflammatory	CCR3
MCP-4/CCL13	9.79 (5.51-12.21)	10.25 (8.42-11.98)	Dual function	CCR4
MDC/CCL22	4.1 (3.45-4.23)	3.64 (3.32-3.66)	Inflammatory	CXCR2 and CXCR4
MIF	5641.13 (3484.61-7186.57)	27998.05 (6310.59-29388.055)	Dual function	CXCR3
MIG/CXCL9	158.11 (116.46-283.72)	185.99 (98.24-234.11)	Inflammatory	CCR1 and CCR5
MIP-1α/CCL3	2.9 (0.93-3.05)	0.74 (0.67-1.32)	Inflammatory	CCR1 and CCR3
MIP-1δ/CCL15	3.49 (3.2-19.8)	4.27 (4.07-6.35)	Dual function	CCR6
MIP-3α/CCL20	5.15 (3.45-5.79)	2.79 (2.59-7.15)	Homeostatic	CCR7
MIP-3β/CCL19	3.76 (3.42-4.44)	3.73 (3.73-3.97)	Dual function	CCR1
MPIF1/CCL23	5.15 (3.89-5.55)	4.64 (4.14-5.27)	Dual function	CXCR6
SCYB16/CXCL16	15.18 (7.19-16.81)	17.98 (14.61-27.02)	Homeostatic	CXCR4
SDF1α+β/CXCL12	17.45 (15.07-17.96)	15.84 (14.72-16.47)	Dual function	CCR4
TARC/CCL17	4.92 (3.88-4.92) *	3.34 (3.29-3.61)	Dual function	CCR9
TECK/CCL25	45.06 (42.07-47.38)	74.02 (61.09-79.19)*	Inflammatory	CXCR1 and CXCR2
IL-8/CXCL8	332.58 (116.85-434.37)	209.94 (149.33-267.32)	Inflammatory	CXCR1 and CXCR2

Appendix 6. SDS-PAGE gel of saliva samples of LAgP and HLAgP subjects. Gel left column (column 1) shows the standard bands used as reference of the molecular weights of proteins, and the subsequent columns (columns 2 to 15) show the bands formed by the separation of proteins of (A) stimulated and (B) unstimulated, according to their molecular weight.



Appendix 7. Cationic-PAGE

Figure 1. Cationic-PAGE. The left column (column 1) shows histatins standard bands and the subsequent columns (columns 2-9) show histatin 1 (A) and histatin 5 (B) incubated in water throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).

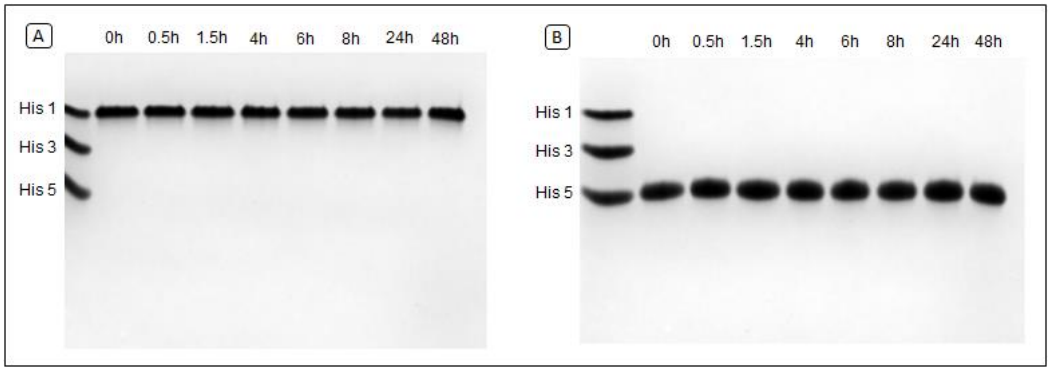
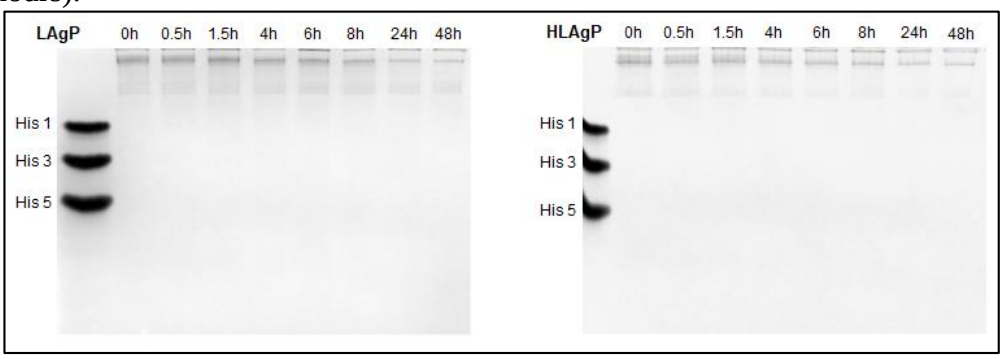


Figure 2. Cationic-PAGE. The left column (column 1) of each gel shows histatins standard bands and the subsequent columns (columns 2-9) show stimulated saliva of LAgP and HLAgP pooled samples incubated in water throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).



Appendix 8. Cationic-PAGE

Figure 1. Cationic-PAGE. The left column (column 1) of each gel shows histatins standard bands and the subsequent columns (columns 2-9) show the products of the incubation of histatins 1 in stimulated saliva of LAgP subjects throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).

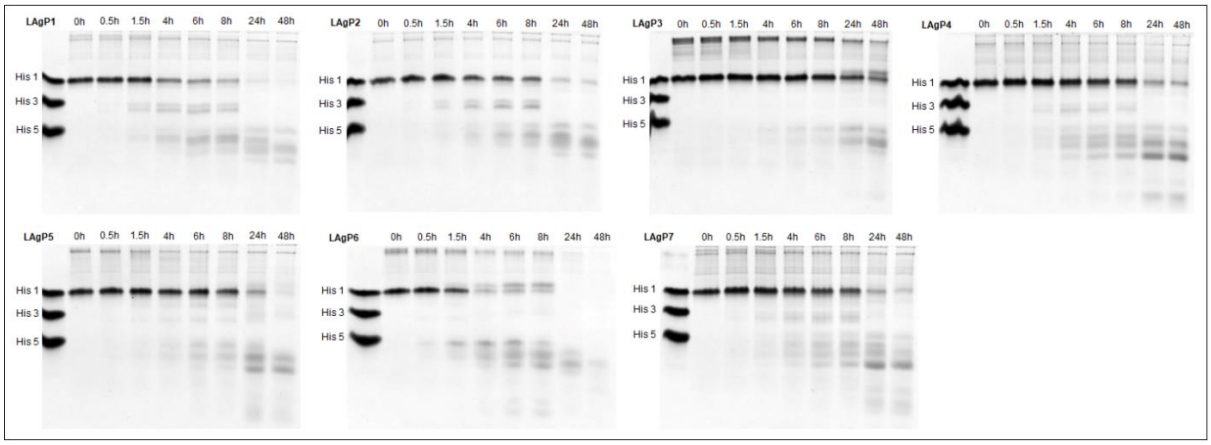
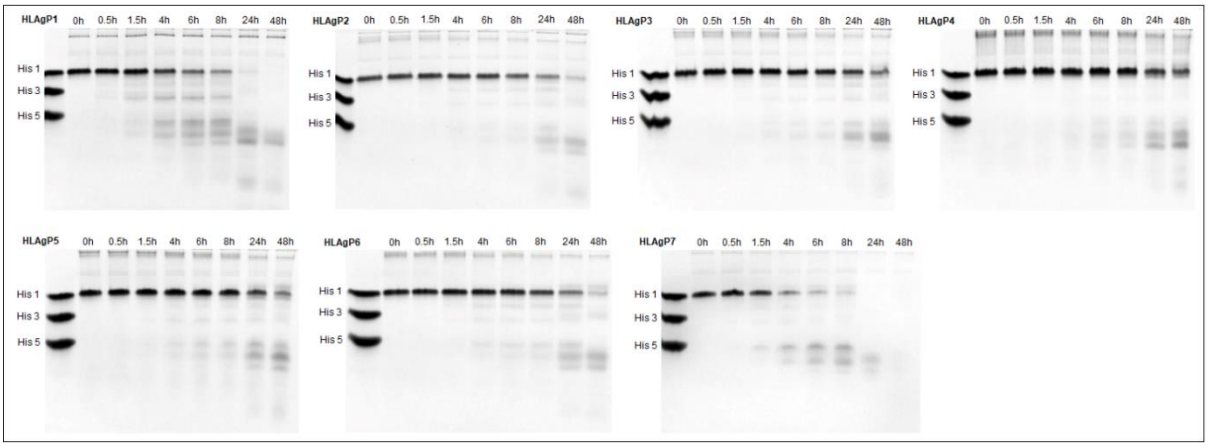


Figure 2. Cationic-PAGE. The left column (column 1) of each gel shows histatins standard bands and the subsequent columns (columns 2-9) show the products of the incubation of histatins 1 in stimulated saliva of HLAgP subjects throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).



Appendix 9. Cationic-PAGE

Figure 1. Cationic-PAGE. The left column (column 1) of each gel shows histatins standard bands and the subsequent columns (columns 2-9) show the products of the incubation of histatins 5 in stimulated saliva of LAgP subjects throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).

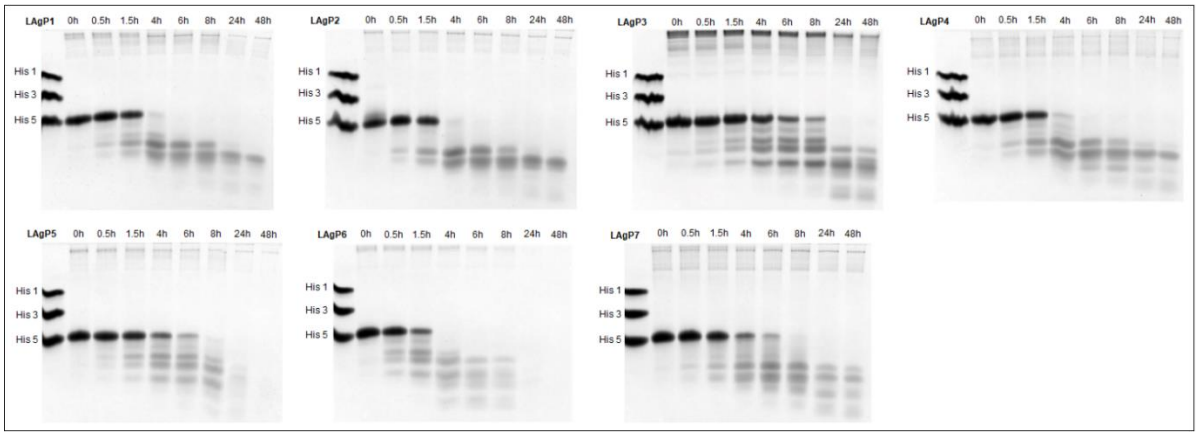
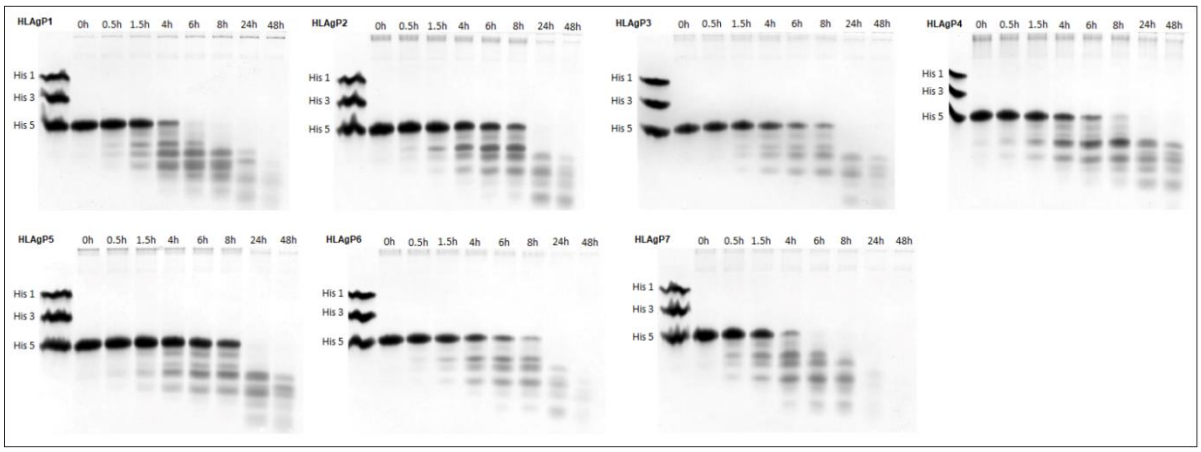


Figure 2. Cationic-PAGE. The left column (column 1) of each gel shows histatins standard bands and the subsequent columns (columns 2-9) show the products of the incubation of histatins 5 in stimulated saliva of HLAgP subjects throughout different time-points (t=0, 0.5, 1.5, 4, 6, 8, 24 and 48 hours).



Appendix 10. Cationic-PAGE. Red dashes indicate the histatins 1 and 5 peptide bands that were chosen to be analyzed by LC-ESI-MS/MS.