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Metagenomic analysis of the periodontal and peri-implant microbiome: a link between health and disease status

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TESI DI DOTTORATO

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ABSTRACT

The oral cavity has been becoming one of the most well-studied microbiomes after the introduction of new genomic technologies, including next-generation sequencing and bioinformatic analysis. Metagenomic studies of the oral microbial community have revealed the complexities of the oral cavity, that contains hundreds of different bacterial, viral and fungal species. Most species are commensal, but they can become pathogenic in responses to environmental changes. The oral microbiota analysis could provide a better understanding of the bacterial communities' role in both physiology and pathophysiology, identifying micro-organisms that cause infections and others that are crucial for successful treatment and recovery of patients.

The main focus of this project is to identify the healthy and disease profiles of the oral microbiome in periodontal and peri-implant diseases by 16S rRNA gene sequencing, to have a broader and more overall view of the trends of the oral microbial community. Data have shown the greatest bacterial richness in health sites, where *Lautropia* appeared as a health biomarker, while *Olsenella*, *Mongibacterium* and *Dialister* were the most discriminative taxa for disease groups. The identification of "*healthy core microbiome*" and a "*diseased core*" led us to the potential definition of specific biomarkers of health or disease. Moreover, we found a higher abundance of *Streptococcus salivarius* species in "*healthy core microbiome"*.

This discovery led us to preliminary evaluate further properties of *Streptococcus salivarius* 24SMBc, which is an oral probiotic already commercialized and used in the prevention of recurrent acute otitis media, but that could represent also an oral probiotic candidate to be used in the prevention and treatment of these pathologies.

The probiotic properties of this strain were tested against oral pathogens chose by supporting our background experience. Firstly, the absence of cytotoxicity on epithelial cell HEp-2 was confirmed, secondly, the ability to interfere in cellular adhesion and to co-aggregate with streptococcal pathogens was assessed. Moreover, a complete genome sequencing analysis of *S. salivarius* 24SMBc was performed by using Illumina sequencing technology to investigate the genetic background of the probiotic potential.

Even if the efficacy of *S. salivarius* 24SMBc has to be proved against a wider sample of periodontal pathogens and in randomized clinical trials, it has responded positively to all test on the list, showing good adhesion interference of pneumococcal colonization and good co-aggregation ability with them. It is also free of streptococcal virulent factors and has got a bacteriocin-like protein (blp) locus.

Thereby, the present work could improve the knowledge of the oral microbiome and its influence on host health and disease, providing fundamental information on wide-ranging interactions among oral bacteria in terms of positive and negative impacts on host health, enriching the literature in this regard and giving new potential diagnostic tools to the clinicians as well as new therapeutic options.

Key Words: Oral Microbiome, 16S ribosomal RNA gene, oral diseases, peri-implantitis, periodontitis, probiotic.

1. INTRODUCTION

1.1 The oral microbiota

Humans and microbes have been co-evolving together for two million years and have been establishing a mutualistic relationship, where there is a benefit to both (Ley et al. 2008). This intimate association with commensal and symbiotic microbes is known as *" microbiota"* (Gill et al. 2006; Turnbaugh et al. 2007). The human body is host to microbial communities that carry out many biological functions, protect us from invasion by pathogenic bacteria and influence human physiology through processes related to development, nutrition and immunity (Mirmonsef et al. 2011; Kau et al. 2011). The characterization of the genomes of these microbial symbionts (collectively defined as the "*microbiome*"), who form human "*supraorganism*", was done only since 2007, when was launched the Human Microbiome Project (HMP) to improve understanding of the microbial flora involved in human health and disease (Turnbaugh et al. 2007).

By metagenomic analysis of human microbiome has been emerged a great variety between anatomical sites and between individuals at the same body habitats (Ursell et al. 2012). This dynamic nature is due to environmental factors, dietary changes or exposure to antibiotics (Benson et al. 2010), for this reason, the major effort of scientists has been to determinate the *''microbiome core''*, that represent a set of microbes consistently presents over long periods of time that contributing effectively to development, health and functioning of its host (El-Chakhtoura et al. 2015).

To date, the best-described microbiota was the human gut (Turnbaugh et al. 2010; Turnbaugh et al. 2009). But here, I would focus on the oral microbiota's bacteria, that for a long time were not be regarded as noteworthy and only successively are being sequenced (McGuire et al. 2008).

The oral microbiota was found to be second only to that of the colon in terms of species-richness and undoubtedly novel species are expected to be identified yet (Bik et al. 2010; Griffen et al. 2012). Recently the oral microbiota became interesting of a growing number of microbiologists for its complexity because it contains around 1000 bacterial species (Dewhirst et al. 2010) and also different viral and fungal species (Wang, Gao, and Zhao 2016; Diaz et al. 2017). Among bacterial taxa, the most abundant is *Streptococcus* genus (Butler et al. 2017), followed by *Haemophilus* in the buccal mucosa, *Actinomyces* in the supragingival plaque and *Prevotella* in the immediately adjacent subgingival plaque (Gao, Xu, et al. 2018).

Already from birth, the mouth is colonized by mostly aerobes and obligate anaerobes related to the genera *Streptococcus, Actinomyces, Veilionella, Neisseria* and some yeasts. After the dentition appears anaerobic forms such as *Prevotella* and *Fusarium* that colonized the space between gums and teeth. Whereas, enamel, gingival epithelial surfaces and saliva are colonized from *Streptococcus species.* such as *Streptoccus parasanguis* and *Streptococcus mutans* (Sampaio-Maia and Monteiro-Silva 2014). The presence of these distinct bacterial communities in the same area (Figure 1) is mainly because the mouth has seven different surfaces (Figure 2) with different niches (Segata et al. 2012).

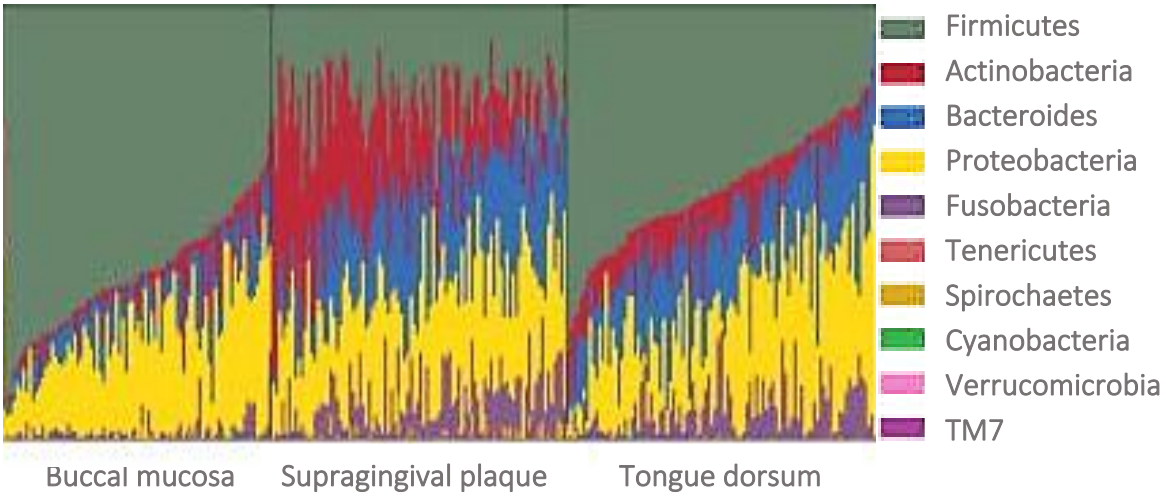


Figure 1. *Predominant phyla of three different surfaces of the oral cavity (Human Microbiome Project, 2012).*

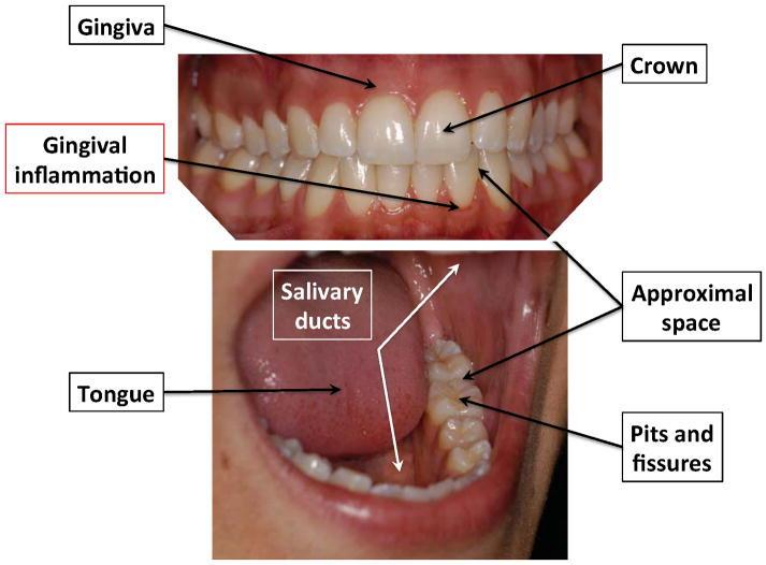


***Figure 2**. Anatomy and ecological niches of the oral cavity (Costalonga and Herzberg 2014).*

Furthermore, the easy availability of epithelial debris as nutrients, water, suitable temperature and pH allows a large microbial variety. This diversity confers versatility to the oral community and the ability to respond to environmental stresses in several ways, in fact, oral bacteria community can cooperate to protect each other from atmospheric stresses, developing complex biofilm, commonly called *"dental plaque"* (Marsh 1994).

Considering the complexity of the oral bacterial, assigning a role for each organism within the community is impossible. Above all oral bacteria vary widely in their sensitivity to oxygen, relatively few species such as *Neisseria* and *Rothia* are obligate aerobes (Diaz et al. 2006), but many others are facultative anaerobes including *Streptococcus* and *Actinomyces*. Moreover, bacteria can be grouped by the main function, because bacteria cooperate, carrying out nutritional function like degradation of complex substrates (Wickström et al. 2009). For example, certain streptococci have glycosidic and endopeptidase activity, that play a nutritional role by releasing carbohydrates which are essential for proliferation from host glycoproteins (Homer, Whiley, and Beighton 1990; Homer et al. 2001).

At last, the oral cavity is the first gateway of the human body and is exposed to exogenous bacteria in food, in water, in air and social contact like kissing, so defining the precise structure is extremely complicated, but a comprehension of the oral microbiome composition and its association with human diseases has been becoming necessary to identify and characterize micro-organisms that cause infections and are crucial for successful treatment and safety of patients (Krishnan, Chen, and Paster 2017).

1.2 The oral microbiota and diseases

The human microbiome plays an important role in maintaining health, whereas dysbiosis is associated with various diseases and conditions (Krishnan, Chen, and Paster 2017; Pflughoeft and Versalovic 2012). Oral microorganisms are not confined to just contribute to the human local oral diseases but there are pieces of evidence that oral microbiota is also closely related to systemic diseases (Graves, Correa, and Silva 2019) (Figure 3).

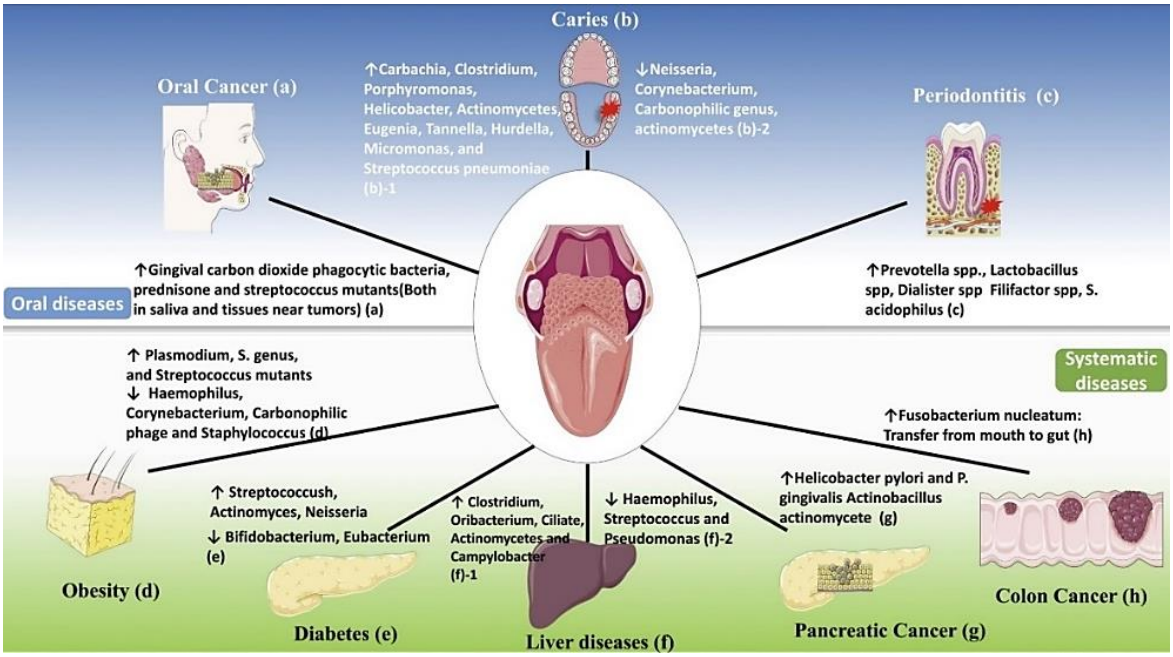


***Figure 3**. Oral microbiota related to oral and systematic diseases (Lu, Xuan, and Wang 2019).*

Among local disease, must be quoted periodontal diseases (Takahashi and Nyvad 2011), peri-implantitis (Ata-Ali et al. 2011), halitosis (Seerangaiyan et al. 2017), oral cancer (Farrell et al. 2012) and dental caries (Gross et al. 2010).

Some of these pathologies, especially dental disease, if are untreated can progress and lead not only to the tooth loss but also the spread of bacteria in the systemic circulation. The oral cavity is a reservoir for dissemination of pathogenic bacteria and their toxins that can enter the systemic circulation through the periodontal blood and determine the systemic dissemination of oral bacterias (Bahrani-Mougeot et al. 2008; Rautemaa et al. 2007), that can invade and persist in the host cells, to escape host immune surveillance and to adapt to niches at extra-oral sites. Regardless of their pathogenic potentials in the oral cavity, some of them can transit from a commensal relationship to one of pathogenicity, for reasons that are still not understood. When oral bacteria colonized extra-oral sites become "*bona fide"* pathogens, leading to the rise of serious pathologies (Jia et al. 2018).

Notably, the oral bacteria carried by saliva through the digestive tract presented a particularly close relationship with digestive diseases (Ray 2017). The oral microorganisms go directly into the intestinal tract through the oesophagus, affecting the digestive system and the intestinal microflora (Arimatsu et al., 2014; Nakajima et al., 2015). Recent studies have shown that oral bacteria such as *Porphyromonas gingivalis, Fusobacterium nucleatum* and *Klebsiella* can induce intestinal inflammation and can colonize the intestines and persist there (Flemer et al. 2018; Zhang et al. 2019; Matsha et al. 2020), despite the physiological distance between the oral cavity and digestive system.

Moreover, recently is appeared a worthy link also with neurological diseases (Bell et al. 2019). Certain oral bacteria may proliferate and trigger a sustained tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Tarkowski et al. 1999), causing the weakening of the blood-brain barrier (BBB) such as in Alzheimer's disease (Kamer et al. 2009; Sparks Stein et al. 2012). Effectively, the oral bacteria can enter into the brain through many routes, because there are many nerves lead from the oronasal cavity directly to the brain such as trigeminal and olfactory nerves

(Riviere, Riviere, and Smith 2002; Mann, Tucker, and Yates 1988). The effects of the oral dysbiosis on the neural function, have currently documented from several studies on Alzheimer's syndrome and Parkinson disease (Stein et al. 2007; Shoemark and Allen 2015; Pereira et al. 2017).

Additionally, salivary microbiota has been recognized also as physio-pathologically relevant for the diagnosis of some neuro-psychiatric pathologies (Iorgulescu 2009) and oral squamous cell carcinoma (OSCC) (Pushalkar et al. 2011; Mager et al. 2005). Saliva is the reservoir also of microbial metabolites, which can enter in the bloodstream and the systemic circulation, elicit an inflammatory state (Pickard et al. 2017; Kamada and Kao 2013). The metagenomic and metatranscriptomic analysis of salivary microbiota could be an indicator of oral health status, revealing disease-associated microbiota. Moreover, the metagenomic analysis of salivary microbiome is more practical than gut microbiota, because saliva can be taken non-invasively and represent the starting point since that members of mouth reach the stomach through swallowed saliva, nutrients and drinks.

Specifically, in autism spectrum disorders (ASD), the tools used for the diagnosis of ASD are limited by the fact that these deficits cannot be observed before 18-24 months of life. Presently there are no efficient molecular diagnostic tools for ASD, but the scientific researchers have given thought and energy to researching therapeutic target biomarkers like microbial dysbiosis.

The first study that has investigated salivary and dental microbiome of ASD children by 16S rRNA sequencing was conducted by a research group of Qiao et al. in 2018. They had found no difference between the salivary microbiota but statistically significant differences in dental samples, with an increase in potential pathogens such as *Haemophilus, Corynebacterium,*

Cardiobacterium, Kingella, Streptococcus and *Rothia* as well as a reduction of native bacteria (Qiao et al. 2018).

Besides, my research group and I have participated in a collaborative study with a research group of Department of Biomedical and Biotechnological Sciences and of Department of Clinical and Experimental Medicine of University of Catania, showing that microRNA and microbiome dysregulations found in the saliva of ASD children are potentially associated with cognitive impairments of the subjects (Ragusa et al. 2020).

Despite great efforts to study oral microbiota implicated in systemic and neurological disease, one of the most well-researched areas remain the ones related to local disease, specifically about dental pathologies, who affected people of any age throughout their lifetime, causing pain and discomfort (Millenium 2000). Below I will report in details the two commonest human dental diseases at this time: periodontal diseases and peri-implantitis.

1.2.1 Periodontitis

The periodontitis occurs because of the attachment' loss between the gingivae and the teeth with the formation of a periodontal pocket, which is suddenly colonized by anaerobic bacteria, also the alveolar bone which supports the teeth is resorbed contributing to the loss of this last. Periodontitis is also associated to dental plaque (Armitage 1999) and gingivitis (Coventry et al. 2000) (Figure 4), which is usually considered a precursor of this disease and may also contribute to the development of mucositis and peri-implantitis.

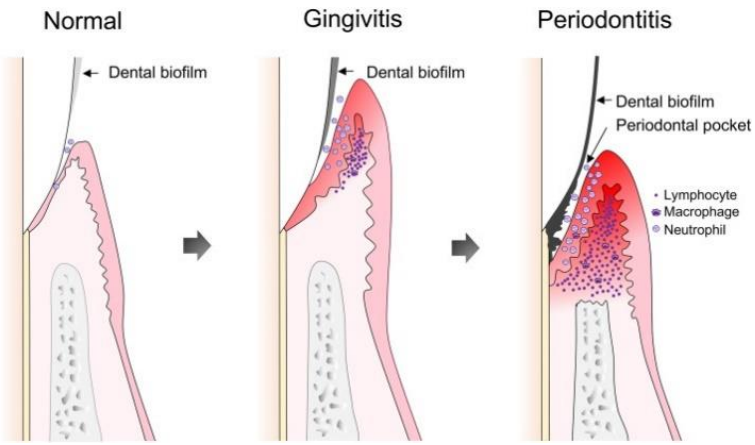


***Figure 4**. Differences between health gum, gingivitis and periodontitis (Kriebel et al. 2018).*

The host inflammatory response instead of helping it leads to worsening the situation because contributes to the development of the lesion with tissue damage through the release of host proteases (Darveau 2010). Periodontitis is the most common infectious disease affecting tooth-supporting structures too. Moreover, the periodontal disease may differ in severity and impact also Oral Health Related Quality of Life (OHRQoL) (Sischo and Broder 2011). Antibiotics were proposed as the mode of treatment, but usually, they are not effective against biofilms and a recent work on ampicillin-resistant of *Fusobacterium nucleatum* strain in dental plaques (Al-

Haroni, Skaug, Bakken, & Cash, 2008), led all scientific community to worry about the potential development of resistance to all β-lactamases, that could create oral pathogens more virulent trough the horizontal transfer and affected the therapeutic success rates (Rams, Degener, and van Winkelhoff 2014).

Left untreated, periodontitis can lead to, or aggravate, existing systemic conditions (Scannapieco, Dasanayake, and Chhun 2010), such as cardiovascular disease, diabetes, pulmonary diseases, obesity and various forms of lung disease (Nazir 2017; Elter et al. 2003).

In cardiovascular disease was found a higher prevalence of *Prevotella, Porphyromonas* and *Clostridiales* (Greenwell 2000). In particular, *Streptococcus mutans* and *Porphyromonas gingivalis* contribute to the development of atherosclerosis by altering the function of epithelial cells and to the production of inflammatory cytokines (Oliveira et al. 2015).

Other clinical studies suggested that periodontopathic bacterial species are associated to organ abscesses, rheumatoid arthritis, nosocomial pneumonia and even to adverse pregnancy outcomes (Heo et al. 2008; Heo et al. 2011; Martinez-Martinez et al. 2009; Dissick et al. 2010; Gonzales-Marin et al. 2011; Han et al. 2009; Han et al. 2010; Wang et al. 2013). Specifically, *Fusobacterium* is the most common oral genus detected in brain, lung, liver, splenic abscesses and appendicitis (Han 2011; Swidsinski et al. 2011).

All these evidences suggest invasiveness ability of oral microorganisms to translocate from the oral cavity to the other body district and to spread to different compartments, leading to a different kind of diseases. However, the precise role of bacteria and "*putative pathogens*" in periodontitis remains still unknown. Thereby, detailed knowledge of the microbiome and its function can improve diagnosis and therapy in patients with periodontitis and associated pathologies.

1.2.2 Peri-implantitis

The partial or total absence of the teeth impacts on social contacts and creates uncomfortable aesthetic conditions, but above all functional problems. Among the main functional problems, there are severe headaches caused by the upsetting of the normal structure of the jaw, the decompensation of the first digestion, the occurrence of maxillary resorption and also phonetics problem, in fact, the edentulous have a "*blown*" pronunciation (Heitz-Mayfield 2008). Furthermore, mandibular and maxillary teeth perform an extremely important function in the regulation and postural control, through the receptors of the periodontium (Kohli et al. 2018). Thereby, if the teeth are lost, the periodontal receptors remain free, not allowing the central nervous system to receive precise information. Thus there will be a wandering jaw with an unstable posture, that will lead to disharmonious and uncertain movements (Dahiya, Sharma, and Kaur 2012). The loss of teeth is not only an aesthetic problem but it regards the well-being of the whole organism.

The National Institute of Dental and Craniofacial Research (NIDCR), has found that 90% of Americans are going to lose at least 3 functioning teeth before 50 years of age (Eke, Dye, Wei, Thornton-Evans, & Genco, 2012) and the implants being placed every year are around 400.000 and their costs are considerable. Implants have a survival rate of 95% over 10 years; however, the past 3 decades have seen the emergence of 2 contemporary diseases: peri-implantitis and peri-implant mucositis (Zitzmann and Berglundh 2008). Implant devices are anchored to the bone to reproduce the missing natural teeth, but despite the continuous improvement in the orthodontic practice in the last 40 years (Branemark 1983), the anchorage to the bone is different in dental implants, because of the miss of periodontal ligament and Sharpey's fibre extremities. Moreover, biofilm formation on the implant surface can trigger the inflammatory

destruction of the peri-implant tissue, inducing peri-implantitis disease (Charalampakis and Belibasakis 2015).

So far, early diagnosis and identification of risk factors are of extreme importance to prevent the disease in the first place. Nowadays probing and radiographic assessment are the primary diagnostic means (Figure 5), that allows the detection of disease only when it has produced some level of destruction (Lang and Berglundh 2011).

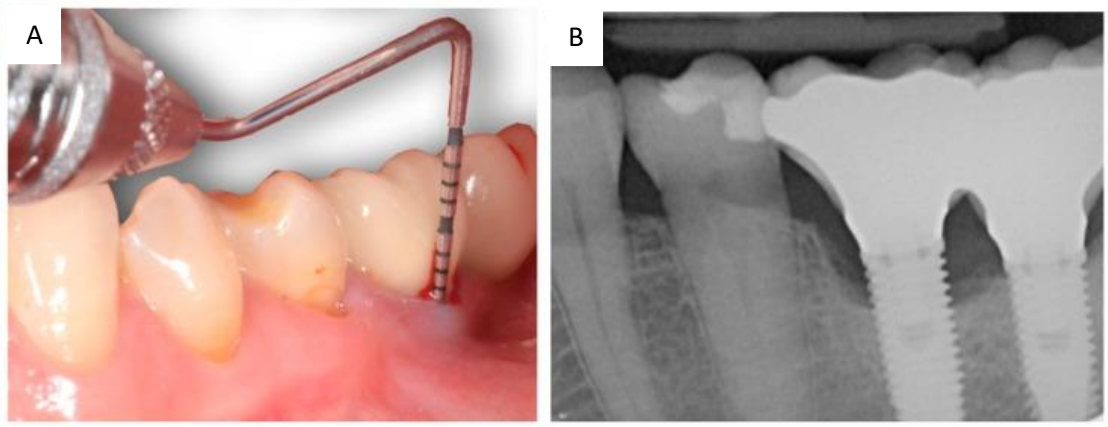


***Figure 5**. Clinical sign of peri-implantitis. A) Bleeding on probing and increased probing pocket depth. B) radiographic assessment of bone loss (Monje, Insua, and Wang 2019).*

The **diagnosis** of peri-implant infections is the object of continuous update. The peri-implant disease has come to the attention of the major international dental associations such as European Federation of Periodontology (EFP), European Association for the Osseointegration (EAO) and American Academy of Periodontology (AAP) who investigated several aspects of this disease. The AAP guidelines refer to the diagnostic findings of bleeding on probing (BOP) and bone loss at a probing depth (PD) of ≥4 mm (AAP 2013), but the criteria for a correct diagnosis of the peri-implant disease have been clearly defined by Heitz-Mayfield in at the 6th EFP Consensus, who provide that the probing should be performed using a slight force (0.25 N) in

order not to damage the peri-implant tissues and should be detected the presence of BOP (Heitz-Mayfield 2008). Furthermore, the diagnosis should be considered PD and radiographs to assess the possible increase in bone loss. Froum and Rosen proposed a classification of different degrees of severity of the peri-implant disease, based on PD and bone loss, dividing the disease into three clinical stages: early, moderate and advanced (Froum and Rosen 2012).

Additionally, several **risk factors** have been associated with the development of the peri-implant disease such as lack of regular supportive therapy, plaque accumulation, smoking, history of periodontal disease and excess cement. Usually, a history of **periodontitis** has a higher rate of implant loss and sites with ≥6 mm PD around implants (Roccuzzo et al. 2014), Sgolastra et al. showed a higher and significant risk for both implant loss and implant bone loss in patients with periodontitis (Sgolastra et al. 2015a). All patients with a previous history of periodontitis that had implants with extra coronal **cement residuals** developed peri-implantitis (Linkevicius et al. 2013), because the rough surface structure of cement remnants may facilitate retention and biofilm formation, increasing the risk for peri-implantitis (Staubli et al. 2017). Another risk factor is represented by **smoke**, but it is much discussed because there are conflicting studies. On the one hand, none association between smoking and peri-implant disease are found (Koldsland, Scheie, and Aass 2011; Roos-Jansaker, Lindahl, et al. 2006; Roos-Jansaker, Renvert, et al. 2006), but on the other hand seems that smokers have a higher and significant risk of peri-implantitis compared with non-smokers, because current smokers harbour more periodontal pathogens in the peri-implant sulci, thus implying a potential risk for the onset of peri-implant disease (Sgolastra et al. 2015b). Beyond the several factors that cause peri-implantitis there is also the **host response** (Lamont and Hajishengallis 2015), in fact, Sgolastra et al. has found a significantly higher increase of matrix metalloproteinase-8 (MMP-8) at implant crevicular fluid, but no differences in levels of interleuckin IL-1β, and no

differences in the detection of periodontal pathogens between implant and natural tooth sites (Sgolastra et al. 2015a). Finally, **oral hygiene** plays a key role on implant survival rate, it must be maintained daily care with mechanical plaque control (with toothbrushes) and professional intervention like mechanical debridement to reduce peri-implant mucositis and its progression to peri-implantitis (Serino and Strom 2009), but also to keep an eye on **dental plaque** development, that is formed mainly around peri-implant mucosa (Karoussis et al. 2003; Sgolastra et al. 2015a). Thus, the importance of better oral hygiene as a safer and effective barrier to infection remains the best prevention factor of disease related to dental plaque around implants (Peterson et al. 2013).

Traditional studies on the pathogenesis of peri-implant infections have analyzed the role of single bacterial species, but recently peri-implantitis is related to polymicrobial nature for the complex interaction and diversity of the microbiota colonizing the implant surface (Kumar et al. 2012; Dabdoub, Tsigarida, and Kumar 2013). The placement of orthodontic appliances increases the pre-periodontal bacteria such as *Aggregatibacter actimonycetemcomitans, Tannerella forsythia* and various *Streptococcus spp.* (Leung, Chen, and Rudney 2006). Despite, the disinfection approach of the contaminated implant surface following by re-osseointegration, the outcomes have been modest (Renvert, Polyzois, and Maguire 2009), with high rates of disease recurrence (Esposito, Grusovin, and Worthington 2012), suggesting the necessity of a deepen studies of the microbiological different niches in the oral cavity; mostly, because information about peri-implantitis are limited and the microbiota have been treated as the same as those of periodontitis.

Moreover, from the analysis of dental implants by 16S rRNA sequencing has emerged that the presence of the cluster of the so-called *"red complex"* (*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*) was higher in peri-implantitis sites than healthy ones

(Koyanagi et al. 2010), however, these same species are considered the strongest indicators of aggressive periodontitis (Schulz et al. 2019). Regarding microbial communities, different studies investigated the ecological shift, in particular, with increasing PD and gingival inflammation, the high rates of coccoid gram-positive cells and few spirochetes found in healthy implants were lost (Rams et al. 1984), then *Synergistetes* of cluster A were found highly associated with peri-implantitis (Belibasakis et al. 2016) and *Eubacterium minutum* levels were greater at peri-implantitis locations (Zheng et al. 2015).

However, Rakic et al., pointed out the quantitative rather than qualitative aspect of microbial composition between peri-implantitis, periodontitis and healthy tooth sites (Rakic, Grusovin, and Canullo 2016).

But despite the great efforts of the implant research community was revealed a high rate of inhomogeneity and the absence of consistent evidence among various studies. This is due to the different techniques used, the different protocol sampling and the different database of references employed.

In this context, the present work is designed to analyze through metagenomic 16S rRNA approach three different dental sites (periodontal, peri-implant and health sites), using a specific database: the Human Oral Microbiome Database (HOMD). This analysis could lead to new considerations that may allow the better diagnosis and treatment before the appearance of clinical manifestations like tissue damage in periodontal pockets or dental hard tissue loss. Also, the knowledge of the healthy implant microbiome could lead to creating new therapeutic approaches such as the use of probiotics to maintain or restore the microbial flora, avoiding the use of antibiotics.

Since the transition from health to disease could be attributed to the loss of homeostasis of the microbial flora rather than to the specific pathogens (Lamont, Koo, & Hajishengallis, 2018) as suggested by Friedrich et al., who affirmed that "*the pathogen might be a disturbed microbial community rather than a single organism*" (Friedrich, 2008), thus the manipulation of the microbiome should use in the reestablishment of the dysbiosis to optimize personal health.

1.3 Probiotics and Oral Health

The cooperative interactions between microbes and their hosts typically aid the host functions such as defence and metabolism (Cornejo Ulloa, van der Veen, and Krom 2019). The resident commensal oral bacteria gave significant benefit to the host by blocking pathogen colonization and by influencing the normal development of cell structure and the immune system with the enhancement of the adaptive immune response (Zhang et al. 2018). In particular, the simple presence of the oral microbiota in the mouth inhibits colonization by pathogens, because when commensals colonize all surfaces of the mouth, keep pathogenic species in check by not allowing them to adhere to mucosal surfaces and preventing the spreading in the bloodstream too (Jenkinson and Lamont 2005; Pennisi 2005). This phenomenon is called of "*colonization resistance*" (Vollaard and Clasener 1994) and its importance can be seen when the commensal microbiota is disrupted by antimicrobials (Sullivan et al. 2001). Commensal bacteria of the oral cavity prevent pathogenic agents and among these, the members of the genus *Streptococcus* have been proposed to protect against them (Tagg and Dierksen 2003). In particular non-pathogenic Streptococci, are the most abundant bacterial species at the oropharyngeal level (Zhou et al. 2010) (Figure 6).

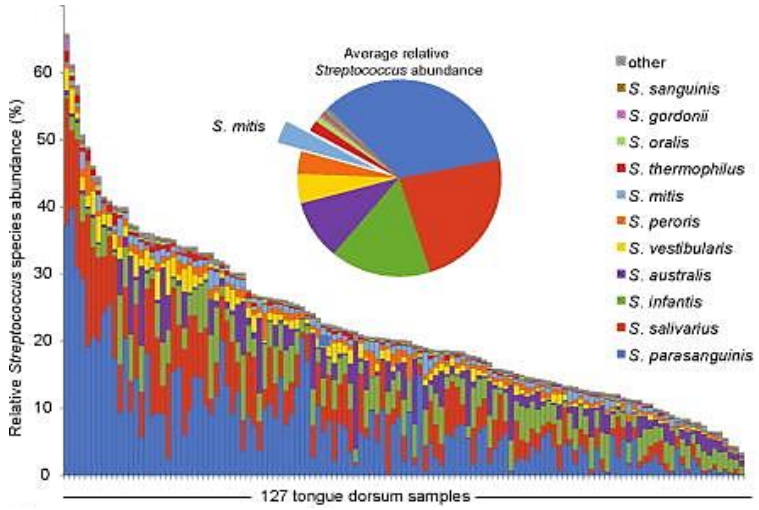


Figure 6. *Relative Streptococcus species abundance in the oral cavity (Human Microbiome Project 2012).*

Despite a lot of studies support the role of probiotics in gastrointestinal health, nowadays researchers have committed themselves to prove its validity in oral health. The first studies of oral probiotics regard the use of live lyophilized acidophilic lactobacteria in patients, who had periodontitis and showed significant improvement or recovery, with no side effects (Pozharitskaia et al. 1994). After, in 2001, Roos at al. were the first to investigate the effect of alpha-hemolytic streptococci, isolated from the human pharynx and administered as a nasal spray containing two *Streptococcus sanguinis*, two *Streptococcus mitis* and one *Streptococcus oralis*, on the incidence of otitis media (OM) in otitis prone children (Roos, Hakansson, and Holm 2001).

Other probiotic strains from the human oral cavity belong to species of *Streptococcus salivarius* that is a lactic acid bacterium and has extremely low pathogenic potential and produce bacteriocin-like inhibitory substances (BLIS) that principally target against relatively similar bacteria but antagonize many oral pathogens (Wescombe et al. 2010). *Streptococcus salivarius spp.* is the first commensal bacterium that appears in the oral cavity of newborns where it colonizes the upper respiratory tract (Aas et al. 2005) and persists here as a predominant member of the native microbiota throughout the life of its human host (Favier et al. 2002). According to several studies, large populations of *S. salivarius* efficiently adhere to the oral epithelial cells, especially on the papillary surface of the tongue that is a strategical location to carry out a population surveillance and modulation role within the oral microbiota (Wescombe et al. 2010; Nobbs, Lamont, and Jenkinson 2009).

One of the most famous among *Streptococcus salivarius* species is *Streptococcus salivarius* K12, which has been used as an oral probiotic (BLISK12TM Throat Guard) and marketed internationally by the New Zealand company BLIS Technologies Ltd (Burton, Chilcott, Moore, Speiser, & Tagg, 2006; Guglielmetti et al., 2010; Hyink et al., 2007; Ishijima et al., 2012). It

is recognized for its antagonistic activity against *Streptococcus pyogenes*, its anti-inflammatory effect, good adhesion to epithelial cells, inhibition of Gram-negative anaerobes implicated in halitosis and for its protective effect against *Candida albicans* (Hyink et al. 2007; Guglielmetti et al. 2010; Burton et al. 2006; Ishijima et al. 2012).

Another *Streptococcus salivarius* commercialized as a nasal spray (Rinogermina®, DMG Italia Srl, Pomezia, Italy), without systemic adverse effect in all healthy subjects is *Streptococcus salivarius* 24SMBc (Santagati et al. 2015). *S. salivarius* 24SMBc was characterized from Santagati's research group and specifically selected by screening of 81 α-hemolytic streptococci isolated from 62 nasal/oropharyngeal swabs of healthy children (Santagati et al. 2012), for its potential to interfere with the colonization of upper respiratory tract (URT) pathogens, indeed has an excellent application in the prevention of recurrent acute otitis media (rAOM) in infants and children for its strong inhibitory ability *versus Streptococcus pneumoniae* (Marchisio et al. 2015). It shows great adhesion to HEp-2 cells, bacteriocins production (Santagati et al. 2012), ability to inhibit the biofilm-producing strains of *Staphilococcus aureus, Staphilococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Moraxella catarrhalis* and *Propionibacterium acnes* and even to disperse their pre-formed biofilms, through diffusible molecules secreted and the lowered pH of the medium (Bidossi et al. 2018).

These characteristics led my research group to patent (Pat. num: WO 2011/125086) and register as DSM 23307 our *S. salivarius* 24SMBc strain. Moreover, my research group and I have been attaining with the further characterization of probiotic properties of this strain, to validate it also in other oral diseases such as peri-implantitis and periodontitis, since related strains are already used in the reestablishment of dental problems like *S. salivarius* TOVE-R and M18, implicating in the reduction populations of *S. mutans* and *S. sobrinus* in dental caries (Tanzer, Kurasz, and Clive 1985a, 1985b; Burton et al. 2013).

2. AIM OF RESEARCH

Metagenomics is currently in its pioneering stages of development and the high throughput technologies are undergoing rapid evolution. My study has the aim to enrich the knowledge of the oral microbiome, identifying health and disease profiles. In particular, the main research objective of this PhD thesis carried out at the Microbial Molecular Antibiotic Resistance (MMAR) laboratory under the careful supervision of Prof. Maria Santagati, is to investigate the microbial composition of the oral microbiome in the periodontal and peri-implant disease.

Most of the knowledge of peri-implant microbiota derives from periodontitis and currently few metagenomic studies were done. Several studies have supported the theory that peri-implantitis and periodontal diseases share the same microbiota, but in recent years it has been demonstrated that the *core* microbiome of these diseases is different. The current unawareness of the microbial aetiology and pathogenesis of peri-implantitis is translated in the lack of effective treatment and the possible complications arising from this therapeutic option. For this reason, we collected samples from healthy, periodontal and peri-implant dental sites of 24 participants for microbiome analysis using 16S rRNA sequencing to determine a specific "*core microbiome"* related to these dental sites, intending to open the way of new therapeutic approaches, detecting specific microbial strains by metagenomics approach as biomarkers of infection before the bone loss and as indicators of health status.

On the other hand, the second research object of this PhD thesis is identify the contribution of microorganisms living in the oral cavity, which impact positively on the health of the individual, to develop personalized treatment in periodontal and peri-implant disease. Ideally, manipulation of the microbiome should use in the reestablishment of the dysbiosis, to optimize personal health. In fact, from the analysis of "*healthy core microbiome"*, was detect a higher

abundance of *Streptococcus* genus, with a prevalence of *Streptococcus salivarius* and *Streptococcus oralis* species. These goals gave rise to follow on a further characterization of *S. salivarius* 24SMBc, that has already shown a role in the prevention of URT diseases, but it could represent a good candidate as oral probiotic also in peri-implantitis and periodontitis. Thus, we've carried out a preliminary investigation of other beneficial abilities, examining the mechanism behind the already known antagonistic activity exhibited against oto-pathogens in order to find new potential features to be exploited. In particular, we focused on the ability of *S. salivarius 24SMBc* to interfere with oral pathogen strains adhesion in human epithelial type 2 (HEp-2) cells and the ability to co-aggregate with these. Moreover, the whole-genome sequencing of *S. salivarius* 24SMBc and preliminary analysis were performed.

Before proceeding to the evaluation of adhesion interference, despite the safety of *S. salivarius* is well documented and in general, the pathogenic potential of these normal colonizers of the oral cavity is considered quite low, with very rare cases of infection, we couldn't preclude *in vitro* assays to assess the safety of probiotics on epithelial cells, measuring LDH release. Then we assessed the ability of pathogenic strains to adhere to HEp-2 by *in vitro* adhesion assay. The preliminary tests were conducted on *Streptococcus pneumoniae* spp. and *Streptococcus pyogenes* spp., because they were already experienced in the antagonistic activity of *S. salivarius* 24SMBc. We chose, three *S. pneumoniae* strains, belonging to the serotype 19A (*S. pneumoniae* BT), serotype 15C (*S. pneumoniae* C2), serotype 9V (*S. pneumoniae* A3) and a strain with non-typable serotype (*S. pneumoniae* M4), isolated from a nasopharyngeal swab. Whereas, *S. pyogenes* strains include two strains of serotype M1 and one of serotype M18 well known for their virulence, that are respectively *S. pyogenes* 35370, *S. pyogenes* 5005 and *S. pyogenes* 2812A. Even if these species don't play an etiologic role in peri-implant disease, they inhabit the oral cavity and are considered "*microbial reservoir*" for the transmission from teeth

to another district. Moreover, future goals will provide a wider sample of periodontal pathogens and a clinical trial that will look into real efficacy against peri-implant and periodontal pathogens.

3. MATERIAL AND METHODS

3.1 Study population

For this clinical study, we enrolled 24 subjects (15 females and 9 males) aged between 48 and 80 years, whose 7 subjects had a history of smoking, while the remaining subjects were classified as "non-smokers" (Table 1).

Patient	Age (years)	Gender	Periodontal site (n=20)	Peri-implant site (n=24)	Healthy site (n=10)	Smoking status *	Abutment Material
1	62	F	34 PA	33 PI	57 HE	0	CrCo
2	52	F	7 PA	10 PI	-	0	titanium
3	52	F	46 PA	47 PI	56 HE	0	CrCo
4	58	F	44 PA	45 PI	60 HE	0	titanium
5	49	M	8 PA	9 PI	58 HE	1	titanium
6	55	F	25 PA	17.1 PI	-	0	titanium
7	51	F	27 PA	28 PI	-	0	titanium
8	80	F	47.1 PA	48 PI	-	0	titanium
9	64	M	40 PA	41 PI	-	0	CrCo
10	59	F	31 PA	32 PI	-	0	CrCo
11	66	F	43 PA	42 PI	-	0	titanium
12	65	M	18 PA	20 PI	-	0	titanium
13	63	F	13 PA	14 PI	53 HE	1	titanium
14	70	M	-	23 PI	-	1	titanium
15	73	F	39 PA	38 PI	-	0	titanium
16	80	M	-	15 PI	-	0	titanium
17	68	M	29 PA	30 PI	54 HE	0	titanium
18	58	F	12 PA	11 PI		1	titanium
19	66	M	35 PA	36 PI	55 HE	0	titanium
20	58	F	35.1 PA	21 PI	61 HE	1	titanium
21	57	F	-	16 PI	-	1	titanium
22	62	F	50 PA	49 PI	62 HE	0	titanium
23	69	M	52 PA	51 PI	-	0	titanium
24	48	M	-	5 A -PI	59HE	1	titanium

Table 1. *Demographic details and general information of the 24 patients. * Peri-implantitis sites: PI; Periodontitis sites: PA; Healthy sites: HE. **smoker: 1, non-smoker: 0.*

All the individuals were diagnosed with at least 2 non-adjacent teeth affected by periodontitis and 1 implant by peri-implantitis. All participants enrolled had the following **criteria of inclusion**:

i) history of periodontitis, ii) at least 1 implant with peri-implantitis, iii) 1 tooth with periodontitis and 1 healthy tooth, iv) implants for at least 1 year, and v) implants inserted in native bone.

Were excluded from the study those participants that had the following **exclusion criteria**: i) post-extractive implant and/or past regenerative procedures, ii) assumption of antibiotics and/or immune suppresses in the 3 months before enrollment, iii) need for antibiotic prophylaxis, iv) pregnancy and/or lactation and/or hormonal therapy, v) uncontrolled systemic diseases and conditions counter-indicating implant therapy.

Written consent was obtained from all subjects enrolled in the study and the study was approved by the Ethical Committee of the University of Catania (47/2018/CECT2).

For the detection of teeth affected by periodontitis and implants by peri-implantitis, we applied a new classification of periodontal and peri-implant disease (Tonetti, Greenwell, and Kornman 2018). While healthy tooth (HE) was defined as a tooth with an intact periodontium, absence of clinical signs of inflammation such as redness, swelling, bleeding on probing, and the presence of normal bone levels ranging from 1.0 to 3.0 mm apical to the cement-enamel junction (CEJ) (Schwarz et al. 2018) and normal gingival sulcus depth with PPD $\leq$ 3 mm.

3.2 Sample collection

Samples from healthy sites, periodontitis and peri-implantitis implants from each subject included in this study were all collected on the same day, following the previously defined inclusion criteria, from workmates of Department of General Surgery and Medical Surgery Specialties, School of Dental Medicine of University of Catania. Before the collection of samples by paper cones, a session of professional hygiene was carried out to remove the supragingival/supramucosal biofilm and plaque deposits.

Sampling was carried out inserting 4 sterile endodontic paper cones with tip diameter 0.25 mm and 2% taper in the gingival/mucosal sulcus in periodontitis and peri-implantitis sites with greatest PPD/PPDi for 2 min. After, the paper points were inserted in sterile Eppendorf tubes containing 2 ml saline solution of NaCl 0.9% and stored in a hermetically sealed refrigerated container and delivered to our laboratory of Molecular and Microbiological Medical Microbiology Resistance (MMAR) in the Department of Biomedical and Biotechnological Sciences (BIOMETEC) of the University of Catania within 2 h for further microbiological analyses.

A total of 58 sites from 24 patients were collected, where 10 from healthy (**HE**), 24 from periodontitis (**PA**) and 24 from peri-implantitis (**PI**) sites. Among healthy tooth sites, only 10 were chosen to maintain the group variability as well as in PA and PI samples (HE-abutment CrCo, HE-abutment titanium-smoking, HE abutment titanium- no smoking), whereas 4 samples were excluded for insufficient amount of DNA to sequence.

3.3 16S rRNA amplicon sequencing

3.3.1 DNA extraction

DNA from sterile paper cone samples was extracted with the PureLink® Genomic DNA Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions with slight variation. Before proceeding with the actual extraction, the samples were subjected to sonication for 120 minutes and two centrifugations, each of 45 minutes. The pellets obtained from each sample were resuspended in 200 µl PBS and then we proceeded following the manufacturer's instructions.

The DNA extraction was verified by the electrophoretic run on 1% agarose gel in 0.5X TBE, containing 1% SYBR Safe (Sigma) and the integrity of the extracted DNA was visualized in gel by UVITEC (Cleaver Scientific) UV transilluminator.

Furthermore, the extracted DNA was checked for quality and quantity by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) based on the A260/A280 ratio. A negative control containing only the buffer was included during each DNA extraction. The final purified genomic DNA was quantified by the Qubit 2.0 fluorometer (dsDNA HS assay, Invitrogen). All genomic DNA was frozen at -80°C until sequencing preparation.

3.3.2 Library preparation and sequencing

Extracted DNA (10 ng) was prepared for 16S amplicon sequencing by MiSeq platform using the Illumina protocol (Part # 15044223, Rev. B) following the workflow using the 16S Library preparation Protocol (Figure 7).

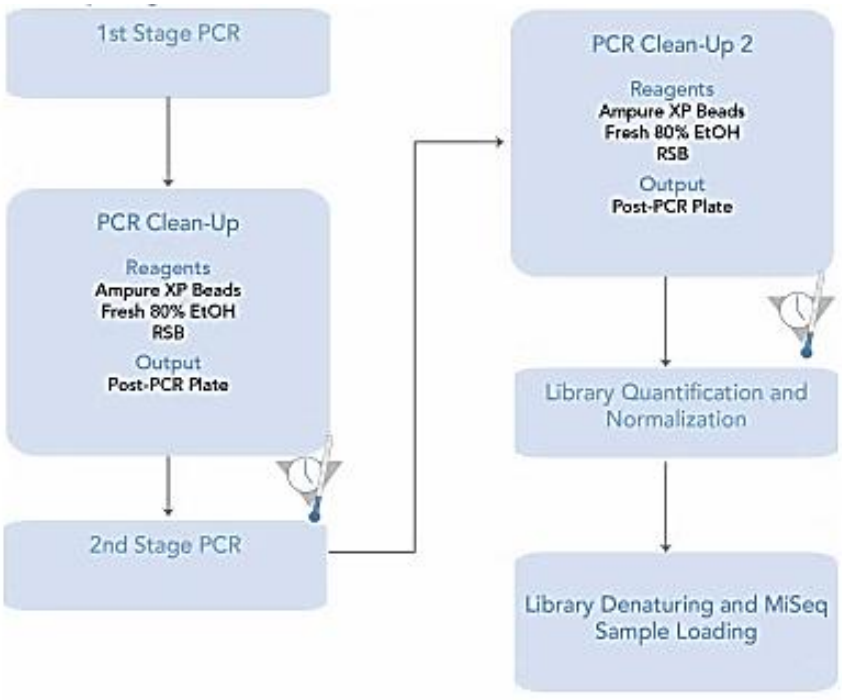


Figure 7. *16 S Library preparation workflow.*

The first step provides for an amplification step by PCR to amplify template out of a DNA sample using region of interest. In this study we used the V3-V4 region of the 16S ribosomal RNA that was amplificated using the following primers (Klindworth et al. 2013):

- forward 5'-CCTACGGGNGGCWGCAG-3'
- reverse 5'-GACTACHVGGGTATCTAATCC-3'.

The amplimers were verified for the size that should be ~550 bp as illustrated in Figure 8, through running of 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip (2100 Agilent Bioanalyzer).

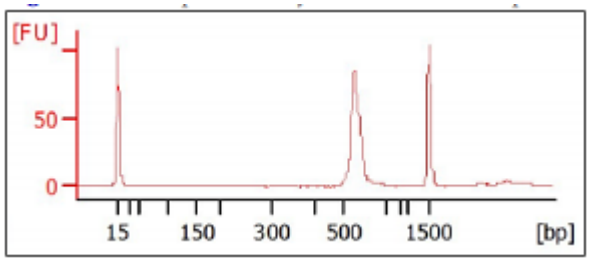


Figure 8. *Amplimer size of amplicon PCR (2100 Agilent Bioanalyzer).*

The second step of the workflow is the PCR-clean up in which all PCR products were purified by Agencourt AMPure XP magnetic beads (Beckman Coulter) (Figure 9).

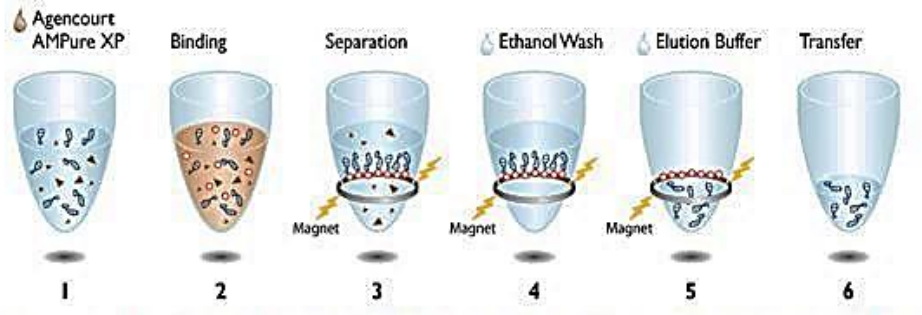


Figure 9. *PCR-clean up workflow.*

The third step of library preparation of 16S is the Index PCR. This step is fundamental because had allowed us to give an index to our samples to identify at the end of sequencing, in particular,

the samples were barcoded by Illumina's dual indexing strategy following default barcode layout. The quality of the Index PCR products was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) as previously explained but the size of amplimer this time should be around ~630 bp as shown in Figure 10. The index libraries underwent further purification with a further PCR-clean up as previously explained.

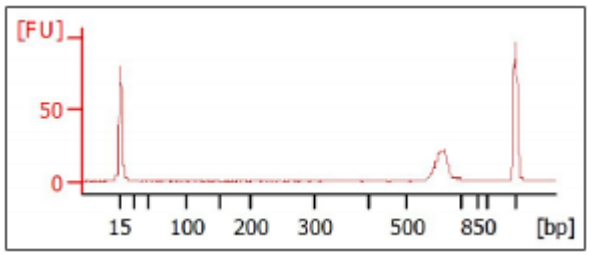


***Figure 10**. Amplimer size of Index PCR (2100 Agilent Bioanalyzer).*

For the library, quantification was used the Qbit, but to calculate DNA concentration in nM, was considered also the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace, calculating as follow:

$$\frac{(concentration\ in\ ng\ \mu l)}{(660\frac{g}{mol}x\ average\ library\ size)}x\ 10^6$$

The final library was normalized and was pooled to obtain a pooling library 4 nM with unique indices. The last step, before the sample loading, is the denaturation; after the pooled library and the internal control, PhiX (20%) were combined, then denatured, placed in the ice water bath and subsequently they were loaded into the MiSeq reagent cartridge previous keep at room temperature. Finally, 12 pM of the library mixtures, spiked with 20% PhiX control, was paired-end (2× 300) sequenced using the MiSeq platform (Illumina, USA) at the Service Center -

B.R.I.T. (University of Catania). The sequences are available in the NCBI BioProject database under accession number PRJNA548277.

3.3.3 Processing of sequencing data

Bioinformatic analysis was performed in collaboration with the Department of Clinical and Experimental Medicine of the University of Catania. At the end of the sequencing run on MiSeq, the integrated MiSeq Reporter software has started a primary analysis to perform de-multiplexing and the subsequent alignment of the "*reads*" in double strands for each indexed sample. V3-V4 16S rRNA FASTQ were de-multiplexed using the barcodes. The paired-end sequences were assembled to form a single read of 445 bp length by Fast Length Adjustment of SHort reads to improve genome assemblies (FLASH) (Magoc and Salzberg 2011) and quality-filtered ≥ 80% bases in a read above Q30. The ends of retained (not-merged) forward reads were clipped to a total read length of 270 bp to remove low-quality bases. The high-quality reads were clustered against a reference sequence collection with Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010) version 1.9.1. This tool allows to distinguish and classify the microbial species present in phylogenetic units, called Operational Taxonomic Unit (OTU), using the closed reference-based OTU picking method against the HOMD database at 97% of sequence similarity (Dewhirst et al. 2010). UCHIME algorithm was used to identify and remove Chimaeras (Edgar et al. 2011). QIIME (*filter_otus_from_otu_table.py*) with a filtering of 0.01% at the OTU level was performed to obtein only prominent taxa, in partcular the scripts (*compute_core_microbiome.py*) was used to obtain *core microbiome* and (*core_diversity_analyses.py*) was applied to detect OTUs present in at least 50% of the samples in order to focus on the abundance of different taxonomic categories at genus/species levels. To avoid sample size biases in downstream analyses,

rarefaction curves were generated with QIIME and calculated using Explicet using a maximum depth of 74.469 sequences/sample (Robertson et al. 2013).

3.3.4 Statistical analysis

The OTU tables were used for assessing α-diversity indices (Chao-1, Shannon diversity), that is the variance within each sample calculated from the taxonomic profiles of PA, PI and HE groups. To evaluate α-diversity among the taxonomic profiles and compared across the PA, PI and HE groups were used Independent Student's t-test and Mann–Whitney U test. Whereas, β-diversity show how samples vary against each other, thus it was performed between PA, PI and HE groups by a weighted UniFrac distance matrix *(beta_diversity.py* workflow) and then visualized by principal coordinate analysis (PCoA) plot.

OTU frequencies across sample groups were performed by the Kruskal-Wallis test, a nonparametric ANOVA test. Statistical Analysis of Metagenomic Profiles (STAMP) was employed for statistical analysis of taxonomic profiles (Parks et al. 2014; Parks and Beiko 2010). Extended error bar plots were computed through White's non-parametric t-test to point out the significant difference of bacterial taxa (P-value< 0.05).

3.4 Preparation of epithelial cells HEp-2

The cells used were human laryngeal cancer HEp-2 cells, which were maintained in a minimum essential medium of Eagle modified according to Dulbecco (D-MEM) (Sigma-Aldrich), added with 2 mM L-glutamine and a solution of antibiotics (penicillin and streptomycin 100 U/ml) and antifungals (amphotericin B 0,25 µg/ml) to prevent contamination,

supplemented with 6% (v/v) of fetal bovine serum (FBS, Thermo Fisher Scientific). Monolayers were released by incubation with 0.25 % trypsin/EDTA (0.25 % (w/v) trypsin, 0.1 mM EDTA, Life Technologies) for 10 min at 37 °C in 5 % CO_2 and seeded into 24-well trays at a concentration of 1.5×10^5 cells/well (counted through in "Bürker's room" from the previous T-75 flask) in D-MEM supplemented with 2% FBS for use in adherence assays. Before use in the adherence assay, the cells were washed at least twice with 500 µl of saline phosphate buffer (PBS) (Sigma-Aldrich). Finally, 500 µl of DMEM with 2% of FBS and without antibiotic were added. Cellular confluence within the wells will be approximately 80% and was checked under an inverted microscope (Figure 11).

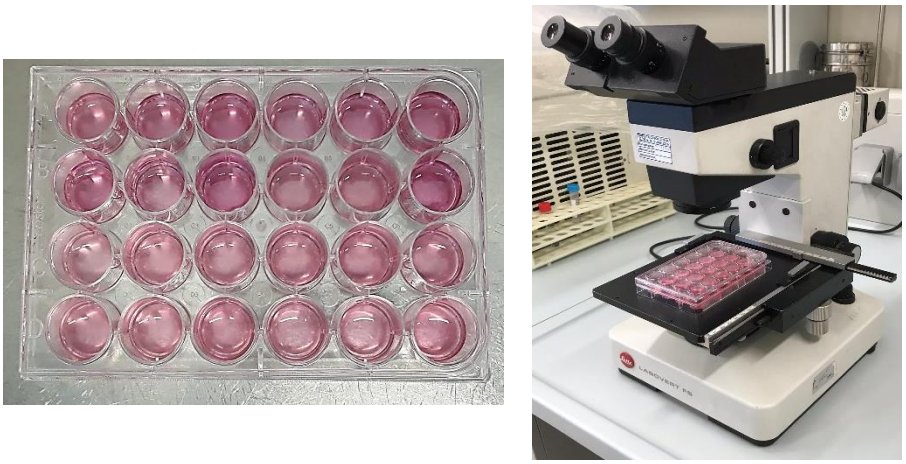


***Figure 11**. 24 well of HEp-2 and inverted microscope.*

3.5 Preparation of Bacterial strains

S. pneumoniae and *S. pyogenes* isolates were selected from our microbial bank at the MMAR lab. All isolates and *S. salivarius* 24SMBc were cultured overnight at 37 °C in 5 % CO_2 on Mueller Hinton agar plates (MH, Oxoid), supplemented by 5% defibrinated horse blood (Thermo Fisher). After, colonies were inoculated in 20 ml of Todd-Hewitt broth (THB; Oxoid), supplemented with 0.5 % (w/v) yeast extract (THY; Oxoid), briefly vortex and incubate at 37°C

in 5 % CO_2 for not more than 16h. From bacterial cultures, 100 µl are re-inoculated in 20 ml of THB + 0.5% of THY to reach mid-log phase (4h for *S. pneumoniae* spp. and around 3h for *S. salivarius* 24SMBc and *S. pyogenes* spp.).

3.6 *In vitro* adhesion of *S.salivarius* and bacterial interference test on HEp-2

Adherence assays were performed as described by Dunne et al., with some modifications (Dunne et al. 2014). Bacteria were grown to reach log phase in THB + 0.5% of THY and resuspended in 0.85 % (w/v) NaCl (Merck) to obtain the appropriate concentrations. The concentrations of 1.5 x 10^9 CFU/ml were used for *S. salivarius* 24SMBc (optical density OD_{600}≈0.30) and 1.5 x 10^8 CFU/ml for *S. pyogenes* (optical density OD_{600}≈0.30) and *S. pneumoniae* spp. (optical density OD_{600}≈0.35). 10 µl of *S. salivarius* 24 SMBc were administered to wells, following by centrifugation at 114 × g for 3 min to promote bacterial adherence to the cell monolayer. Plates were incubated at 37 °C in 5% CO_2 for 1 h. *S.pneumoniae* and *S. pyogenes spp.* were administered as above and plates were incubated for a further hour. PBS was used as a negative control and 100 U/ml heparin was used as positive control for blocking pneumococcal adhesion.

After 1 h incubation, the medium was removed from each well, then cells (and adherent bacteria) were washed two times with phosphate buffered saline (PBS) 1X (Gibco) to eliminate the bacteria unable to adhere to epithelial cells. Finally, cells were added with 100 µl 0.25 % trypsin/EDTA to each well and incubated for 5 min at 37 °C in 5% CO_2 and then were detached using 900 µl of THB added with 0.5% of THY to remove the cells with bacteria attached on the surface.

Adherent bacteria were quantified by determining viable counts (serial dilution and plating on Tryptic Soy Agar with Sheep blood 5%, MEUS) and expressing as mean % adherence ± standard deviation, normalizing the number of adherent microorganisms to 100%, using GraphPad Prism 6 software (GraphPad software Inc., San Diego, CA, USA). The adhesion interference of probiotic *S. salivarius* 24SMBc *versus* pathogens was assessed by comparing the viable count of pathogen strains alone and that obtained from the treatment with probiotic. The detection of streptococcal strains was obtained by observing features and morphology of bacterial colonies as showed in Figure 12 A-B.

These experiments were performed in triplicate and the statistical analysis of adherence' antagonism was performed by Multiple t-test, P value: * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$.

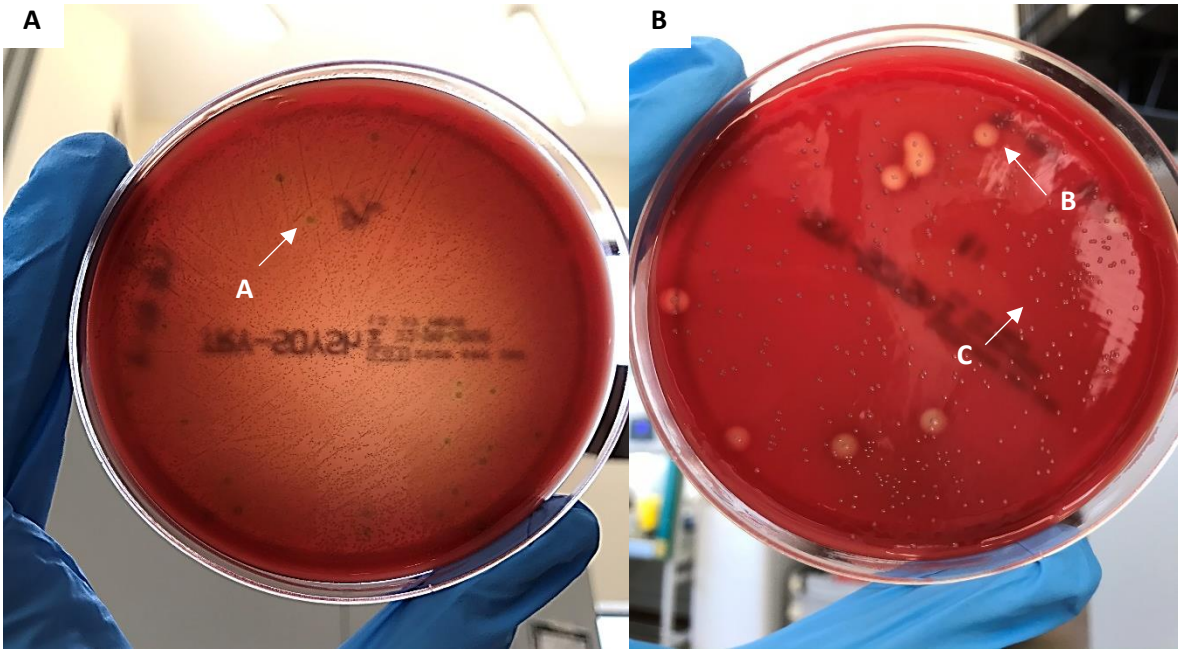


***Figure 12**. **A) S. salivarius 24SMBc and pneumococcal colonies on sheep blood agar**. Pneumococcal colonies are typically larger, flatter and greenish in color, as indicated by the white arrow and letter A. **B) S. salivarius 24SMBc colonies and S. pyogenes colonies on sheep blood agar.** S. pyogenes colonies are small, shiny, and translucent with total hemolysis, as indicated by the white arrow and letter B. S. salivarius SMBc colonies are small and white, as indicated by the white arrow and letter C.*

3.7 LDH assay

To evaluate the cytotoxic effect of *S. salivarius* 24SMBc on HEp-2, lactate dehydrogenase (LDH) release was misured. After 3 h of incubation with *S. salivarius* 24SMBc the supernatants from HEp-2 monolayers grown on 24-well tissue culture plates were collected. The levels of LDH in supernatants were assayed in triplicate using a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. LDH is a stable cytosolic enzyme of eukaryotic cells, indicator of necrotic cell death when released. HEp-2 cells exposed to Triton x100 (0.9%) were used as a control of total release (100% LDH release). The background level (0% LDH release) was determined with bacteria free culture medium (Cosseau et al. 2008). One-way ANOVA was used for analysis of LDH assay, P value: * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$.

3.8 Co-aggregation test

The co-aggregation test was conducted following the protocol of Chaffanel et al. with slight modification (Chaffanel et al. 2018). Briefly, all the strains were grown to reach log phase in THB added with 0.5% of THY as described above. Pellets were harvested after centrifugation at 3000g for 15 ' at 4 °C and resuspended in a peptone water solution (casein peptone 0.1% (p/v), Sigma-Aldrich) to obtain an optical density $OD_{600}\approx0.8$. Suspension of *S. salivarius* 24 SMBc to be assayed for co-aggregation were combined with an equal volume of indicator strains and incubate at room temperature for 60 minutes. Subsequently, 100 µl of the bacterial suspensions alone and in co-aggregation are dispensed in triplicate in a 96 wells plate for the first reading at T0 with the microplate reader (BioTek Synergy™ H1) (BRIT Biometec of Catania). After 1 hour of incubation at 150 rpm at room temperature (Kamaguchi et al. 1994) a

second reading at T1 was taken, but before of a centrifugation at 650g X for 2' to precipitate the bacteria that have not aggregated, in fact, 100 µl of the supernatant of the bacterial suspensions were dispensed in the microtiter.

Finally, data have been evaluated with the following formulas:

Self-aggregation

$$1 - \frac{AT1}{AT0} x100$$

Co-aggregation

$$\frac{AT0 - AT1}{AT1} x100$$

The co-aggregation of *S. salivarius* 24SMBc towards pathogens have been considered significant when the percentage of co-aggregation was higher than the percentage of self-aggregation of each pathogenic strain (De Gregorio et al. 2014). Both tests were repeated in triplicate. Co-aggregation assay were analyzed using ANOVA with Fisher's significant difference (LSD) test, P value: * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$.

3.9 Whole-genome sequencing of *Streptococcus salivarius* 24SMBc

3.9.1 DNA extractions

Genomic DNA of *S. salivarius* 24SMBc was extracted using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Finally, the DNA was quantified by Qubit 2.0 fluorometer (dsDNA HS assay, Invitrogen).

3.9.2 Whole-genome sequencing and preliminary analysis

The whole-genome of *S. salivarius* 24 SMBc was sequenced by using Illumina sequencing technology at Probiogenomics lab (Parma, Italy). The preliminary annotation was conducted with Rapid Annotations using Subsystems Technology (RAST) (Aziz et al. 2008) and the detection of putative bacteriocin gene clusters was carried out with BAGEL4 webserver (van Heel et al. 2018).

4. RESULTS

4.1 Patients' clinical characteristics

Participants clinical characteristics are summarized in Table 2. We collected a total of 54 sites from 24 subjects: 24 peri-implant (**PI**), 20 periodontal (**PA**) and 10 healthy (**HE**). From the healthy site population (n=10) mean age was 59 years (SD 6.95 years), Male/Female (M/F) Ratio was 4/6; 4 subjects (40%) were smokers and only 2 subjects had CrCo implant abutments. From the periodontitis site population (n=20) mean age was 62 years (SD 7.84 years), M/F ratio was 6/14; 4 subjects (20%) were smokers and only 4 subjects had CrCo implant abutments. From the peri-implantitis site population (n=24) mean age was 62 years (SD 8.80 years), M/F ratio was 9/15; 7 subjects (29.1%) were smokers and only 4 subjects had CrCo implant abutments.

Parameters	Healthy (n=10)	Periodontitis (n=20)	Peri-implantitis (n=24)
Age (Years ± SD)	59 ± 6,95	62 ± 7,84	62 ± 8,80
Sex (M/F)	4/6	6/14	9/15
Smoking (%)	40%	20%	29,1%
Abutment Material (CrCo/Titanium)	2/8	4/16	4/20

***Table 2**: Characteristics of the periodontal (PA), peri-implant (PI) and healthy (HE) sites.*

4.2 Microbial profile of healthy, periodontal and peri-implant sites.

A total of 54 samples were sequenced and a total of 7,414,811 valid reads, with an average of 137,311 reads/participant, were generated 70,265- 266,973 range, which were clustered in 376 operational taxonomic units (OTUs), with 97% similarity level using HOMD database. The β-

diversity between the three groups revealed variation in all groups. PI and PA samples were clustered together (PERMANOVA, p-value: 0.001) as showed in a PCoA plot (Figure 13).

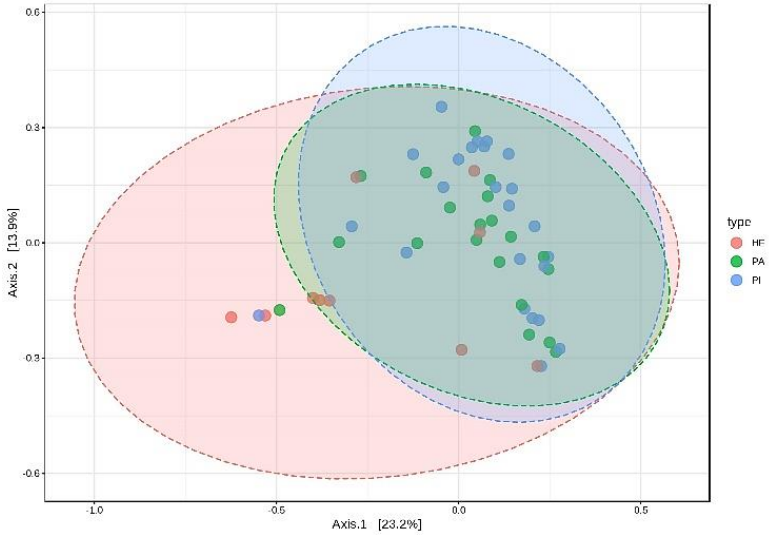


***Figure13. β-diversity**. A principal coordinate analysis (PCoA) plot generated using weighted UniFrac distances based on abundance of OTUs of periodontal (PA)(n:20)(green), peri-implant (PI)(n:24)(blu) and healthy (HE)(n:10)(red) samples (PERMANOVA, p-value: 0.001).*

The α-diversity within each group was evaluated by the Chao-1 index and the Shannon H index. OTU richness calculated with Chao-1 index was higher in the HE group (p-value: 0.70468; ANOVA), while the microbial diversity estimated by the Shannon diversity index showed a greater variety in HE and PI respect to PA sites (p-value: 0.44315; ANOVA) even if there were no statistically significant in both analyses (Figure 14).

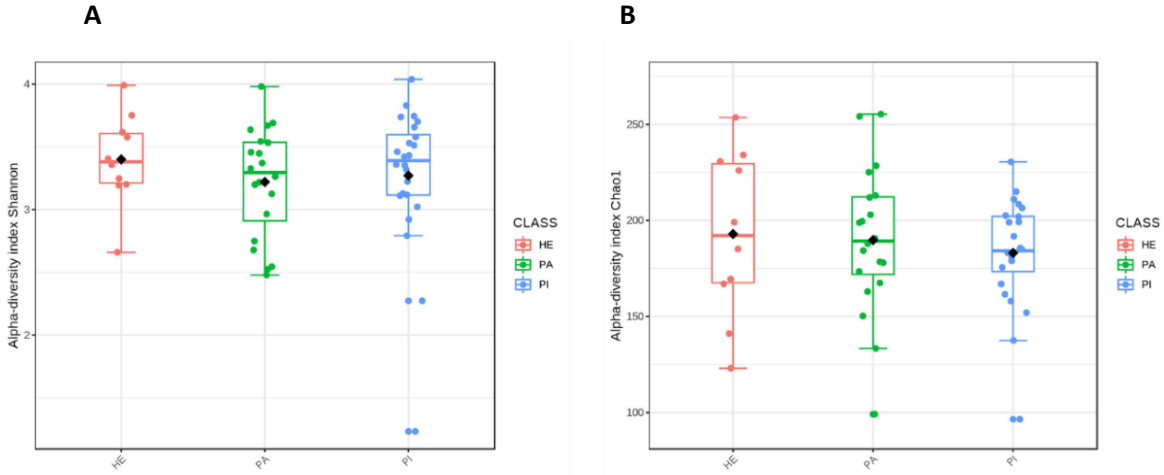
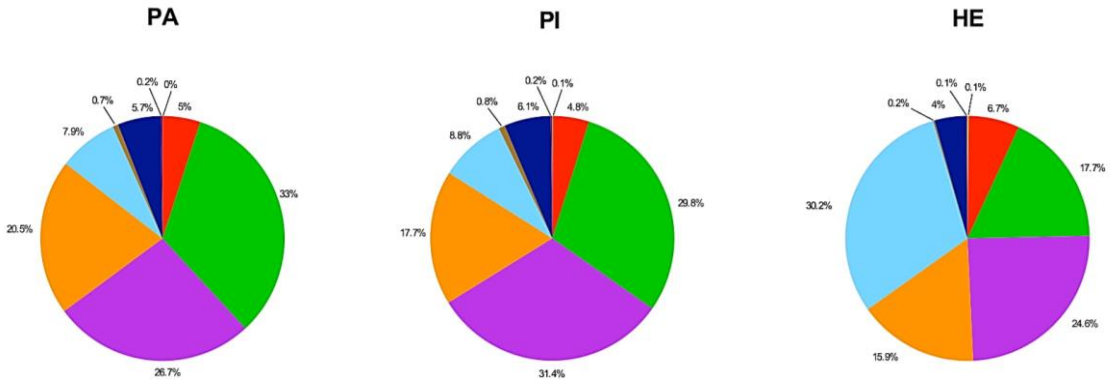


***Figure 14. α-diversity**. A) α-diversity by Chao-1 index (community richness) (p-value: 0.70468). B) α-diversity by Shannon H index (diversity) (p-value: 0.44315). Blu: peri-implant (PI); Green: periodontal (PA) and red: healthy (HE) sample.*

Regarding phyla, among PA, PI and HE sites, 10 are the most predominantly: *Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Absconditabacteria* (SR1), *Actinobacteria, Fusobacteria, Saccharibacteria* (TM7), *Spirochaetes*, and *Synergistetes*. *Proteobacteria* (29.15) was more abundant in the HE group, while *Bacteroidetes* (33%) and *Firmicutes* (31.4%) were more abundant in the PA and PI groups (Figure 15).



	PA	PI	HE
p__Absconditabacteria_(SR1)	0.0%	0.1%	0.1%
p__Actinobacteria	5.0%	4.8%	6.5%
p__Bacteroidetes	33.0%	29.8%	17.1%
p__Firmicutes	26.7%	31.4%	23.6%
p__Fusobacteria	20.5%	17.7%	15.3%
p__Proteobacteria	7.9%	8.8%	29.1%
p__Saccharibacteria_(TM7)	0.7%	0.8%	0.2%
p__Spirochaetes	5.7%	6.1%	4.1%
p__Synergistetes	0.2%	0.2%	0.1%

***Figure 15**. Different bacterial abundance between periodontal (PA), peri-implant (PI) and healthy (HE) groups at the phylum level.*

The microbiome analysis of *core* composition at the genus/species level showed a preponderance in HE sites of *Actinomyces, Rothia, Capnocytophaga* (*C. gingivalis*) *Streptococcus* (*S. oralis*), *Kingella, Lautropia* (only *L. mirabilis), Neisseria, Campylobacter, Aggregatibacter* and *Haemophilus* (only *H. parainfluenzae*) respect to diseases conditions.

In PA sample groups the most abundant taxa were *Porphyromonas, Tannarella, Peptoniphilaceae*_[G-1] and *Fusobacterium,* while in PI sites were abundant the genera *Atopobium, Alloprovetella* (*A. tannerae), Prevotella (P. intermedia), Parvimonas (P. micra), Filifactor (*only *F. alocis), Mogibacterium, Peptostreptococcaceae*_[XI], *Dialister* (only *D. invisus*) and *Treponema (T. denticola and T.HMT_237)*. The shift towards a pathological condition was associated with a significant abundance of *Porphyromonas gingivalis* (13%) in PA and *Porphyromonas endodontalis* (4.7%) in PI samples. Interestingly, in HE was detected a higher abundance of *Streptococcus* genus, with a prevalence of *Streptococcus salivarius* (0.5%) and *Streptococcus oralis* (8.8%) species as shown in Table 3.

LEVEL	GROUP		Relative abundance		
			PA (%)	PI (%)	HE (%)
genus	**↑ *Actinomyces***		**2.8**	**1.3**	**3.5**
species		*Actinomyces gerencseriae*	0.2	0.1	0.3
		Actinomyces _HMT_180	0.3	0.4	0.7
	↑ *Rothia*		**0.7**	**0.6**	**2.1**
		Rothia aeria	0.6	0.5	1.5
	↓ *Atopobium*		**0.2**	**1.2**	**0.0**
	↓ *Porphyromonas*		**17.1**	**11.5**	**6.9**
		Porphyromonas endodontalis	3.7	4.7	1.4
		Porphyromonas gingivalis	13.0	6.6	4.7
	↓ *Tannerella*		**3.4**	**2.1**	**2.1**
	↓ *Alloprevotella*		**1.4**	**2.5**	**0.6**
		Alloprevotella rava	0.1	0.2	0.1
		Alloprevotella tannerae	1.1	2.0	0.3
	↓ *Prevotella*		**7.8**	**9.4**	**3.9**
		Prevotella denticola	0.4	1.1	0.1
		Prevotella intermedia	2.2	2.7	1.3
		Prevotella nigrescens	1.1	1.0	0.1
		Prevotella oris	0.9	1.7	0.4
	↑ *Capnocytophaga*		**1.0**	**0.4**	**1.9**
		Capnocytophaga gingivalis	0.2	0.1	0.8
		Capnocytophaga granulosa	0.2	0.0	0.2
		Capnocytophaga sputigena	0.3	0.2	0.5
	↑ *Streptococcus*		**5.6**	**7.5**	**10.4**
		Streptococcus oralis	3.9	4.2	8.8
		Streptococcussalivarius	0.1	0.1	0.5
	↓ *Parvimonas*		**2.3**	**2.7**	**1.2**
		Parvimonas micra	1.6	2.0	0.3
		ParvimonasHMT_110	0.4	0.4	0.8
	↓ *Peptoniphilaceae_[G-1]*		**2.1**	**1.3**	**0.5**
		Peptoniphilaceae [G-1]_HMT_113	2.1	1.3	0.5

	↓ *Filifactor*		**3.2**	**4.2**	**1.1**
		Filifactor alocis	3.2	4.2	1.1
	↓ *Mogibacterium*		**1.5**	**1.8**	**0.3**
	↓ *Peptostreptococcaceae_[XI]*		**2.2**	**2.9**	**0.2**
	↓ *Dialister*		**1.5**	**2.4**	**0.3**
		Dialister invisus	0.7	1.4	0.2
	↓ *Fusobacterium*		18.6	16.6	14.6
		Fusobacterium nucleatum_subsp.vincentii	0.1	0.1	0.1
		Fusobacterium periodonticum	0.0	0.0	0.5
	↓ *Leptotrichia*		**1.1**	**1.0**	**0.7**
	↑ *Kingella*		**0.6**	**0.2**	**2.0**
	↑ *Lautropia*		**0.1**	**0.1**	**3.7**
		Lautropia mirabilis	0.1	0.1	3.7
	↑ *Neisseria*		**1.2**	**1.0**	**5.0**
		Neisseria bacilliformis	0.3	0.0	3.2
	↓ *Desulfobulbus*		**1.4**	**1.4**	**1.0**
		Desulfobulbus HMT_041	1.4	1.4	1.0
	↑ *Campylobacter*		**2.1**	**1.5**	**3.2**
		Campylobacter rectus	1.3	0.6	1.6
	↑ *Aggregatibacter*		**0.6**	**0.1**	**1.7**
		Aggregatibacter actinomycetemcomitans	0.0	0.0	0.1
		Aggregatibacter aphrophilus	0.5	0.0	0.2
		Aggregatibacter HMT_898	0.0	0.0	0.4
	↑ *Haemophilus*		**1.0**	**3.8**	**5.2**
		Haemophilus parainfluenzae	1.0	3.8	5.2
	↓ *Treponema*		**5.7**	**6.1**	**4.0**
		Treponema denticola	2.3	2.3	1.0
		Treponema maltophilum	0.4	0.3	0.3
		Treponema socranskii	0.7	0.7	0.3
		Treponema sp._HMT_237	1.4	1.5	1.2

***Table 3**. Relative abundances of the top 25 microbial genus and species of periodontal (PA), peri-implant (PI) and healthy (HE) sites. Arrows indicate the direction of the variation with respect to HE: ↓, decreased; ↑, increased; =, unchanged.*

Among genera, 30 showed statistically significant differences in relative abundance between HE and PA groups (Figure 16), 46 between HE and PI groups (Figure 17). From the comparison of the relative abundance of genera made first between HE and PA, and then between HE and PI groups, we found *Prevotella (P. nigrescens, P. oralis* in both PA and PI groups, while *P. oris, P. denticola* and *P. fusca* only in PI), *Filifactor* (*F. alocis* in PA and PI sites), *Porphiromonas (P. endodontalis* only at the species level in PI sites), *Parvimonas micra* (only at the species level in both PA and PI groups), *Eggerthia* (*E. catenaformis* in both PA and PI groups), *Slaxkia* (*S. exigua* in both PA and PI groups), *Peptoniphilus* (*P. lacrimalis* in both PA

and PI groups), *Dialister* (*D. invisus* only in PI group) and *Alloprevoltella* (*A. tannerae* in both PA and PI groups) more abundant in infected sites, while *Rhizobiales, Bacillus, Klebsiella, Bergeyella, Clostridiales_[F-1][G-2], Afipia, Microbacterium, Granulicatella (G. adiacens)* and *Lautropia (L.mirabilis)* were more abundant in HE sites (Figure 18 and 19).

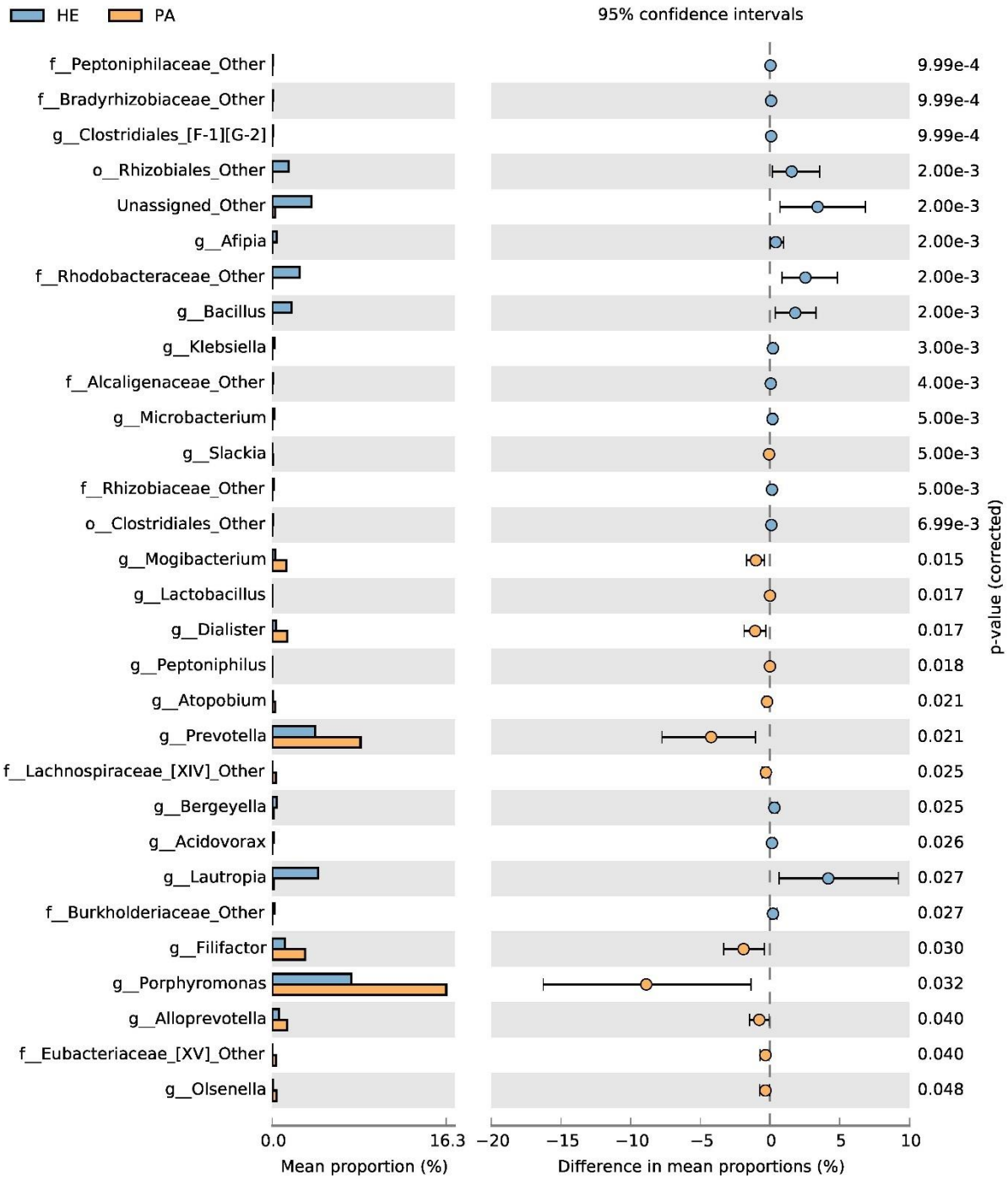


Figure 16. Different relative abundances at the genus level between healthy (HE) and periodontal (PA) sites. *Different bacterial abundance between HE and PA groups (White's non-parametric t-test; p-value <0.05).*

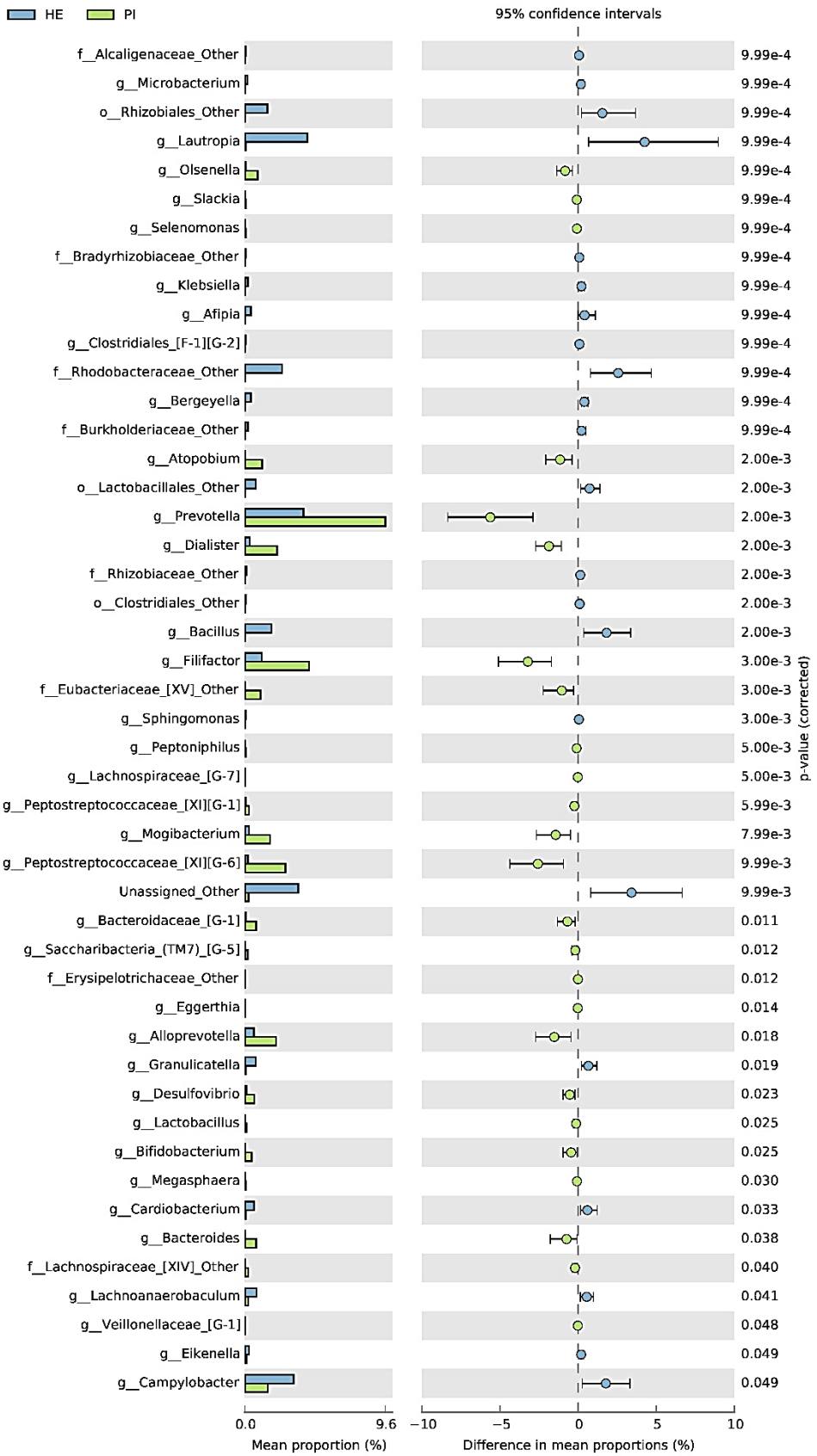


Figure 17. Different relative abundances at the genus level between healthy (HE) and peri-implant (PI) sites. *Different bacterial abundance between HE and PI groups (White's non-parametric t-test; p-value <0.05).*

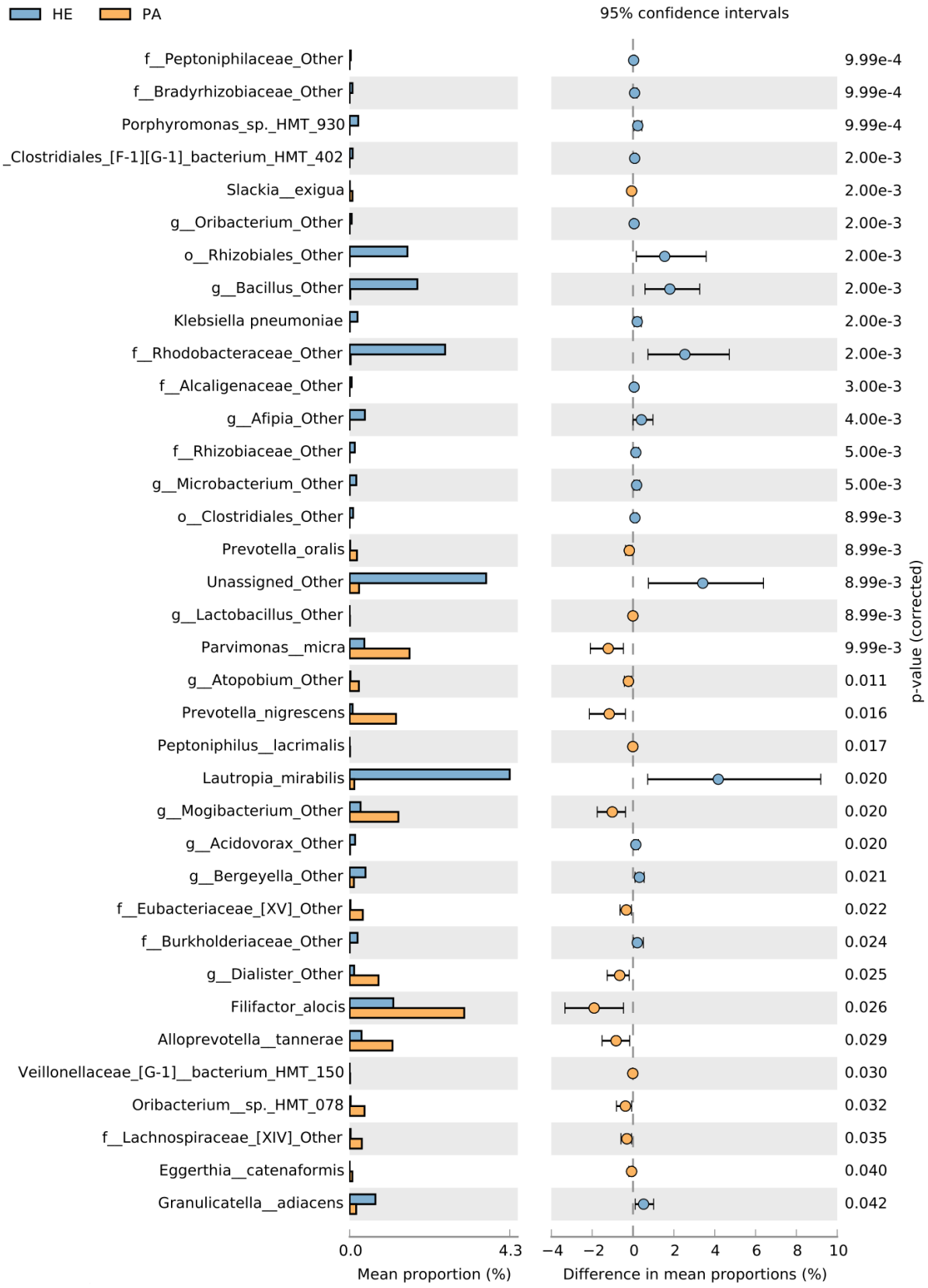


Figure 18. Different relative abundances at species level between healthy (HE) and periodontal (PA) sites. *Different bacterial abundance between HE and PA groups (White's non-parametric t-test; p-value <0.05).*

Figure 19*. *Different relative abundances at species level between healthy (HE) and peri-implant (PI) sites. *Different bacterial abundance between HE and PI groups (White's non-parametric t-test; p-value <0.05).*

Indeed, the microbial analysis across the three groups displayed 22 genera statistically significant (one way-ANOVA p-value <0.05), including *Klebsiella*, *Escherichia*, *Cardiobacterium*, *Acidovorax*, *Lautropia* (the most prevalent), *Burkholderiaceae*, *Alcaligenaceae*, *Rhodobacteraceae*, *Afipia*, *Bradyrhizobiaceae*, *Clostridiales*, *Bergeyell*a and *Corynebacterium* that were more abundant in HE groups, while *Slackia*, *Bacteroidaceae*[G-1], *Eubacteriaceae*[XV], *Peptostreptococcaceae*[XI], *Selenomonas*, and *Lacnospiraceae*[G-7] were more abundant in PI samples, whereas *Mogibacterium*, *Olsenella* and *Dialister* had a similar relative abundance in PI and PA groups (Figure 20).

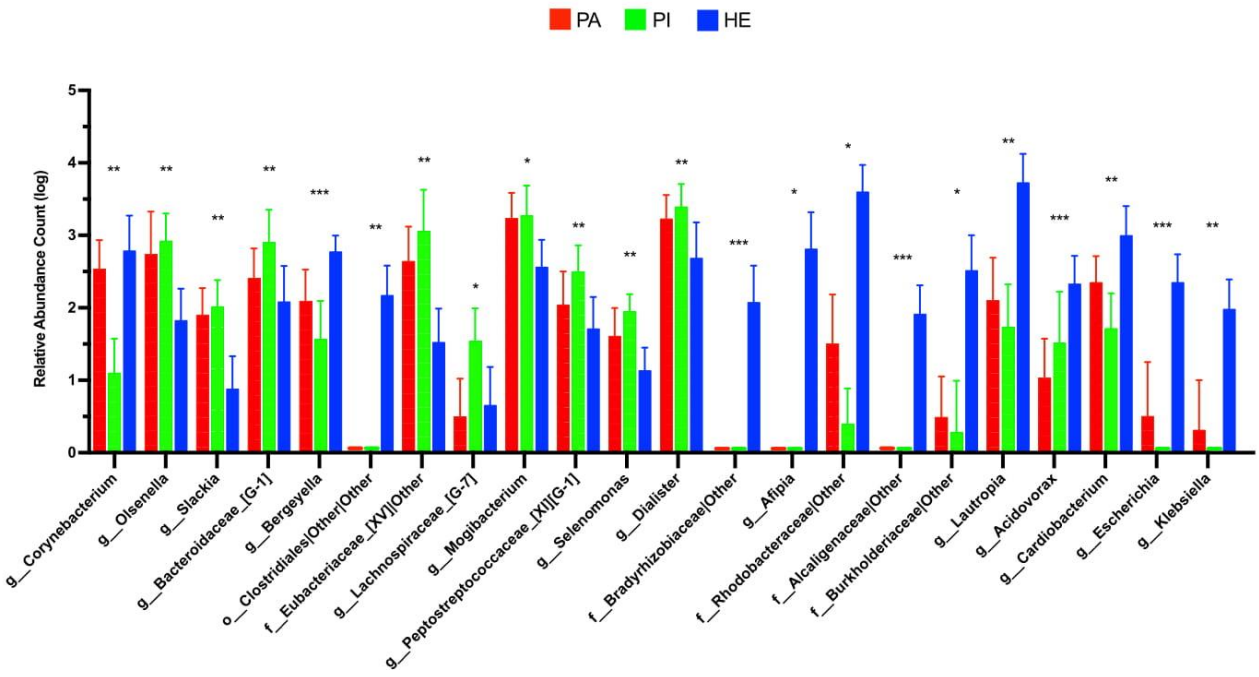


***Figure 20**. Different bacterial abundance across the three groups. The significant differences in terms of abundant of genera by ANOVA (one-way, p-value <0.05).*

4.3 Activity of *S. salivarius* 24SMBc *vs S.pneumaniae* and *S. pyogenes* on HEp-2

S. salivarius 24SMBc was showed a good adhesion to HEp-cell lines as previously demonstrated by Santagati et al (Santagati et al. 2012). In this test, we used an inoculum of

1.5x10^9 CFU/ml of *S.salivarius* and we obtained the streptococcal adhesion of 7.10x10^8 CFU/ml on HEp-2. The ability to adhere to HEp-2 cell lines was assessed against four *S. pneumoniae* strains (BT, C2, A3 and M4) and three *S. pyogenes* strains (*S. pyogenes* 2812A, *S. pyogenes* 35370 and *S. pyogenes* 5005) using an inoculum of 10^8 CFU/ml for both. The quantification of viable count has shown that all the strains can adhere and among pneumococci strains, all showed a good ability to adhere with a mean count of 1,88 x 10^5 CFU/ml, while among *S. pyogenes* spp. of 3,57 x 10^6 CFU/ml.

The adhesion's interference on HEp-2 cell lines of *S. salivarius* 24 SMBc (10^9 CFU/ml) against pneumococci showed a significant reduction (Figure 21A). The greatest reduction was seen against pneumococci BT and C2 belonging respectively to serotype 19A and 15C. Regarding the adhesion of *S. pyogenes* strains, no significant reduction was found (Figure 21B). The viable count and the percentage of adherence were reported in Table 4.

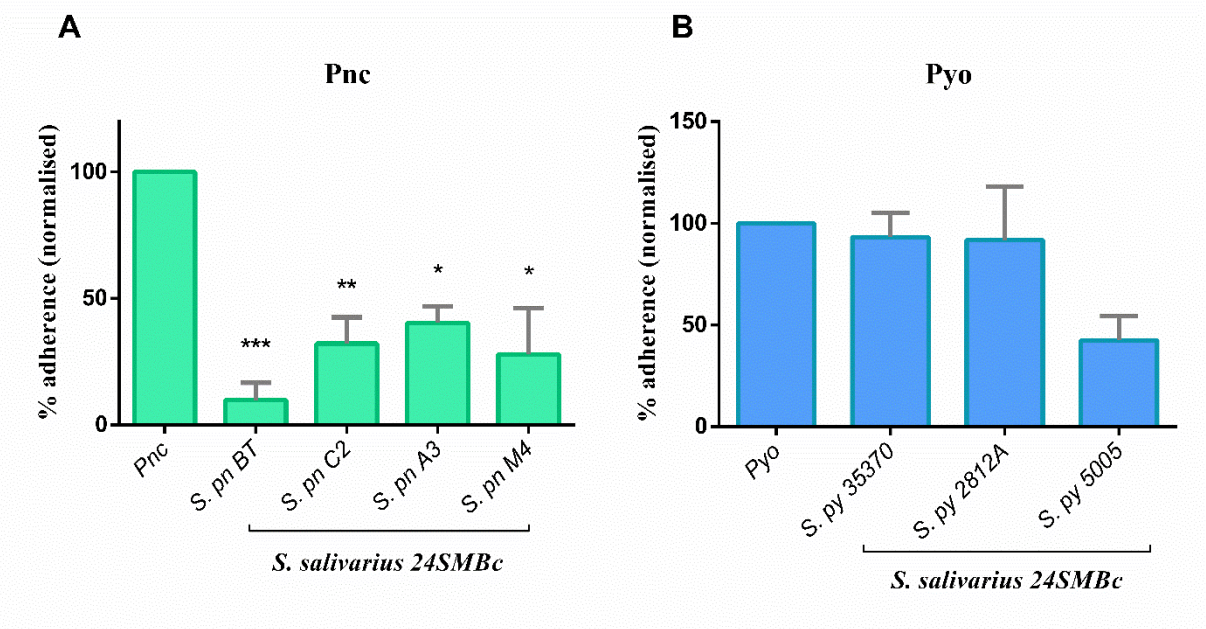


Figure 21. Activity of S. salivarius 24SMBc vs S.pneumaniae and S. pyogenes on HEp-2*. A). Effect of S. salivarius 24SMBc(10^9 CFU/ml) on pneumococcal (10^8 CFU/ml) adherence to HEp-2. Pnc: pneumococci; normalized to 100%. B). Effect of S. salivarius 24SMBc (10^9 CFU/ml) on S. pyogenes spp. (10^8 CFU/ml) adherence to HEp-2.* Pyo: S. pyogenes; *normalized to 100%.* * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ when compared to pneumococci and S. pyogenes spp. alone (Multiple t test).*

	Assay condition	CFU/ml Pnc/Pyo (viable count)	% adherence (viable count)
Pnc alone (10^8 CFU/ml)	*S. pneumoniae BT*	1,30E+05	100
	S. pneumoniae C2	4,27E+05	100
	S. pneumoniae A3	4,77E+04	100
	S. pneumoniae M4	1,14E+05	100
Pnc (10^8) + *Sal* (10^9 CFU/ml)	*S. pneumoniae BT*	1,33E+04	9.88
	S. pneumoniae C2	1,37E+05	32.16
	S. pneumoniae A3	1,92E+04	40.31
	S. pneumoniae M4	4,00E+04	27.80
Pyo alone (10^8 CFU/ml)	*S. pyogenes 35370*	1,20E+06	100
	S. pyogenes 2812A	4,68E+06	100
	S. pyogenes 5005	4,83E+06	100
Pyo (10^8) + *Sal* (10^9 CFU/ml)	*S. pyogenes 35370*	1,12E+06	93.99
	S. pyogenes 2812A	4,30E+06	91.81
	S. pyogenes 5005	2,05E+06	42.41

***Table 4. S. pneumoniae and S. pyogenes spp. adherence to HEp-2 cells determined by viable* count**. * Pnc = *pneumococci*; Sal = *S. salivarius* 24SMBc and Pyo= *S. pyogenes* spp.

4.4 Cytotoxic effect of *S. salivarius* 24 SMBc on HEp-2

S. salivarius 24SMBc (10^9 CFU/ml) had no cytotoxic effects on HEp-2 cells, as indicated by microscopic observation (Figure 22A) and by measuring LDH release from cells incubated with the bacteria for up to 3 h. The results had shown that the amount of LDH release in the supernatant of HEp-2 cultured in the absence or presence of the bacteria was unchanged, indicating that *S. salivarius* 24SMBc (10^9 CFU/ml) didn't cause cell lysis (Figure 22B).

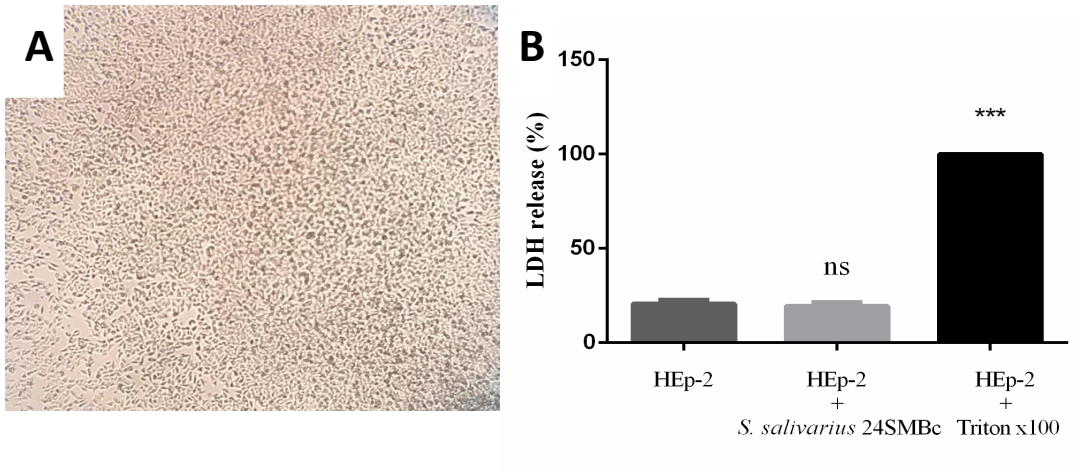


Figure 22. Cytotoxicity assay. *A) Observation of HEp-2 cells treated with 10^9 CFU/ml of S. salivarius 24SMBc with inverted microscope. B) LDH release. Mean SD. NS (not significant) and ***P <0.001 compared to HEp-2 control.*

4.5 Auto-aggregation and Co-aggregation ability

Aggregation properties were assayed with the auto-aggregation and co-aggregation tests measuring two different characteristics of the strains. The auto-aggregation rate of *S. salivarius 24SMBc* measured after 1 h of incubation, gave the following value 81.8% ± 0,012. The degree of *S. salivarius* 24SMBc co-aggregation with pathogen streptococci was very high, ranging between 78.6% ± 0.005 and 52.5% ± 0.015.

S. pyogenes spp. (S. pyogenes 2812A, S. pyogenes 35370 and S. pyogenes 5005) despite a strong value of selective interactions *versus S. salivarius* 24SMBc (78.6%, 68.8% and 69.9%), possessed a strong auto-aggregation property (78.6 %, 68.8% and 69.9% respectively).

Despite the co-aggregation percentage of all bacteria strains being higher than self-aggregation percentages, significant co-aggregation was found only for pneumococci spp. as illustrated in Figure 23.

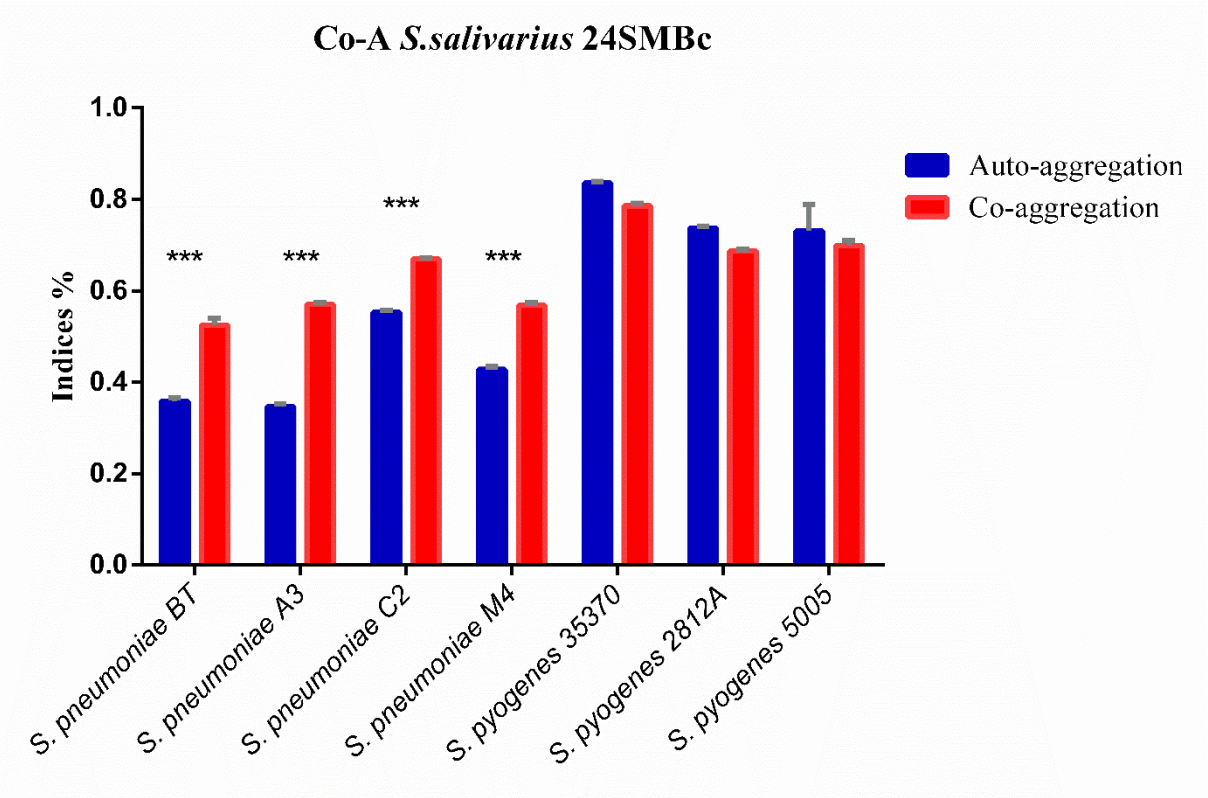


Figure 23. Co-aggregation ability of S. salivarius 24 SMBc*. Results are presented as average of at least three independent experiments and the error bars correspond to standard deviations. Statistical significance was evaluated by ANOVA with Fisher's least significant difference (LSD) (*** $p \leq 0.001$).*

4.6 Preliminary genome analysis of *S. salivarius* 24SMBc

The genome of *S. salivarius* 24SMBc was of 2,131,204 bp and contains 32 contigs, displaying a GC content of 39.85%, carries 1954 open reading frames (ORFs). The genome sequence analysis evaluated by RAST software predicts 2 subsystems in the genome and 28 metabolic networks as shown in Figure 24.

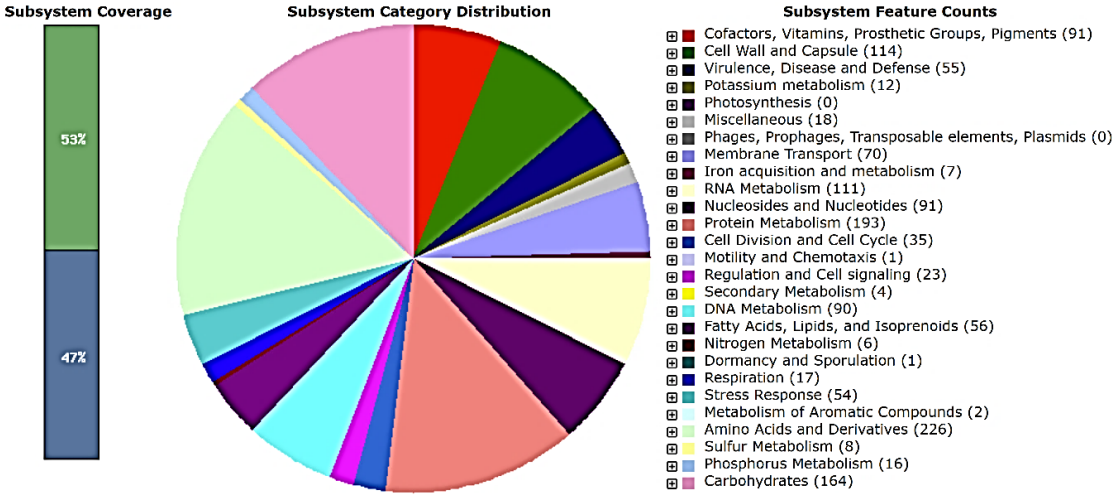


Figure 24. Subsystems category distribution of Streptococcus salivarius 24SMBc revealed by genome annotations based on the RAST server. *The pie graph indicates the subsystem distribution statistics of S. salivarius 24SMBc. Each color represents a subsystem category with the feature counts of which listed on the right of the graph.*

The genome sequencing analysis confirmed the presence of blpU-like genetic locus in contig 24 of 8.023 bp carrying the blpU-like, which was involved in the bacteriocin production. The blp cassette organization has 11 orfs and presents three main modules: the ABC-transporters, the bacteriocin immunity module and the leader peptide that contains a double-glycine motif. The blp peptide belongs to the clan GG-leader CL0400 that carries a distinctive GG-cleavage motifC-terminal of Class IIc (bacteriocin_ IIc, ComC, blpD) (Figure 25).

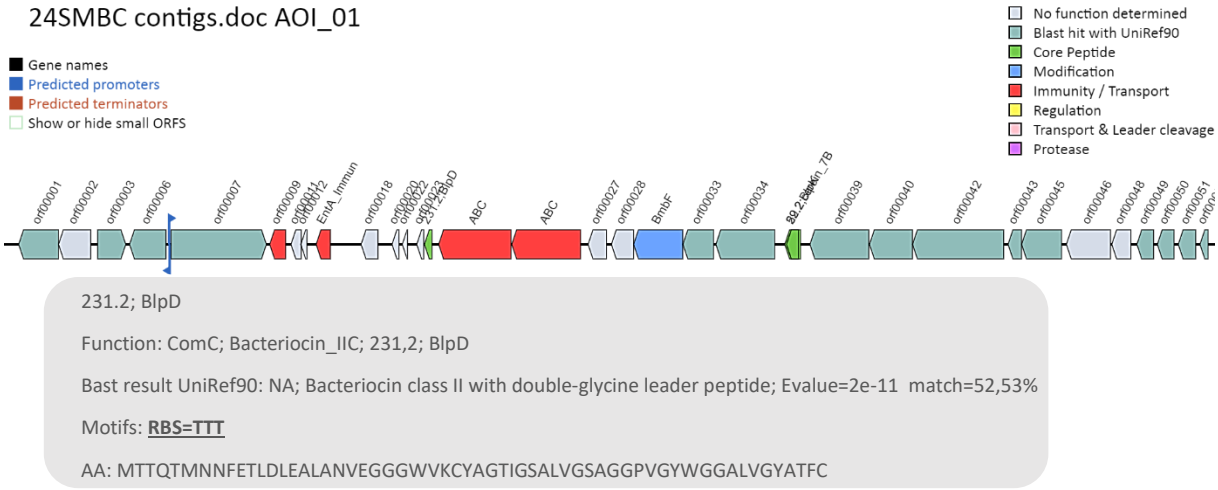


Figure 25*. Genetic organization of the blp-like cassette of S. salivarius 24SMBc (BAGEL4 software).*

5. DISCUSSION AND CONCLUSION

The oral microbiome research has been revolutionized by high-throughput sequencing technology and has furthered our understanding of the bacterial communities impact on the pivotal role playing in health and disease status.

Peri-implantitis disease is the result of the complex interaction between the commensal microbiota, host susceptibility and environmental factors such as diet and smoking (Zitzmann and Berglundh 2008). The real problem of peri-implantitis and periodontal is that often the damage does not remain limited to the mouth, but invades other organs, compromising the remaining oro-pharyngeal parts or even the cardiovascular system and gut (Beck and Offenbacher 2005). Besides, the diagnosis and treatment of peri-implantitis represent an important issue because of its clinical implications.

This study highlights the quantitative and qualitative differences of the microbial species presenting health and disease status, identifying a biomarkers that could be used in the diagnosis but also show a new approach of diagnosis.

In addition, the clinical protocol used a new classification of severity for inclusion criteria to provide greater reliability of the results obtained, considering that all samplings for each patient were performed on the same day. We believe that a thorough knowledge of this disease, supported by the data presented here, which will allow the clinician to better diagnosed and prevent its occurrence before bone damage and spreading of the inflammatory process.

Our data demonstrated different grades of variability among each group of samples, in particular a greater diversity was found in the healthy group in according with Apatzidou et al. (Apatzidou et al. 2017). Conversely, comparing the α-diversity by Chao-1 seems that PA and PI groups overlapped each others.

At the phylum level, *Proteobacteria* was overrepresented in HE sites according to Yu et al. (Yu et al. 2019). At the genus level, the greatest abundance of *Proteobacteria* phylum was statistically confirmed with the prevalence of *Clostridiales, Bradyrhizobiaceae, Alcaligenaceae, Burkholderiaceae, Lautropia, Escherichia* and *Klebsiella.* These genera were revealed almost exclusively in healthy sites, so these findings encourage us to speak of "*healthy core microbiome".* In addition, in HE sites was detected a higher abundance of *Lautropia mirabilis* that was supported also by recent literature (Gao, Zhou, et al. 2018; Tsigarida et al. 2015). Furthermore, in the HE *core* there were also predominant but not statistically significant taxa: *Actinomyces, Rothia, Streptococcus, Haemophilus* and *Neisseria.*

Whereas, in PI and PA was found a high prevalence of *Treponema denticola, Tannella* spp. and *Porphyromonas gingivalis* which are the important periodontal pathogens of the "*red complex*" in according to previous microbiome studies (Rakic, Grusovin, and Canullo 2016; Yu et al. 2019; de Melo et al. 2020). The diseased sites, indeed revealed a microbial ecosystem shared by both in which *Mogibacterium, Dialister, Prevotella, Filifactor, Alloprevotella* and *Olsenella* were the most prevalent. These results are in agreement with the knowledge that one of the principal risk factors for the onset of peri-implantitis is the periodontitis (Heitz-Mayfield 2008).

Despite this, we went into more detail, analyzing different signatures between diseased sites, in which in peri-implant infections were identified the most abundant taxa: *Lachnospiraceae[G-7], Selenomonas, Peptostreptococcaceae [XI][G-1], Mollicutes [G-2], Peptoniphilaceae, Bacteroidaceae [G-1]* and *Atopobium*, while discovering of *Corynebacterium* and *Cardiobacterium* in PA sites has confirmed a previous hypothesis of distinct ecosystems existence among the two diseased sites (Chen et al. 2018). In fact, PA and PI had significantly different abundance levels of species. *Porphyromonas gengivalis* was more abundant in PA than PI sites, on the contrary of *Porphyromonas endodontalis*. Among *Prevotella* genus that

belongs to the orange complex of periodontal pathogens and are considered to be important for the progression of the periodontitis, there was *Prevotella nigrescens* in both diseases, while *Prevotella intermedia* and *Prevotella denticola* were significantly abundant in peri-implantitis. However, *Fusobacterium nucleatum* was found only at a low prevalence as reported to Maruyama et al. (Maruyama et al. 2014). Interestingly, the presence of *Parvimonas micra*, *Filifactor alocis*, *Dialister invisus* and *Alloprevotella tannerae* were more abundant in PI samples (Rakic, Grusovin, and Canullo 2016). To date, this is the first study in which *Alloprevotella*, specifically *Alloprevotella tannerae* and *Atopobium* were significantly abundant in peri-implantitis.

Moreover, the microbial population increase in abundance from PA to PI. Thus, these results have suggested that periimplantitis is the results of a dysbiosis process involved in significant changes in the abundance of predominant bacterial. The role of microbiome should be studied as suggested by Rakic et al. considering the quantitative rather than qualitative aspects of microbial composition (Rakic, Grusovin, and Canullo 2016).

Finally, although, it is very difficult identity a microbiome strictly related to periimplantitis disease, these data at present led us to define a "*healthy core microbiome*" in which the genus *Lautropia* appears solely related to healthy status and a "*diseased core microbiome*" with taxa sharing in PI and PA. Next to these statistically significative species in *healthy core microbiome*, in terms of relative abundance was detect a higher abundance of *Streptococcus* genus, with a prevalence of *Streptococcus salivarius* and *Streptococcus oralis* species, who conducted us to further characterize the probiotic properties of *Streptococcus salivarius* 24SMBc, that could be suitable as new candidate probiotics to improve peri-implant and periodontal microbial dysbiosis.

If these results will be confirmed in an increasing number of patients, the hypothesized biomarkers identified here could be validated and used for developing new strategies to prevent and treat specifically PI and PA and not generically as a unique condition, using 16S metagenomic approach as means of diagnosis. This could be paving the way towards a certainly wider future goal to be able to prevent also other pathologies related to periodontitis and peri-implantitis that can cause damage to the level of the oesophagal mucosa such as oesophagal cancer and squamous oral cancer (Peters et al. 2017; Gould 2002). Moreover, the knowledge of the variations in healthy and diseased implant microbiome could lead to creating new therapeutic approaches such as the use of probiotics to maintain or restore the microbial flora, avoiding the use of antibiotics

In this context, my research group has investigated the concept of "*bacteriotherapy*" that seems to be related to bacterial interference. Mechanisms contributing to microbial interference might typically include the greater ability to adhere to the epithelial surface, excluding by competition the pathogen's adhesion and nutrition. Another desirable mechanical property for probiotics is their capacity to aggregate among themselves (auto-aggregation), or with pathogens (co-aggregation) (Wescombe et al. 2012). Aggregation of commensal strains appeared to be necessary for adhesion to epithelial cells and enabled the formation of a barrier that protects the host's epithelium from colonization by pathogens. Moreover, the ability to co-aggregate with a pathogen allows the probiotics to entrap it. These two features are considered desirable property for probiotics strains, in fact, auto-aggregation ability test together with co-aggregation could be used for preliminary screening identifying potentially adherent bacteria with properties suitable for commercial purposes (Collado, Meriluoto, and Salminen 2007).

Since, *Streptococcus salivarius* 24 SMBc was already known for the prevention of rAOM in children and marketed in a commercial product (Rinogermina®, DMG Italia Srl, Pomezia,

Italy) with the strain *Streptococcus oralis* 89a. We hypothesized that it could be used as an oral probiotic employed in the reestablishment of the dysbiosis. Thus, we started with the further characterization of this strain, firstly screening the probiotic properties above mentioned against oral pathogens chosen by supporting our background experience. It has shown no cytotoxic effect on HEp-2, an extraordinary ability to adhere to the host epithelium and it has a very high value of 81.8% auto-aggregation, which justifies its permanence and predominance in the respiratory microbiota. It has significantly co-aggregated with pneumococci and *S. pyogenes spp.*, even if the co-aggregation with the last one can't be considered significative. *S. salivarius* 24 SMBc, when inoculated at concentrations of 10^9 CFU/ml, determined a significative reduction of pneumococci CFU counts. The mechanism behind this is not yet well know, but this reduction could be due to competition of binding sites and to the mutual exclusion by co-aggregation. *S. salivarius* 24SMBc not only determined a reduction of pneumococci CFU but showed also a good and significative co-aggregation with them. Conversely, the results obtained for *S. pyogenes* spp. showed no significant reduction of adhesion in the presence of *S. salivarius* 24SMBc and none significative co-aggregation, probably the the *S. salivarius* inhibitory activity in this case could be linked to the production of BLIS and not for competition of binding sites. In fact, *S. pyogenes* is a producer of Salivaricin A1 (SalA1), but the SalA peptide sensing system does not discriminate between subtypes of SalA. Thus, SalA1 produced by *S. pyogenes* might stimulate production of SalA by commensals strains as *S. salivarius*, leading to modulation of the number of *S. pyogenes* cells and determining a way of regulation for the coexistence of streptococcal populations in the oral microbial community (Upton et al. 2001). *S. salivarius* 24 SMBc could represent an oral probiotic candidate and also an alternative to antibiotics used in the prevention and treatment of oral infections. The extraordinary ability to inhibit pneumococcal colonization represents a good property not only for respiratory

infection but for other oral diseases, since pneumococci inhabit the oral cavity and are considered "*microbial reservoir*" for the transmission from teeth to another district.

Secondly, this study provides preliminary structural and functional genomic information of *S. salivarius* 24SMBc. The whole genome sequencing showed that it is free of streptococcal virulent factors and confirmed the detection of a blp (bacteriocin-like protein) locus with characteristics similar to peptides produced by *S. pneumoniae*. Therefore, the peptide homology shared by both microorganisms may explain the strong inhibitory activity of *S. salivarius* 24SMBc against this pathogen and thus its crucial role in interspecies competition within the nasopharynx (Santagati et al. 2015; Santagati, Scillato, and Stefani 2018). The analysis of the complete genome of S. *salivarius* 24SMBc can help in further understanding the beneficial properties and improve the identification of genes implicated in the mechanism of competition like adherence to the surface and the release of metabolites. In particular, the analysis at the genome level may facilitate the selection and application of strain for specific biotechnological purpose in several oral related diseases. To conclude, future goals will allow validating *S. salivarius* 24SMBa as a therapeutic strategy against peri-implant and periodontal pathogens, through a wider sample of pathogens and clinical trial.

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