Research Article

Functional Active Microbiome in Supragingival Biofilms in Health and Caries

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Abstract

The oral microbiome is unique at inter and intra- individual levels at various sites due to physical and biological factors. This study aimed to compare the bacterial composition of supragingival biofilms collected from enamel sites with different caries activity, from active and inactive-caries subjects, and from caries-free subjects. Twenty-two individuals (aged between 13 and 76 years old; med=23.5 years old) were allocated into three groups: caries-active (CA) (n=10); caries-inactive (CI) (n=6); and, caries-free (CF) (n=6). From the caries-active group, 3 sites were sampled: caries-active (ANCL), caries-inactive (INCL), and sound enamel surface (S). From the subjects of the caries-inactive group, biofilm from caries-inactive lesion was collected (INCL); while for the caries-free subjects (S), a pool of biofilm from sound enamel surfaces were sampled. The total RNA was extracted, cDNA libraries were prepared and paired-end sequenced (Illumina HiSeq 3000). The final dental biofilm samples analysed from CA was 16 (ANCL-CA=6, INCL-CA=4, S-CA=6); from CI, three (INCL-CI=3); and from CF, six (S-CF=6) (some samples were lost by insufficient genetic material). Read sequences were processed and analysed using MG-RAST (Metagenomics Analysis Server). High-quality sequences (3,542,190) were clustered into operational taxonomic units (OTUs) (97% identity; SILVA SSU), representing 915 genera belonging to 29 phyla (higher abundant: *Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria*). The presence of a core microbiome was observed (123 shared genus). The alpha diversity analysis showed less bacterial diversity in disease (S-CA) compared to health (S-CF). The dominant genera included *Actinomyces, Corynebacterium, Capnocytophaga, Leptotrichia, Veillonella, Prevotella, Streptococcus, Eubacterium*, and *Neisseria*. *Veillonella* and *Leptotrichia* were related with disease, and *Prevotella* with health. *Corynebacterium, Capnocytophaga,* and *Actinomyces* clustered together presenting high abundance in health and disease. The Metric Multidimensional Scaling Ordination analysis shows that sites from active subjects (ANCL-CA, INCL-CA and S-CA) are closer to each other than either INCL-CI subjects or S-CF subjects. In conclusion, supragingival bacterial communities presented intra-individual similarities, but inter-individual diversity and difference in bacterial composition reveal that the subject's caries activity status matters more than sites.

Introduction

The oral cavity harbours diverse niches for microbial colonization, supporting distinct site-specific microbial consortia combination, which responds and reflects to ecological determinants at each site, [Marsh, 1994; 2018; Aas et al., 2005]. Investigating the total microbial population, which is metabolically active in dental health and disease sites is necessary to understand mechanisms leading to dysbiosis [Benítez-Páez et al., 2014; Henne et al., 2016; Nascimento et al., 2017]. Although the microbial composition of these biofilms remains stable over time, an increase in dietary fermentable carbohydrate induces a shift in the microbial ecosystem leading to low pH in the biofilm environment, and resulting in dental demineralization [Loesche, 1986; Marsh, 1994; Paes Lemes et al., 2006, Bjørndal et al., 2019]. Microbial homeostasis can be restored if changes occur in the dental plaque ecosystem, for instance by increasing pH to neutral conditions which could result in dental remineralization [Marsh, 1994; Paes Lemes et al., 2006]. Both active and inactive conditions, as well as non-affected sites, can be found in the same subject [Marsh, 1994; 2018; Aas et al., 2005; Filoche et al., 2010; Simón-Soro et al., 2014; Arweiler and Netuschil, 2016].

Culture-based studies of dental caries [Orland et al., 1954; Fitzgerald and Keyes, 1960; Gibbons et al., 1964; Krasse, 1966; Krasse et al., 1967; Loesche et al., 1975; Loesche, 1986] have failed to explain the aetiology of the disease. Organism interactions are essential to understand ecosystem dynamics of oral microbial communities are more than the sum of the individual species [Mark Welch et al., 2016; Marsh, 2018] justifying biofilms communities' studies. Currently, culture independent molecular biology methods, and recently, next-generation sequencing technologies (NGS) are improving knowledge about diversity, composition and functional aspects of the dental biofilms microbial communities in its natural habitats [Simón-Soro et al., 2013; 2014; Benitez-Páez et al., 2014; Johansson et al., 2016; Xiao et al., 2016; Eriksson et al., 2017; Espinoza et al., 2018; Richards et al., 2017; He et al., 2018; Jiang et al., 2019; Schoilew et al., 2019]. Moreover, the RNA sequencing (RNA-Seq) approach allows the functional characterization of a complex microbial community (microbiome) under a specific condition, revealing both the composition of the metabolically active microbiota and the gene expression levels which provide an insight into the ongoing metabolic pathways within the microbial community [Jiang et al., 2016; Hrdlickova et al., 2017].

Studies have demonstrated substantial differences in biofilm microbiota composition in caries lesions, showing a community stability disruption, with acidogenic and acid-tolerant species enrichment [Aas et al., 2008; Gross et al., 2010; Benitez-Páez et al., 2014; Simón-Soro et al., 2014; Johansson et al., 2016; Xiao et al., 2016; Eriksson et al., 2017; Espinoza et al., 2018; Richards et al., 2017; Jiang et al., 2019; Schoilew et al., 2019]. According to Marsh [2018] there is specificity in terms of caries aetiology biochemical function, despite the lack of bacterial name specificity; but organisms are highly relevant to understand the structure, function, and dynamics of the members in a microbial consortium [Mark Welch, 2016]. Considering the dental caries polymicrobial aetiology and complex dental biofilm ecosystem [Simón-Soro et al., 2014; Mark Welch, 2016; Xiao et al., 2016; He et al., 2017], and that microbiota composition is not the same on different surfaces [Aas et al., 2008; Simón-Soro et al., 2014; Richards et al., 2017; Espinoza et al., 2018; Schoilew et al., 2019], we proposed to characterize the functional active microbiome composition and diversity of supragingival biofilms in caries-free, caries-inactive, and in three different dental conditions from caries-active subjects.

Subjects, Materials and Methods

Study Population and subjects

Subjects (19.3±29.6 years old) were selected at the Federal University of Rio Grande do Sul (UFRGS, Porto Alegre, Brazil). This research is a collaboration between the Biochemistry and Microbiology Oral Laboratories (LABIM/UFRGS) and the Division of Oral Biology, at the University of Leeds, United Kingdom. The inclusion criteria were the absence of using antimicrobial agents for at least two months before sample collections, and complete permanent dentition (age≥12). In the first appointment, caries diagnostic was performed by visual-tactile method (Nyvad criteria), after dental prophylaxis, isolation (cotton rolls) and teeth air-drying, by two calibrated examiners (LDE and NDT). The lesion was diagnosed as follow: Active non-cavitated lesion (ANCL): whitish/yellowish opaque surface, exhibiting a chalky or white appearance; the surface felt rough by probing; Inactive non-cavitated lesion (INCL): shiny and felt smooth surface on gentle probing, and colour varying from whitish to brownish or black [Kidd and Fejerskov, 2004]. After caries diagnosis, subjects were allocated in the following groups, according to their caries activity (Figure 1): CA (caries-active subjects) (n=10): DMF-T/S $\geq$ 1, presenting at least one active non-cavitated caries lesions; CI (caries-inactive subjects) (n=6): DMF-T/S $\geq$ 1, but presenting only inactive non-cavitated caries lesions and any active caries lesion; CF (caries-free subjects) (n=6): DMF-T/DMF-S=0.

Sample collection and storage

The subjects were not treated with topical antimicrobial agents, and any recommendation to change diet or dental hygiene method was done at the first appointment. After one week, subjects returned to collect dental biofilm. They refrained from teeth brushing for 12 hours, and from eating and drinking for at least one-hour prior sample collection. Samples of supragingival biofilm (SB) were collected with sterilized Gracey curette. From CA, 3 conditions were sampled: sites presenting ANCL; sites presenting INCL; and sound enamel surfaces (S) (samples were pooled from enamel surfaces/lesions with the same characteristics from the same subject); from CI, only INCL were collected; and from CF, a pool of biofilm from S were sampled. The samples were immediately treated into 1 mL RNA stabilization solution (RNAlater, Ambion Inc., Cambridgeshire, UK) at room temperature (until 24 hours); centrifuged (5 minutes; 10.000 rpm); pelleted, and frozen at -80^{o}C until further processing.

RNA Extraction and Quantification

UltraClean® Microbial RNA Isolation kit (Mo-bio, San Diego, USA; DNase digestion Qiagen, Inc) as described elsewhere [Damé-Teixeira et al., 2019], with a previous treatment with Lysozyme (10 minutes; 37^{0}C). RNA measurement was performed (Quant-iTTM RiboGreen® RNA Reagent and Kit; Invitrogen, Ltd.; spectrofluorometer with excitation ~480 nm, and emission ~520 nm). Samples with total RNA concentration less than 30 ng/uL were excluded from analysis.

Library preparation and RNA-sequencing

The True Seq® Sample Preparation Guide, Low Sample (LS) Protocol Illumina (Illumina, Inc., San Diego, CA) was used for genomic library preparations and Agilent Technologies 2200 TapeStation was used for genomic library quality validation. The double strand DNA was quantified with Quant-iT^{TM}

PicoGreen® dsDNA Kit (Turner BioSystems, Inc., CA) before sequencing at the Illumina HiSeq3000 (Illumina Inc.) (2x150bp).

Bioinformatic analysis

The sequences were filtered, excluding the ones <150 bp and an expected error >0.5. The resulting FASTA files were submitted to Metagenomics RAST Server (MG-RAST) [Meyer et al., 2008], that generates taxonomic and functional categories graphs. The MG-RAST pipeline options included a sequence data trimming to remove low quality reads (phred score=15) [Cox et al., 2011]. Artificial replicate sequences produced by sequencing artifacts were removed (dereplication) [Gomez-Alvarez et al., 2009]. A screening to remove human host sequences using DNA level matching with bowtie was selected to *H. sapiens*, NCBI v36 [Langmead et al., 2009]. A phylogenetic reconstruction was computed from a set of hits against SILVA SSU database, considering 97% of similarity to genus taxonomic level.

Statistical analysis

Kruskall-Wallis test, post-hoc Bonferroni and Nemenyi, compared the average number of reads for all groups. Friedman test, post-hoc Nemenyi, compared the composition profile and bacterial diversity among CA sites (paired analysis). Mann-Whitney U test with a Benjamini-Hockenberg correction for multiple comparisons compared INCL-CA versus INCL-CI; S-CA versus S-CF; and CA versus CF subjects. The significance level was 95%. Richness estimators (Chao-1; ACE: Abundance-based Coverage Estimator), and indexes (Shannon-Wiener; Simpson; Pielou-Shannon) were used for alpha diversity analysis. The K-means clustering beta-diversity analysis compared healthy and diseased conditions, and metric Multidimensional Distance Scaling (mMDS/PCoA: Principal components analysis) using average of log2 fold change (avg(logFC)) compared all groups. A Venn diagram was generated using the gplots package (RStudio). Shared genera present in all subjects (100% core threshold) were defined as the core microbiome (Xiao et al., 2016). All analyses were conducted in RStudio (version 3.5.0) using packages for ecological data analysis (Vegan, BiodiversityR, Phyloseq).

Results

The characteristics of the included subjects are shown in Table 1. From 22 subjects, a total of 42 SB samples were collected to analysis. However, lost of samples occured due to insufficient total RNA recovered, low quality of the library prep or insufficient reads recovered (Figure 1).

A good coverage >97% was obtained in the sequencing. The rarefaction curve considering the average of sequences count can be observed is shown in the supplementary Figure 1. After data trimming and quality filtering of reads by removing artifacts an average of 3,542,190 high-quality sequences with 147.7±3.7 base pairs (bp) were recovered, corresponding to 16.48% of sequences generated.

There were recovered reads from domain Archaea (mean= 2.76 reads), Bacteria (mean= 8602231.96 reads), Eukaryota (mean= 72118.12 reads), Viruses (mean= 758.28 reads) and unclassified sequences (mean= 65841.56 reads) (supplementary Figure 2). Bacteria were the most abundant domain.

The $\geq$ 1% relative abundance of OTUs to phylum are detailed in Table 2. Of 29 phyla recovered (supplementary Figure 3 and supplementary Table 1), *Bacteroidetes* was less abundant in INCL-CI compared to INCL-CA ($p<0.05$) (Table 2; supplementary Table 1). We recovered 915 genera, 18

representing >1% relative abundance (Table 3); *Capnocytophaga* was less abundant in INCL-CI compared to INCL-CA ($p<0.05$) (Table 3). Also, 123 genera were shared in all sites and subjects, revealing a common core microbiome (Figure 2; supplementary Table 2). The low abundant microbiome (≥0.1% and <1%) corresponded to 74 genera (8.09%).

Paired analysis from sites of the CA (ANCL, INCL and S) revealed *Actinomyces* genera higher abundant in CA-ANCL (relative abundance = 20.83%) compared to CA-S (relative abundance = 9.63%) (Friedman test; Nemenyi post-hoc; $p<0.05$). *Capnocytophaga* showed significative higher abundance in CA-INCL (relative abundance = 14.88%) compared to CA-ANCL (relative abundance = 4.82%) (Friedman test; Nemenyi post-hoc; $p<0.05$). No statistical difference was observed for other genera in these group (CA), considering relative abundance cutoff point of 0.1% (data not showed).

The Chao1, ACE (Abundance-based Coverage Estimator) did not reveal difference in OTU's richness between the sample groups (Figure 3). Shannon-Wiener, Simpson, and Pielou-Shannon alpha diversity indices revealed similar intrapersonal bacteriome among ANCL, INCL and S sites from CA subjects; and, between INCL-CA and INCL-CI groups (Figure 4). The bacteriome between sound sites from CA (S-CA) and CF (S-CF) subjects, and CA and CF groups was less diverse in diseased condition and showed less evenness compared to the healthy ones (Figure 5).

Sites from CA were closer each other compared to CI and CF (Figure 6). The MDS1 explain 37.4% of the variation observed, and MDS2 explain 28% of the variation. However, the samples did not form well-separated clusters corresponding to the five groups, suggesting that the bacterial structures in healthy and caries groups were similar. *Corynebacterium, Capnocytophaga,* and *Actinomyces* clustered together presenting high abundance in health and disease conditions; *Veillonella* was associated with disease, and *Prevotella* to health (supplementary Figure 4).

Functional analysis revealed 2,467 unique KO numbers found for all the genes. In the CA, 2,364 unique KO numbers were expressed (ANCL-CA=1,877; INCL-CA=1,464; S-CA=2,100); in the CI, 1,325 from INCL; and, in the CF, 1,662 (supplementary Tables 3 and 4). Essential components of the glycolytic pathway, as glyceraldehyde 3-phosphate dehydrogenase, enolase, formate C-acetyltransferase, fructose-bisphosphate aldolase and phosphoglycerate kinase, were among highly expressed genes in dental biofilm microbiome from active group (supplementary Table 4).

Discussion/Conclusion

This study revealed, for the first time, the metabolically active bacteriome from whole SB in dental health and enamel caries. We demonstrate the bacteriome composition and diversity from caries-active, caries-inactive, and caries-free subjects. Our study confirms that high bacterial diversity in the biofilm samples, identified from RNA-seq analysis (RNA-based) is related to live organisms in SB and not due to dead/inactive species, highlighting polymicrobial dental caries aetiology, where multispecies microbial consortia are metabolically active in lesions [Simón-Soro et al.; 2014]. The functional analysis of unique KO numbers confirms a functionally active state of the microbiome in the dental biofilms (supplementary data).

An average of 21,855,554 high-quality sequences were obtained from all groups, which was higher than previously reported [Benitéz-Páez et al., 2014; Simon-Soro et al., 2014]. The good coverage of

>97% suggested that sequencing depth was sufficient to full SB diversity estimation, indicating that extremely low rare genera abundance could be detected. We observed few >1% relative abundance genera in all groups, and a great portion of very low abundant genera. The functional redundancy feature of microbiota, inherent to related species, may explain the human microbiome inter-individual variability. The interpersonal microbial composition variability extension, within and across varied dental plaque niches, is largely uncharacterized, but these factors are likely to directly contribute to the disparate results obtained from various studies examining dental caries [Benitéz-Páez et al., 2014; Simon-Soro et al., 2014; Mark Welch et al., 2016, Xiao et al., 2016; Espinoza et al., 2018; Wolff et al., 2019].

The abundance differences observed in microbial communities may result from real differences among individuals, fluctuations within a single individual over time, or a combination of the two [Mark Welch et al., 2016]. The dental plaque "hedgehog" structured consortia described by Mark Welch et al. [2016], indicate that, its structure composition and organisms across many individuals are highly relevant to understand organization, function, and dynamics of consortium members. In our research, 123 genera formed a "core microbiome". Its existence was first proposed by Turnbaugh et al. [2007] and referred to organisms, genes, or functions shared by all or most individuals in a given human habitat, such as the oral cavity. Considering the polymicrobial aspect of caries we can suggest that a shared community should be modulated during metabolic alterations in host and in local niche driving to healthy or diseased conditions, corroborating with the ecological plaque hypothesis proposed by Marsh [1991] and the extended concept proposed by Takahashi and Nyvad [2011].

Our study supports that supragingival ecological niche is a highly selective environment once we observed four phyla higher than 10% abundance, among 29 phyla in these bacteriomes [Keijser et al., 2008; Benitéz-Páez et al., 2014; Xiao et al., 2016; Eriksson et al., 2017; Jiang et al., 2019]. The dominant phyla were *Actinobacteria, Firmicutes, Bacteroidetes* and *Fusobacteria*. *Bacteroidetes* was higher abundant in INCL from CA than CI subjects. *Bacteroidetes* had been retrieved among abundant phyla from dental plaque microbiome, but show a variable abundance, sometimes first or second most abundant [Johansson et al., 2015; Xiao et al., 2016; Eriksson et al., 2017; Jiang et al., 2019], sometimes moderate or low abundant [Gross et al., 2010; He et al, 2017], suggesting a high genera and species adaptability that make up this phylum.

Actinomyces, Corynebacterium, and *Capnocytophaga* presented >10% relative abundance from all 915 recovered genera. *Capnocytophaga* was more abundant in INCL from CA than CI subjects, indicating that this genus was the most abundant of *Bacteroidetes* phyla. In DNA-based approaches it has been highly retrieved from health conditions [Aas et al., 2008; He et al., 2017; Jiang et al., 2019]. Eriksson et al. [2018] demonstrated that dental plaque members, including *Capnocytophaga*, can be disease-related in microbiomes with extreme low or no detectable *Streptococcus mutans*, suggesting microbial community mutualistic relationship. When a metabolite is used by different microorganisms, a metabolic communication that drives a positive or negative regulatory effect into the microbiome is generated [Hojo et al., 2009]. The fact that biofilms are found in healthy and diseased subjects, the presence of commensal bacteria, playing important role for the microbiome equilibrium, is suggested. This fact can be an important explanation for high abundance of *Capnocytophaga* in both diseased and healthy oral conditions. In the RNA-based study from Benitéz-Páez et al. [2014], *Corynebacterium, Actinomyces* and *Neisseria* were the most abundant genera in a community from a 24-hour dental

plaque in one dental healthy subject. Our RNA-based study also recovered *Corynebacterium* with high abundance in health subjects. March Welch et al. [2016] suggest that *Corynebacterium* is the foundation taxon of dental plaque bacterial consortium, structuring the environment, creating habitat for other organisms and nucleating a plaque-characteristic consortium. Nyvad and Fejerskov [1987] observed scattered filamentous cells oriented perpendicularly to the primarily coccus-covered surface at 24-hour biofilm and a mixed community of abundant filamentous organisms by 48 hours, suggesting that colonization with *Corynebacterium* may take place around the 24-hour stage in plaque development. Our samples recovered high abundance of *Corynebacterium* from all sites, and can represent a well-established microbial community, once the subjects were oriented to remain, at least, 12 hours without dental hygiene. *Actinomyces* was highly represented in ANCL than INCL in CA subjects. Benitéz-Páez et al. [2014] found *Actinomyces* overrepresented in healthy conditions from RNA-based community. Eriksson et al. [2018] found *Actinomyces* related with disease in microbiome with extreme low or no detectable *S. mutans*. These results can indicate that these genera have an important capability for metabolic modulation, adapting itself in different host conditions. *Streptococcus* and *Actinomyces* are among the early colonizers in dental biofilm formation [Keijser et al., 2008; Dige et al., 2009; Marsh and Zaura, 2017]. Mark Welch et al. [2016] demonstrated that *Actinomyces* can be found near the "hedgehog" structure base, and that *Corynebacterium* attaches in sites with pre-existent biofilm consisting of *Streptococcus* and *Actinomyces*, and not directly on the tooth surfaces. This observation can explain the *Actinomyces* high abundance in both, diseased and healthy conditions.

The richness estimators Chao1 and ACE did not differ among all sites and subjects, suggesting a similar number of genera recovered from all sites. The richness indexes, Shannon-Wiener, Simpson and Pielou-Shannon revealed similar alpha diversity among sites from CA subjects, and from INCL from CA and CI subjects. However, sound sites from CA subjects were less diverse and presented less evenness than CF-S. Higher diversity has been described in healthy sites by several ecologic studies [Gross et al., 2010; Benitez-Paez et al., 2014; Simón-Soro et al., 2014; Xiao et al., 2016; Schoilew et al., 2019; Wolff et al., 2019]. The lowering of the pH, from lactate produced by acid-producing species, could lead to suppression of acid-sensitive species and overgrowth of acid-tolerant species, resulting in decreased bacterial diversity in supragingival plaques as caries progresses, as well as a decreasing number of species capable of surviving harsh conditions [Gross et al., 2010]. Bacteriome in diseased condition presented significantly less diversity, higher dominance of rare genera, and showed less evenness than healthy ones, suggesting interpersonal variability.

In our study, *Veillonella* was associated with disease, and *Prevotella* to health conditions. *Prevotella* and *Veillonella* species could be recovered from both healthy and diseased conditions [Gross et al., 2012; Richards et al., 2017; Wolff et al., 2019]. These findings support that the degree of dominance of pathogens depends on environmental factors during progression of the disease and not only of the disease status [Wolff et al., 2019]. *Corynebacterium*, *Capnocytophaga*, and *Actinomyces* clustered together presenting high abundance in both conditions. Utter et al. [2016] described plaque microbiome characterized by a community stability showing variability in the relative abundance of members of the community and between individuals and over time. They found *Corynebacterium, Capnocytophaga, Fusobacterium, Actinomyces* and *Streptococcus* relatively abundant and constant among individuals. In the "hedgehog" structure, Mark Welch et al. [2016] observed nine taxa as regular participants, including these above mentioned. The same nine genera presented higher abundance in

our supragingival microbiome analysis, proving that our analysis obtained a good picture of supragingival active microbiome.

We observed a tendency for CA sites cluster together and distant from CI and CF subjects. The mMDS (betadiversity analysis) could explain 37.4% of distance among groups, by the x-axis, and 28% by the y-axis, exhibiting very similar communities' structures. However, it is important to analyse the composition of genera into different microbiomes. Jiang et al. [2019] found a difference in niches but did not observe clear differences among dental microbiome from active and caries free subjects. Richards et al. [2017] observed more similar CA communities in different site-specific conditions than communities from CF subjects. The authors highlight the concept of plaque communities as a part of a larger ecosystem and that the changes in the structure of one community may eventually affect another, reinforcing the importance of site-specific studies.

Conclusion

We concluded that the functional active microbiome in supragingival bacterial community profiles show intra-individual similarities but were more diverse at inter-individual levels. The differences in bacterial composition may indicate that the individual's healthy/diseased status matters more than sites. We suggest that alterations from supragingival microbial communities should be analysed, from a longitudinal way, in caries diseased subjects for a better comprehensive understanding of this ecological process.

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Statement of Ethics

This study had Ethics Committee of Federal University of Rio Grande do Sul (UFRGS) approval (CAAE 56583316.8.0000.5347). All subjects provided informed consent or assent participation (Resolution 466/2012, National Commission of Ethic in Research, Department of National Health, Brazil). All patients received dental treatment at UFRGS.

Conflict of Interest Statement

"The authors have no conflicts of interest to declare."

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Author Contributions

DJ. Corralo, contributed to design, data analysis and interpretation, drafted and critically revised the manuscript; LD. Ev, contributed to conception, design, samples acquisition, data analysis, and critically revised the manuscript; N. Damé-Teixeira, contributed to conception, design, samples acquisition, data analysis, interpretation, and critically revised the manuscript; M. Maltz, contributed to conception, design, data analysis, interpretation, drafted and critically revised the manuscript; RA. Arthur, contributed to conception, design, samples acquisition, samples, and critically revised the manuscript; T. Do, contributed to conception, design, data analysis, and critically revised the manuscript; CCF. Parolo, contributed to conception, design, samples acquisition, data analysis, interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure Legends

Fig. 1. Flow chart showing the number of subjects enrolled and allocated to groups.

Fig. 2. Venn diagram showing shared and unique OTU abundances at 97% identity, for genus taxonomic level, among supragingival biofilm from caries active subjects (ANCL-CA: active non-cavitated lesion; INCL-CA: inactive non-cavitated lesion; S-CA: sound); caries inactive subjects (INCL-CI: inactive non-cavitated lesion); and caries-free subjects (S-CF: sound).

Fig. 3. Richness estimators Chao 1 and ACE (Abundance-based Coverage Estimator) among different dental surfaces from caries-active (CA) subjects (ANCL: active non-cavitated lesions; INCL: inactive non-cavitated lesions; S: sound surfaces) (A); between inactive sites (INCL) from CA and caries-inactive (CI) subjects (B); and between CA and caries-free (CF) subjects (C). Mann-Whitney U test, 95% confidence level.

Fig. 4. Alpha diversity indexes Shannon-Wiener, Simpson, and Pielou-Shannon among different dental surfaces from caries-active (CA) subjects (ANCL: active non-cavitated lesions; INCL: inactive non-cavitated lesions; S: sound surfaces) (A); and, between inactive sites (INCL) from CA and caries-inactive (CI) subjects (B). Mann-Whitney U test, 95% confidence level.

Fig. 5. Alpha diversity indexes Shannon-Wiener, Simpson, and Pielou-Shannon between sound sites from caries-active (S-CA) and caries-free (S-CF) subjects (A); and, from caries-active (CA) and caries-free (CF) groups (B). Mann-Whitney U test, 95% confidence level.

Fig. 6. Metric Multidimensional Scaling Ordination (mMDS/PCoA) among supragingival biofilms communities from all groups. Each sample is represented by a diamond. Black square represents the active non-cavitated lesions from caries active subjects (ANCL-CA). Horizontal black lines diamond represents the inactive non-cavitated lesions from caries active subjects (INCL-CA). Chess circle represents the sound surfaces from caries active subjects (S-CA). Black diamond represents the inactive non-cavitated lesions from caries inactive subjects (INCL-CI). Gray circle represents the sound surfaces from caries-free subjects (S-CF). mMDS using average of log2 fold change (avg(logFC).