Received: 19 August 2020 | Revised: 7 April 2021 | Accepted: 14 April 2021

DOI: 10.1111/odi.13883

ORIGINAL ARTICLE

ORAL DISEASES Leading in Oral, Maxillofacial, Head & Neck Medicine WILEY

Microbiomes in supragingival biofilms and saliva of adolescents with gingivitis and gingival health

Roland Wirth[1,2] | Gergely Maróti[2] | Lídia Lipták[3] | Mónika Mester[3] | Alaa Al Ayoubi[3] | Bernadett Pap[2] | Melinda Madléna[3] | János Minárovits[4] | Kornél L. Kovács[1,4]

[1]Department of Biotechnology, University of Szeged, Szeged, Hungary

[2]Institute of Plant Biology, Biological Research Centre, Szeged, Hungary

[3]Department of Orthodontics and Pediatric Dentistry, Faculty of Dentistry, University of Szeged, Szeged, Hungary

[4]Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Szeged, Hungary

Correspondence
Kornél L. Kovács, Department of Biotechnology, University of Szeged, Szeged, Hungary.
Email: kovacs.kornel@bio.u-szeged.hu

Funding information
European Regional Development Fund, Grant/Award Number: GINOP-2.3.2-15-2016-00011, GINOP-2.2.1-15-2017-00081, GINOP-2.2.1-15-2017-00033 and EFOP-3.6.2-16-2017-00010; National Research, Development and Innovation Office (NKFIH), Hungary, Grant/Award Number: PD132145 and FK123899; Hungarian Academy of Sciences

Abstract

Background: Important alterations exist in the microbiomes of supragingival biofilm and saliva samples from adolescent patients developing induced or spontaneous gingivitis relative to healthy controls. These and the relationships to dental health are not fully understood yet.

Subjects and Methods: Supragingival biofilm samples (n = 36) were collected from the teeth of 9 adolescents with gingivitis induced by orthodontic appliances, as well as dental plaques (n = 40) from 10 adolescents with spontaneous gingivitis, in addition to similar samples (n = 36) from 9 healthy controls. The bacterial metagenomes were analyzed by 16S rRNA gene amplicon sequencing. Salivary microbiomes of the same persons were characterized by shotgun metagenome sequencing. The data sets were examined using advanced bioinformatics workflows and two reference databases.

Results: The composition and diversity of bacterial communities did not differ extensively among the three study groups. Nevertheless, the relative abundances of the genera *Fusobacterium*, *Akkermansia*, *Treponema*, and *Campylobacter* were prominently higher in gingivitis patients versus controls. In contrast, the genera *Lautropia*, *Kingella*, *Neisseria*, *Actinomyces*, and *Rothia* were significantly more abundant in controls than in either of the two gingivitis groups.

Conclusions: The abundance pattern of certain taxa rather than individual strains shows characteristic features of potential diagnostic value. Stringent bioinformatics treatment of the sequencing data is mandatory to avoid unintentional misinterpretations.

KEYWORDS
amplicon sequencing, gingivitis in adolescents, microbiome, saliva, shotgun sequencing, supragingival biofilm

1 | INTRODUCTION

Gingivitis is a non-destructive, reversible periodontal disease highly prevalent in various human populations (Burt, 2005; Califano, 2003). In most cases, gingivitis develops as an inflammatory host response elicited by the accumulation of bacterial biofilm, called dental plaque, on the tooth surface (Armitage, 1999; Burt, 2005; Theilade et al., 1966; Trombelli et al., 2018). The alterations of the gingival

tissue and the changes in the levels of inflammatory markers usually disappeared after removal of the supragingival biofilm in experimental gingivitis models (Eberhard et al., 2013; Offenbacher et al., 2010). In susceptible individuals, however, persistent gingivitis may lead to periodontitis, a disease associated with irreversible tissue destruction and tooth loss (Könönen et al., 2019; Lang et al., 2009; Schätzle et al., 2003).

Differentially distributed bacterial taxa have been identified in supragingival plaques collected from adults with experimental or naturally occurring gingivitis and healthy individuals, respectively (Huang et al., 2011; Huang et al., 2014, 2016; Kistler et al., 2013; Shaw et al., 2016). Similarly, changes in the relative abundance of various bacteria were observed in subgingival plaque samples from gingivitis-affected teeth versus non-affected teeth (Deng et al., 2017; Park et al., 2015; Schincaglia et al., 2017).

Although local plaque accumulation plays a decisive role in the induction of gingivitis, systemic factors may also modulate the gingival inflammatory response (Murakami et al., 2018). In an experimental gingivitis model, two subpopulations of healthy adults could be distinguished based on the individual variability of the plaque-induced inflammatory response (Trombelli et al., 2004). Thus, important differences in clinical parameters of gingival inflammation between high-responder and low-responder individuals were reported, which were characterized by similar amounts of dental plaque deposits (Tatakis & Trombelli, 2004; Trombelli et al., 2004). Metabolic, genetic and environmental factors may affect the bidirectional interactions between the gingival tissue and the bacterial biofilm (Tatakis & Trombelli, 2004). Smoking suppressed gingival bleeding on probing (BOP), a clinical sign of gingival inflammation in a related study (Dietrich et al., 2004). Other environmental factors may also contribute to an individual's higher susceptibility to gingivitis (Dietrich et al., 2006). Salivary steroid hormones were also suggested as potential modulators of gingivitis prevalence, especially during adolescence (Morishita et al., 1988).

The propensity to develop gingivitis increases gradually from early childhood to adult age (Marsh, 2005; Matsson, 1978). Plaque-induced chronic gingivitis is highly prevalent among adolescents, and even relative small amounts of plaque may elicit an inflammatory reaction in this age group (12–19 years; Murakami et al., 2018; Oh et al., 2002). It was also observed that the chance of developing periodontitis following gingival inflammation is lower in adolescents, compared to adults (Al-Ghutaimel et al., 2014).

Orthodontic treatment with fixed appliances is frequently associated with increased presence of oral pathogens, for example, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Treponema denticola*, that are capable of triggering immune responses in the oral cavity (Gong et al., 2011). In a related study, *P. intermedia*, *T. denticola*, *Fusobacterium nucleatum*, and *Campylobacter rectus* were identified in increased abundance in gingival plaques after 3 months of placement of the braces although a general rearrangement of the core gingival microbiota apparently did not take place (Guo et al., 2017).

Overall, the published depictions of microbial communities associated with gingivitis are incongruent, partly due to methodological differences applied in the various laboratories and the oral hygiene habits of the subjects (Belstrøm et al., 2018). Therefore, the utilization of up-to-date sequencing and bioinformatics workflows is warranted to minimize systemic oversights.

For this reason, and because of the paucity of metagenomics data related to the supragingival biofilm communities of adolescents, we analyzed the microbiomes in supragingival plaque and saliva samples of 15–18 years old patients displaying symptoms of either orthodontic appliances induced or spontaneous gingivitis, and compared them to the microbial communities of age-matched healthy controls.

2 | MATERIALS AND METHODS

2.1 | Study design and recruitment of participants

The study participants were recruited from the population of suitable adolescents visiting the Faculty of Dentistry, University of Szeged. None of the participants had known chronic systemic illnesses, and none were treated with antibiotics at least 6 months prior to sampling. All subjects declared having no habitual smoking or drinking dependencies.

The subjects were divided into 3 study groups as follows. Group A: induced gingivitis patients wearing fixed metal braces (nine patients, three males and sic females, mean age 16.9 years, range: 15–18 years); Group B: spontaneous gingivitis patients diagnosed with plaque-induced gingivitis (10 patients, seven males and three females, mean age 17 years, range: 16–18 years); Group C: healthy individuals without the symptoms of gingivitis (nine subjects, four males and five females, mean age 17 years, range: 15–18 years). The subjects' characteristics, including the Modified Gingival Indices (MGI) indicating the gingival condition (Lobene et al., 1986, 1989), are summarized in Table S1. Fixed orthodontic appliances were placed on the labial tooth surfaces (more than 1.5 mm from the gingival margin) according to the professional guidelines with an acid-etch composite system (Transbond XT, 3 M Unitek), reported previously (Lipták et al., 2018).

2.2 | Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of the University of Szeged, Hungary. Signed informed consent was obtained from each adult participant enrolled into the study at the Department of Orthodontics and Pediatric Dentistry University of Szeged, Hungary. In case of study participants younger than 18 years of age, signed informed consent was obtained from one of the parents.

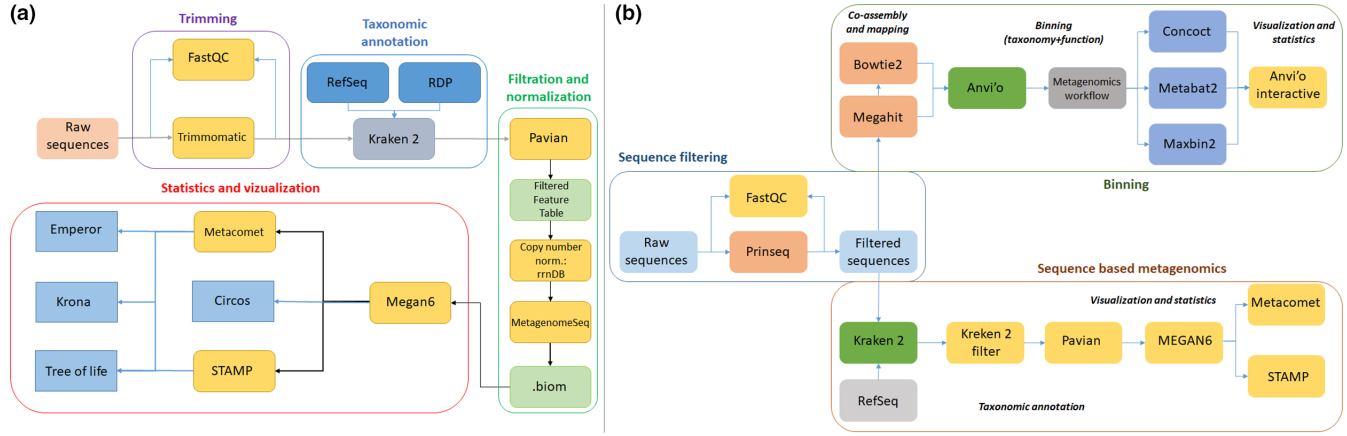


FIGURE 1 (a) Bioinformatics pipeline for amplicon sequence analysis of supragingival biofilm samples. (b) Bioinformatics pipeline for metagenome sequencing of saliva samples

2.3 | Dental examination and sample collection

Each participant underwent dental examination by a qualified staff dentist of the Department of Orthodontics and Pediatric Dentistry. All participants were repeatedly instructed about proper oral hygiene during the orthodontic treatments. The gingival condition of each participant was assessed as described by Lobene et al., using the non-invasive Modified Gingival Index, MGI (He et al., 2018; Lobene et al., 1986, 1989).

Unstimulated whole saliva samples were collected from the participants by the simple drooling method (Bellagambi et al., 2020). The samples were aliquoted and stored at −80°C. Supragingival plaque (biofilm) samples were taken using sterile paperpoints from the surface of four teeth with inflamed gum of each participant diagnosed with gingivitis (Groups A and B) as well as from the surface of the same number of teeth of each healthy controls without the symptoms of gingivitis (Group C). The supragingival plaque samples were stored in sterile plastic tubes (Axygen, MCT-150-C 1.5 ml) at −80°C until DNA isolation.

2.4 | DNA isolation, sequencing and data analysis

2.4.1 | DNA isolation from saliva and supragingival plaque samples

Saliva samples were thawed, and 3 ml of each was centrifuged at 13,000 *g* for 5 min. Supragingival plaque samples taken by sterile paperpoints were resuspended in 500 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and were also pelleted at 13,000 *g* for 5 min. DNA extractions were carried out from both sample types by using the Macherey-Nagel (Düren, Germany) NucleoSpin Soil DNA kit (Macherey-Nagel: 740780.250). The lysis mixture contained 700 µl SL1 and 150 µl Enhancer SX lysis solutions. After lysis (bead beating: maximum speed for 5 min), the kit protocol was followed. The quantity of DNA was determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and a Qubit 2.0 Fluorometer (Life Technologies). DNA purity was tested by agarose gel electrophoresis and on an Agilent 2200 TapeStation instrument (Agilent Technologies).

2.4.2 | Next-generation sequencing of supragingival biofilm and saliva samples

Prokaryotic 16S rRNA gene amplification, purification and sequencing were performed as described in "Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" standard protocol provided by the supplier (Illumina). Briefly, the hypervariable V3–V4 region of the 16S rRNA gene was PCR-amplified by using the forward primer 5′TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGCCTACGGGNGGCWGCAG; and the reverse primer 5′GTCT CGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC HVGGGTATC TAATC.

The PCR products were cleaned up by using the Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel: 740609.50) and checked with Agilent TapeStation 2200. Library preparation was done following the instructions of NEBNext Ultra II DNA Library Prep Kit for Illumina (Cat. Num.: E7645L). DNA sequencing was carried out on an Illumina MiSeq machine using V2 sequencing chemistry (MiSeq Reagent Kit v2; 500 cycles).

"In-vitro" fragment libraries were prepared from the saliva total DNA samples for the whole genome sequencing (shotgun metagenome sequencing) using the NEBNext Ultra II DNA Library Prep Kit by Illumina. Paired-end reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Raw sequences are available on NCBI Sequence Read Archive (SRA) under the submission number: PRJNA650272.

2.4.3 | Bioinformatics pipeline for amplicon sequence analysis of supragingival biofilm samples

A novel system of bioinformatics pipeline consisting of five modules was composed to handle the amplicon sequencing data (Figure 1a). In the Sequence preparation module, fastq interleacer was used to join paired-end fastq reads from two separate files, one with the left mates and one with the right mates, into a single file. Raw sequences were trimmed using the Trimming module (Trimmomatic v.0.36.5; settings: sliding window 4:20; minlen 200; leading 3; trailing 3; Bolger et al., 2014). Raw and processed sequence qualities were checked by FastQC (v.0.11.8). Taxonomic classification of amplicon DNA reads was done in the Taxonomic annotation module by the sensitive and highly accurate Kraken 2 (v.2.0.8) program using the NCBI RefSeq (genome) and RDP (16S rDNA amplicon) databases (Wood et al., 2019). In the Filtration and normalization module, both Kraken feature table outputs were filtered by Kraken 2 filter command (confidence threshold: 0.95, indicated ~95% precision to the lowest common ancestor). Normalization to the 16S rRNA gene copy numbers was done through the rrnDB (v.5.6) database (Roller et al., 2016). Metagenomeseq (v.1.16.0) was used for cumulative sum scaling and to create normalized and scaled output of microbial abundance matrices (Paulson et al., 2013). The filtered and normalized microbial abundance matrix was converted to standard BIOM file format (JSON formatted, taxon table). In the Statistics and visualization module Megan6 (v.6.18.1), we scrutinized the microbial communities and exported data for statistic calculations (Huson et al., 2016). Rarefaction estimation was performed by Megan6. The average composition of microbial taxa was visualized by the Krona (v.2.6.1) program (Ondov et al., 2011). The distribution of the top 10 most frequent microbes between the three groups of samples (A: induced gingivitis; B: spontaneous gingivitis; and C: control) was presented in Circos (v.0.63.9) (Connors et al., 2009). For microbial core and diversity calculation, MetaCoMET (Metagenomics Core Microbiome Exploration Tool), an interactive web tool, was used (Wang et al., 2016). Alpha diversity was estimated by the Shannon index. Emperor program (integrated to MetaCoMET) was employed for principal component analysis. The abundance differences were calculated using Statistical Analysis of Metagenomic Profiles (STAMP v.2.0.9) in the case of whole microbiomes (Parks & Beiko, 2010). Dissimilar taxa were identified with two-sided "*t test*" at 0.95 confidence intervals and the results with *q*-value (corrected *p*-value) of <.05 were retained. Minimum difference between proportions was set to 0.3 in STAMP, and Storey FDR (False Discovery Rate) was used in order to filter out false positive significant differences. UPGMA (Unweighted Pairwise Grouping Method with Arithmetic mean) with Bray–Curtis method was employed to cluster the samples, that is, gingivitis-1 (predominated by "purple" complex bacteria), gingivitis-2 (predominated by "orange" complex bacteria), and healthiest (predominated by "yellow" complex bacteria). Significant differences and the UPGMA clustering of samples were visualized by interactive Tree of Life (iTOL v.5.3) online platform (Letunic & Bork, 2019). Both RDP (Ribosomal Database Project) and RefSeq (NCBI Reference Sequence Database) databases were tested in the identification of microbiome community members.

2.4.4 | Bioinformatics pipeline of whole metagenome sequence analysis of saliva samples

Two strategies were applied for the shotgun metagenome data analysis of saliva samples. Both read-based metagenomics and genome-centric approaches were performed. The workflow of the data analysis is summarized in Figure 1b.

Raw sequence filtering

Galaxy Europe server was employed to pre-process the raw sequences (Afgan et al., 2016). Low-quality reads were filtered by Prinseq (v.0.20.4), using the following parameters: min. length: 150; min. score: 15; quality score threshold to trim positions: 20; sliding window was used to calculated quality score: 1 (Schmieder & Edwards, 2011). The quality of raw and filtered sequences was checked with FastQC program.

Read-based metagenomics

For taxonomic analysis of read-based metagenomics data, the Kraken 2 (v.2.0.8) program was employed using the NCBI RefSeq genome database. The Kraken 2 feature table output was filtered by Kraken 2 filter command (confidence threshold: 0.8 indicated 80% precision to the lowest common ancestor).

The microbial communities were investigated with MEGAN6, and data were exported for statistical analyses. The efficiency of sequencing was monitored by computing rarefaction curves based on the comparison of the reads with sequence data in RefSeq as well as with rRNA sequences deposited in RDP database. Rarefaction estimation was performed by MEGAN6 (Huson et al., 2016) (data not shown). The average composition of microbial taxa was visualized via Krona (Ondov et al., 2011). The distribution of top 10 frequent microbes between the three types of samples was plotted in Circos. Microbial core and diversity were calculated by the interactive web tool MetaCoMET (Metagenomics Core Microbiome Exploration Tool). Shannon statistical method was performed to estimate alpha diversity. The Emperor program (integrated in MetaCoMET) did the principal component analysis.

Genome-based evaluation of the sequencing data (binning)

Filtered sequences produced by Prinseq were co-assembled with Megahit (minimum contig length: 1,000 bp, minimum k-mer size: 21, maximum k-mer size 141; Li et al., 2015). After simplifying the header of contig FASTA file, using the Anvi'o script, Bowtie2 (v.2.3.4) was employed to map back the original sequences to the contigs (Langmead & Salzberg, 2012). Following this step, Anvi'o (v.5.3 "Margaret") was used in the metagenomics workflow (Eren et al., 2015). Briefly, during the first stage a contig database was generated, where open reading frames were identified by Prodigal (v.2.6.1) and each

contig k-mer frequencies were computed. Then Hidden Markov Model (HMM) of single-copy genes was aligned by HMMER (v.3.0; Campbell et al., 2013; Finn et al., 2011; Rinke et al., 2013; Simão et al., 2015). InterProScan (v5.31–70) and the metagenome classifier Kaiju (v.16.0) were used for the functional and taxonomic annotation of contigs (Agarwala et al., 2018; Finn et al., 2014; Jones et al., 2014; Menzel et al., 2016). The outputs were imported into the contig database. BAM files made by Bowtie2 helped to profile the contig database, in this way we generated sample-specific information about the contigs, that is, mean coverage. The sample-specific information was merged together. Three automated binning programs, that is, CONCOCT (v.1.1.0), METABAT2 (v.2.12.1), and MAXBIN2 (v.2.2.7), were employed to reconstruct microbial genomes from the contigs (minimum contig length: 2,000) (Alneberg et al., 2013; Kang et al., 2015; Wu et al., 2015). The resulting picture was complemented by the Anvi'o human-guided binning and "anvi-refine" option (Delmont et al., 2018). All binning results were integrated into the contig database. The Anvi'o interactive interface allowed the visualization and summarization of the data.

3 | RESULTS

3.1 | The adolescent supragingival biofilm microbiota (amplicon sequencing)

The supragingival bacterial communities in 112 supragingival biofilm samples were determined using next-generation amplicon sequencing, in which a short, variable region of the 16S rRNA coding marker gene was amplified by PCR before sequencing.

3.1.1 | Sequencing depth

In all cases, rarefaction curves reached their asymptotes (data not shown). This supported a sufficient sequencing depth to cover all genera in the bacterial communities. In other words, more than enough sequences were obtained from each sample to extract the total taxonomic information of the microbial community in them. Alignment of the quality-filtered reads with sequences in the NCBI RefSeq database allowed the identification of 172 operational taxonomic units (OTUs) in the samples analyzed (Table S2).

3.1.2 | The core microbial community

The richness or alpha diversity of OTUs was lower in the supragingival bacterial communities of induced and spontaneous gingivitis patients relative to healthy controls, but the difference was not significant (data not shown). Comparison of the microbial communities using principal component analysis (PCA) also showed a general overlap between supragingival plaque samples from gingivitis patients and healthy controls (data not shown).

Distinct differences were not evident between the study groups at higher taxonomy levels, that is, genus and beyond, but slight differences were noted depending on the reference databases employed. Characteristic alterations took place among the low-abundance members of the community; however, only very few sequence reads could be associated with those taxa, which made statistical comparisons uncertain and likely could have led to erroneous conclusions. Therefore, the low-abundant species and genera (i.e., relative abundance <0.01%) were excluded from the subsequent analyses. Annotation of the genera by RDP revealed 23 abundant genera (relative abundance >1%) and 17 rare genera (relative abundance <1%), while using the RefSeq database, 20 abundant species (relative abundance >1%) and 21 rare species (relative abundance <1%) were identified (Table S3).

The general similarity of the OTUs in the three study groups allowed the amalgamation of all data and determination of the global microbiota of adolescent supragingival plaque (Figure 2a). *Veillonella parvula* was the most abundant bacterial species in the majority of the samples. At genus level, *Prevotella*, *Veillonella*, *Actinomyces*, *Capnocytophaga*, and *Streptococcus* predominated in the supragingival plaques (Figure 2a).

3.1.3 | The most abundant bacterial species in the supragingival biofilms of the distinct study groups

The identified 10 most abundant bacterial species were confirmed by both RDF and RefSeq databases and represented 75% of the whole community. The following bacterial species were the most prevalent ones in our study (Figure 2, Table S3): *V. parvula* and *V. dispar* (Phylum: *Firmicutes*; Genus: *Veillonella*), *F. nucleatum* (Phylum: *Fusobacteria*; Genus: *Fusobacterium*), *Rothia dentocariosa* (Phylum: *Actinobacteria*; Genus: *Rothia*), *Haemophilus parainfluenzae* (Phylum: *Proteobacteria*; Genus: *Haemophilus*), *C. gracilis* and *C. concisus* (Phylum: *Proteobacteria*; Genus: *Campylobacter*), *Streptococcus sanguinis* (Phylum: *Firmicutes*; Genus: *Streptococcus*), *P. oris* and *P. intermedia* (Phylum: *Bacteroidetes*; Genus: *Prevotella*).

3.2 | Pairwise comparison of study groups

Although the composition of the microbial communities in the supragingival biofilm of patients diagnosed with the two types of gingivitis and healthy controls was similar, the ranking order of predominant bacterial species was different in each group (Figure 2b). *V. parvula* dominated the microbiota in induced gingivitis patients, with a relative abundance higher than 57%, followed by *C. gracilis* and species in lower abundance, that is, *S. sanguinis*, *H. parainfluenzae*, *C. concisus*, and *F. nucleatum*. In contrast, in the supragingival biofilm of patients with spontaneous gingivitis, the predominant *V. parvula* was followed by *F. nucleatum* (relative abundance higher than 21%), *P. intermedia*, and *C. gracilis*. In healthy controls, the dominant *V. parvula* was accompanied by *R. dentocariosa* and *H. parainfluenzae* and the moderately abundant species *F. nucleatum* and *S. sanguinis* (Figure 2b).

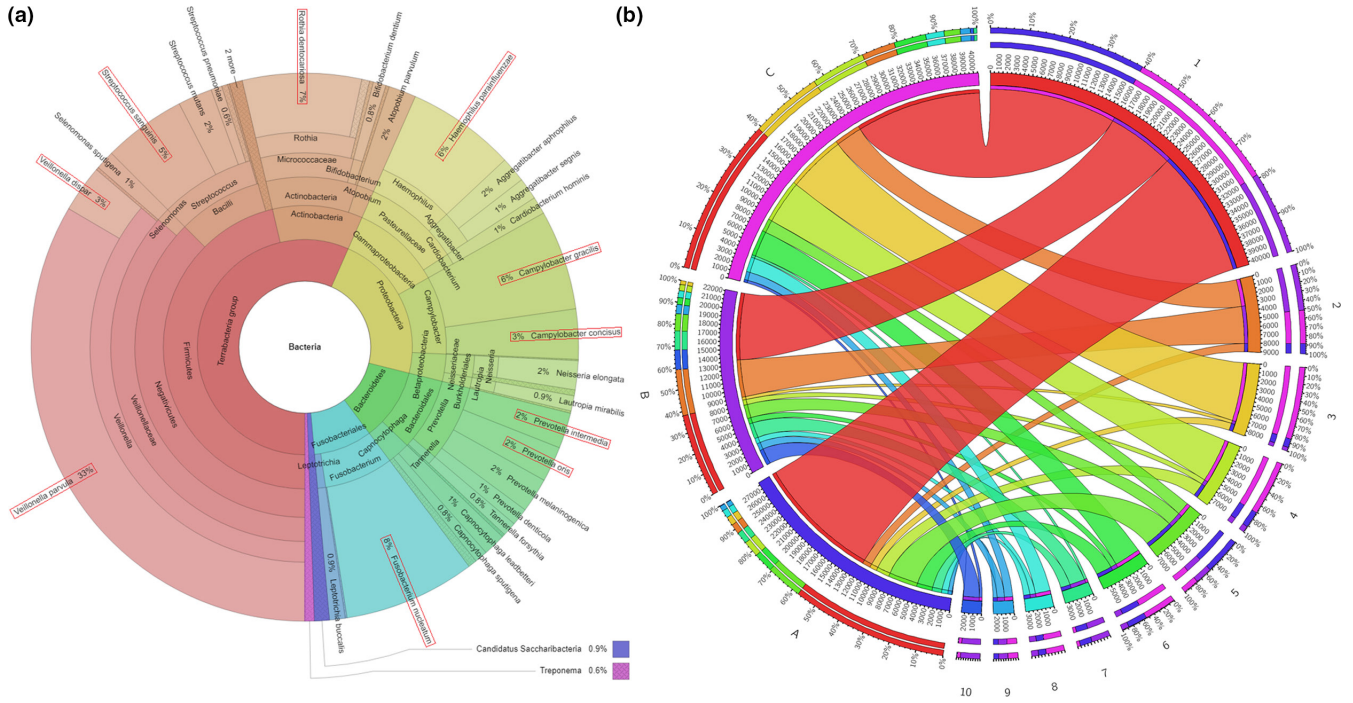


FIGURE 2 (a) Overall composition of the oral microbiota in supragingival biofilm samples derived from gingivitis patients and healthy controls involved in the study. The most abundant species are outlined in red boxes. OTUs were annotated based on the RefSeq NCBI Reference Sequence Database. (b) Relative distribution of the 10 most abundant bacterial species identified in supragingival biofilm samples of the study participants. Designations: 1. *Veillonella parvula*, 2. *Fusobacteriumnucleatum*, 3. *Rothia dentocariosa*, 4. *Haemophilus parainfluenzae*, 5. *Campylobactergracilis*, 6. *Streptococcussanguinis*, 7. *Campylobacterconcisus*, 8. *Veillonella dispar*, 9. *Prevotella oris*, 10. *Prevotella intermedia*

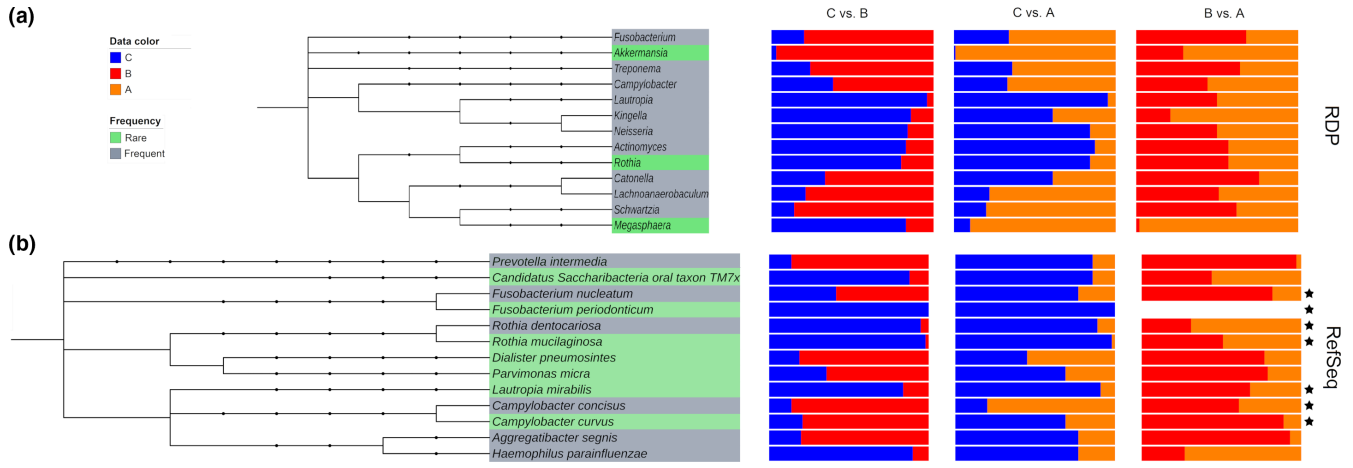


FIGURE 3 Comparison of bacterial communities in supragingival biofilm samples of induced (orange), spontaneous (red) gingivitis patients and healthy controls (blue). Horizontal bars indicate the relative abundances of the given taxon in the study groups. (a) significantly different genera (annotation based on RDP); (b) significantly different species (annotation based on RefSeq). Stars designate OTUs, which were identified by both RDP and RefSeq reference databases

In order to uncover the characteristic and significant differences between the three subject groups, the data sets were compared pairwise (Figure 3). Perhaps, the most pronounced of these was the alterations between the diseased and healthy subjects. We observed an increased relative abundance of the genera *Fusobacterium*, *Akkermansia*, *Treponema*, and *Campylobacter* in supragingival plaques of gingivitis patients versus controls. In contrast, the genera *Lautropia*, *Kingella*, *Neisseria*, *Actinomyces*, and *Rothia* were substantially more abundant in controls than in either of the two groups of gingivitis patients (Figure 3a). The genus *Megasphaera* showed notable relative distribution changes between the control subjects and induced and spontaneous gingivitis patients. In addition, relative abundances between the two groups of gingivitis patients were also noticeable, which might deserve further studies on large cohorts of subjects.

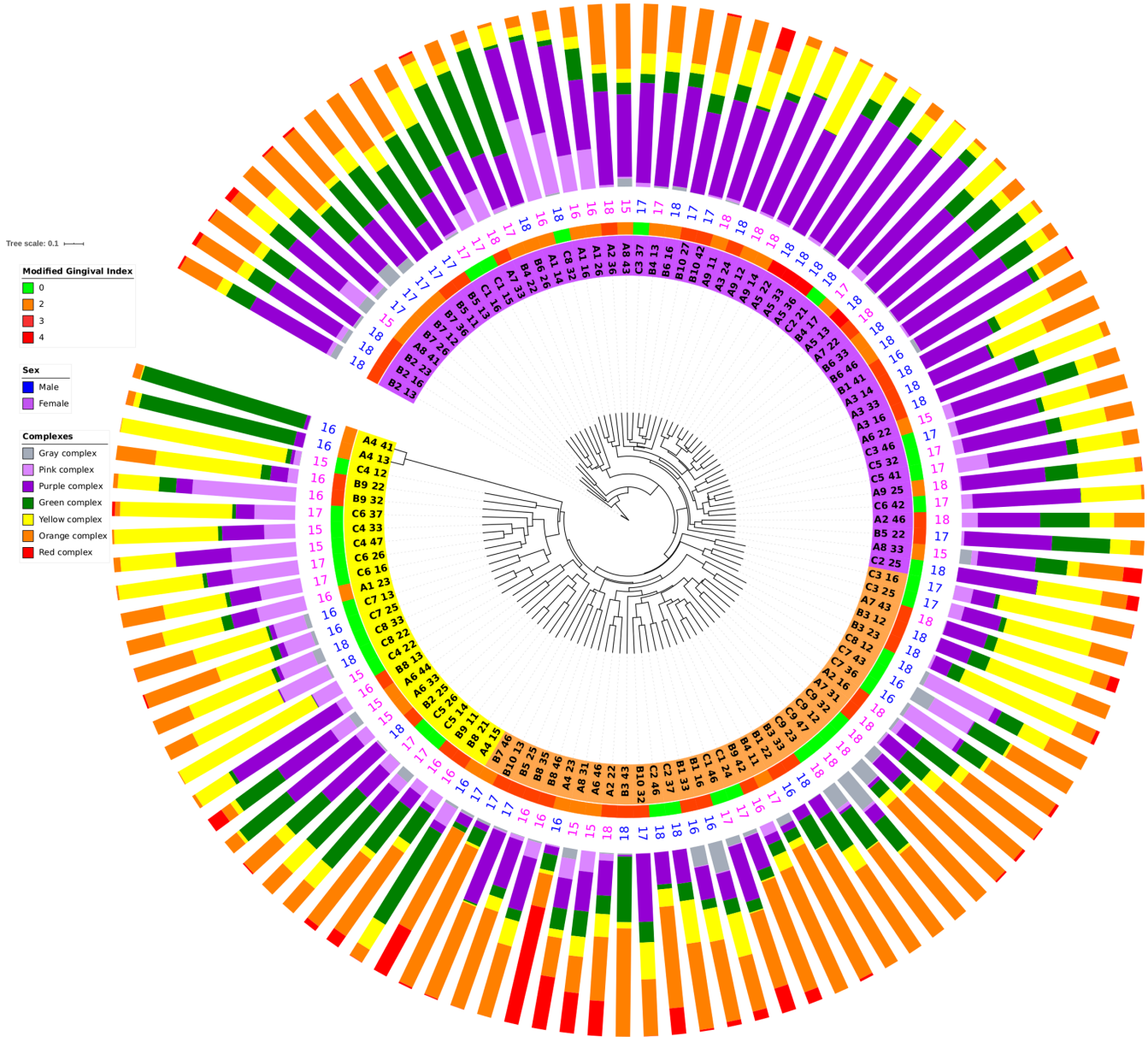


FIGURE 4 UPGMA (unweighted pair group method with arithmetic mean) cluster analysis of supragingival biofilm metagenomes. The study group designation (capital letters), the numbers marking the anonymous individual patients and sampled tooth positions are highlighted in purple, orange, and yellow, according to their position on the major branches of the UPGMA tree (innermost ring). Study groups were as follows. (a) samples from induced gingivitis patients; (b) samples from spontaneous gingivitis patients; (c) healthy controls. Modified gingival indices (MGIs) (Lobene et al., 1986, 1989) are indicated as red, orange, and green sectors in the next ring. Predominant bacterial taxa identified in each supragingival biofilm sample are shown as colored columns at the outer edge of the circular diagram above numbers indicating the age of the patients: Color-coding of the bacterial groups was based on their association with microbial complexes involved in oral pathogenesis, indicating their potential contribution to oral health (Colombo & Tanner, 2019; Haffajee et al., 2008; Socransky et al., 1998) (Supplementary Figure 1)

At the species level, a significantly higher abundance of *C. concisus* was apparent in both gingivitis groups versus the controls (Figure 3b). We also observed that the relative abundance of Candidatus Saccharibacteria oral taxon TM7x, *R. dentocariosa*, *R. mucilaginosa*, *Lautropia mirabilis*, and *H. parainfluenzae* was lower in supragingival biofilms of either gingivitis patients versus healthy controls. Interestingly, *F. periodonticum* was detected in healthy samples only. Comparison of bacterial species in the two groups of gingivitis patients showed that the relative abundances of Candidatus Saccharibacteria oral taxon TM7x, *R. dentocariosa* and *H. parainfluenzae*, were significantly higher in patients with induced versus spontaneous gingivitis. Other species, including *P. intermedia*, *F. nucleatum*, *Parvimonas micra*, *Dialister pneumosintes*, *C. concisus*, *C. curvus*, and *Aggregatibacter segnis*, were less abundant in induced gingivitis versus spontaneous gingivitis group (Figure 3b). Out of the 13 significantly different species, seven were positively confirmed by both RDP (on the taxonomic level of genus) and RefSeq (on the taxonomic level of species) databases, marked with stars in Figure 3b. These

patterns may be useful for diagnostic and future targeted therapy point of view.

3.2.1 | Microbial complexes in the gingivitis and healthy study groups

Similar to PCA and alpha diversity, clustering of supragingival microbiota using UPGMA (unweighted pair group method with arithmetic mean) did not result in a clear separation of the three study groups. Samples from induced gingivitis patients (A), spontaneous gingivitis patients (B), and controls (C) appeared intermingled with each other on the UPGMA tree (see the letters preceding the tooth position numbers in the innermost ring in Figure 4). The samples did not separate into distinct clusters according to the Modified Gingival Index (MGI) reflecting the severity of gingivitis (2nd ring form inside in Figure 4).

Nevertheless, a different clustering was apparent according to the microbial complexes. Oral microbes have been classified on the basis of their roles in pathogenesis and have been arranged in color-coded segments of the microbial complexes (Colombo & Tanner, 2019; Haffajee et al., 2008; Socransky et al., 1998) (Figure S1). One cluster of metagenomes was predominated by the purple complex (mainly *V. parvula*), and these are marked with purple background of the ID tags. The ID tags (innermost ring in Figure 4) indicate the study group letter followed by the subjects' ID number and tooth positions. This cluster comprised 52 of the 112 supragingival microbiota included in this study. *F. nucleatum* (25 out of 36 microbiota) predominated the cluster highlighted in orange, together with other members of the microbial orange complex (Colombo & Tanner, 2019; Haffajee et al., 2008; Socransky et al., 1998). The third cluster, highlighted in yellow, showed a balanced distribution of prevailing species, such as *R. dentocariosa*, *H. parainfluenzae*, and *V. dispar*. 12 out of 25 samples were assigned to this cluster (yellow background of innermost ring in Figure 4).

3.3 | Salivary microbiota of adolescent subjects (whole metagenome sequencing)

In addition to the dental plaques, saliva samples were also collected from the same study participants. In these experiments, we wanted to examine the importance of sampling site, that is, tooth biofilm associated or planktonic microbiota of gingivitis patients relative to the microbiota of healthy controls belonging in the same age group. Saliva samples contained enough DNA to allow whole metagenome sequencing; thereby, we could eliminate the potential random systematic error implied in amplicon sequencing (Ranjan et al., 2016). The analysis and evaluation of sequence data followed a workflow (Figure 1b) similar to the one applied to the amplicon sequencing, although in this case, both read-based and genome-based analyses became possible, which extended the information content considerably.

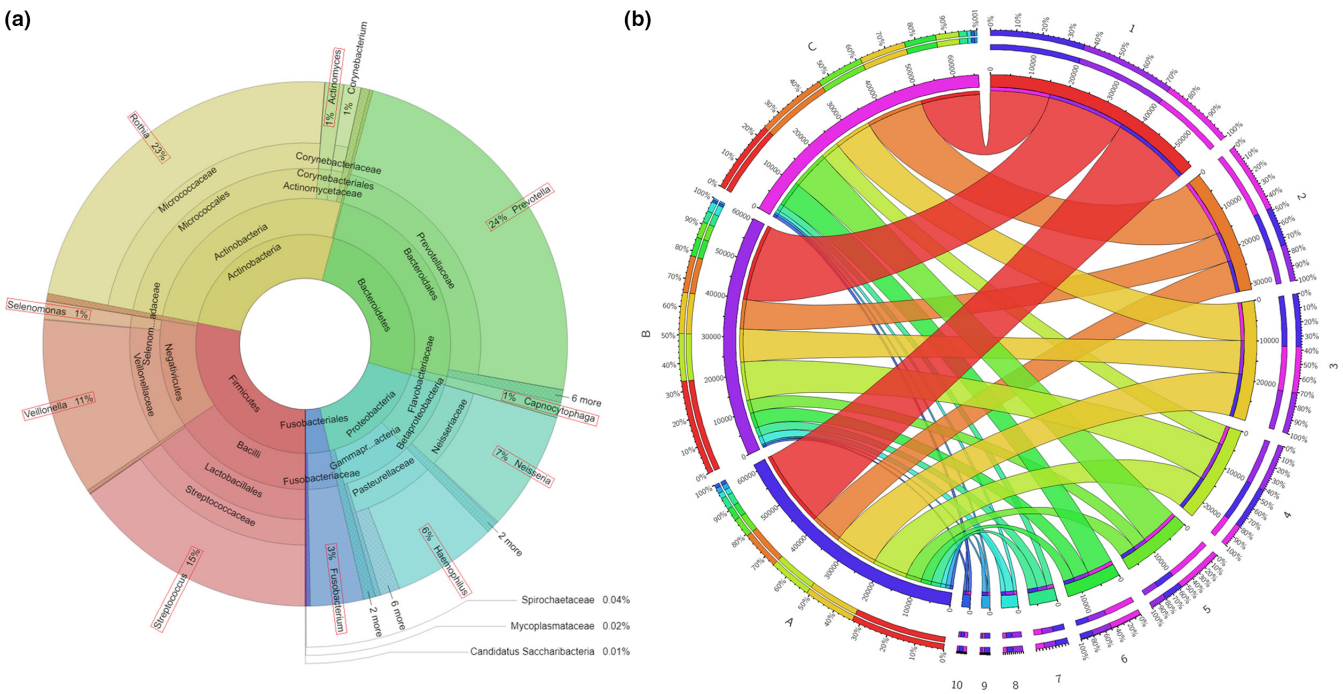
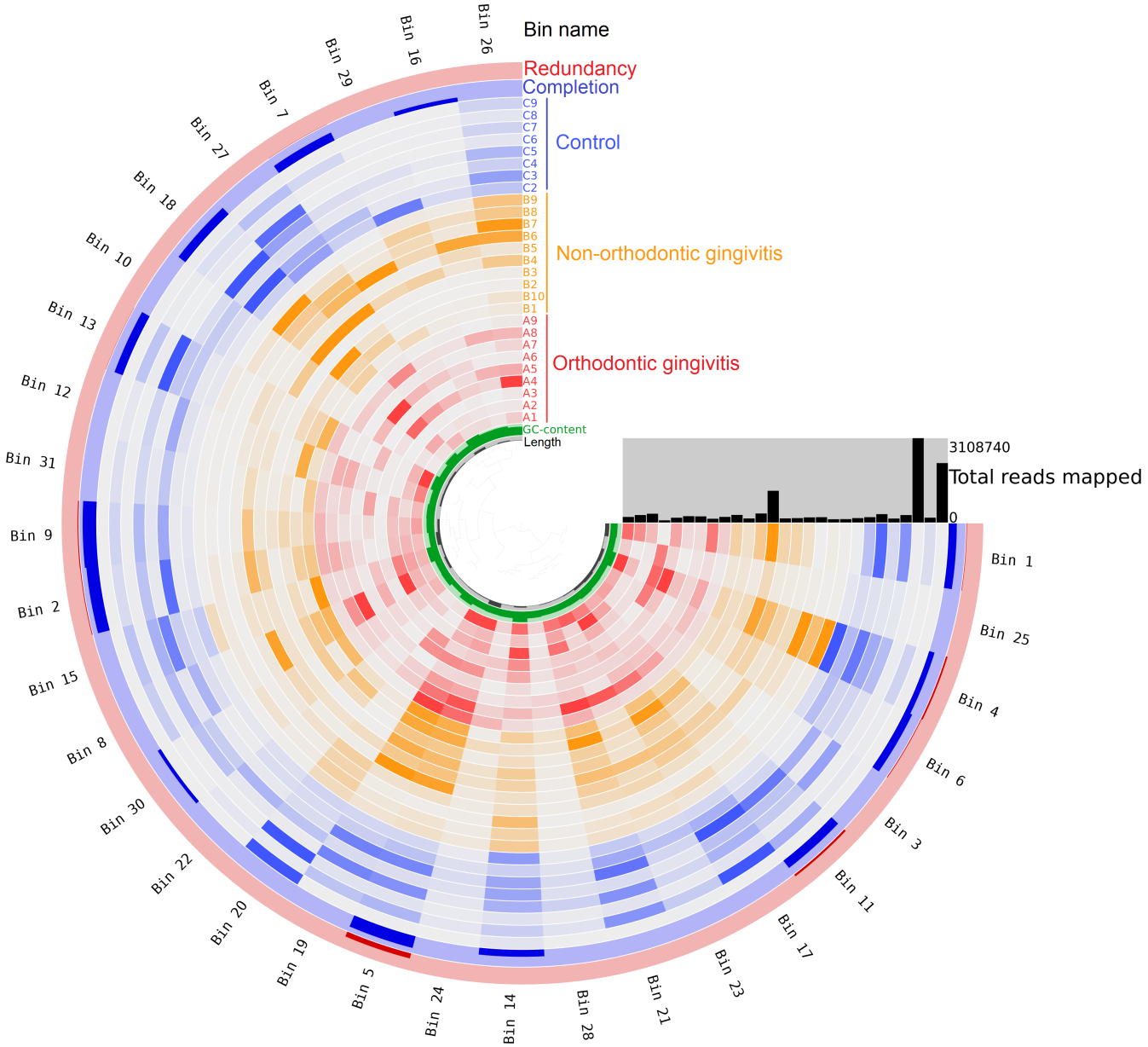


FIGURE 5 (a) Overall composition of saliva samples derived from gingivitis patients and healthy controls involved in the study. The most abundant species are outlined in red boxes. OTUs were annotated based on the RefSeq NCBI Reference Sequence Database. (b) Relative distribution of the 10 most abundant bacterial genera identified in the saliva samples of the study participants. Designations: 1. *Prevotella*, 2. *Rothia*, 3. *Streptococcus*, 4. *Veillonella*, 5. *Neisseria*, 6. *Haemophilus*, 7. *Fusobacterium*, 8. *Selenomonas*, 9. *Capnocytophaga*, 10. *ACTINOMYCES*

3.3.1 | The most abundant bacteria in the saliva of study groups (read-based metadata)

The richness and evenness of the salivary microbial communities did not differ significantly between any combination of gingivitis study groups and healthy controls according to the Shannon indices (data not shown). PCA of the salivary microbiota did not reveal distinct clusters either (data not shown). In this respect, the whole salivary microbiota exhibited the same patterns as those found in the dental plaque biofilms with amplicon sequencing. Therefore, we could



Bin 1: *Prevotella histicola,* Bin 2: *Prevotella pallens,* Bin 3: *Prevotella salivae,* Bin 4: *Unknown Rothia,* Bin 5: *Unknown Veillonella,* Bin 6: *Unknown Streptococcus,* Bin 7: *Lautropia mirabilis,* Bin 8: *Prevotella sp. C561,* Bin 9: *Megasphaera micronuciformis,* Bin 10: *Prevotella nanciensis,* Bin 11: *Unknown Prevotella,* Bin 12: *Prevotella sp. Oral taxon 306,* Bin 13: *Actinomyces graevenitzii,* Bin 14: *Unknown Actinobacteria,* Bin 15: *Actinomyces sp. ICM47,* Bin 16: *Corinebacterium matruchatii,* Bin 17: *Prevotella melaninogenica,* Bin 18: *Unknown Porphyromonadaceae,* Bin 19: *Veillonella atypica,* Bin 20: *Escherichia coli,* Bin 21: *Veillonella dispar,* Bin 22: *Fusobacterium periodonticum,* Bin 23: *Veillonella parvula,* Bin 24: *Streptococcus parasanguinis,* Bin 25: *Streptococcus salivarius,* Bin 26: *Streptococcus pneumoniae,* Bin 27: *Haemophilus parainfluenzae,* Bin 28: *Campylobacter concinsus,* Bin 29: *Rothia dentocariosa,* Bin 30: *Candidatus Saccharobacteria oral taxon TM7x,* Bin 31: *Atopobium parvulum*

FIGURE 6 Bacterial taxa identified in saliva samples of induced and non-induced gingivitis patients and controls using genome-based evaluation of sequencing data. The distribution of contigs is plotted in the circular rings. The grouping of contigs was based on sequence assignments of automated binning programs METABAT2, MAXBIN2, and CONCOCT as well as manually defined bins as presented on the Anvi'o platform. The list of identified bins is given at the bottom part of the figure

combine all saliva microbiota to generate a global picture of the adolescent saliva microbial landscape (Figure 5a).

The genera *Prevotella*, *Rothia*, *Streptococcus*, and *Veillonella* predominated the salivary microbiota. It is noteworthy that *Veillonella* was not the outstanding single genus in saliva, as in the cases of the supragingival biofilm samples (see Figures 2a and 5a).

The relative distribution of the 10 most abundant bacterial genera was determined next. In induced gingivitis, the relative abundance of *Prevotella* was higher than in the two other groups. *Streptococcus* was the most abundant genus in spontaneous gingivitis (Figure 5b). In control saliva samples, the relative abundance of *Rothia* was comparable with that of *Prevotella*. In each study group, 14 genera accounted for >95% of the taxa identified at the genus level.

3.3.2 | Genome-based evaluation of the saliva sequencing data (binning)

In addition to the read-based data, bioinformatics analysis of saliva samples was accomplished by genome-centric binning (Figure 1b). In this approach, the filtered sequences were first assembled in contigs, which were then distributed into virtual bins, based on their inherent sequence features. Inspection of the genetic content of the individual bins supplied detailed information about taxonomy from a viewpoint distinct of the read-based approach and in many cases about the genes coding for possible specific metabolic pathways.

Genomic fragments belonging in the most abundant taxa of the healthy salivary microbiome (Segata et al., 2012) could be detected in the saliva of each study group. In line with the results of read-based metagenomics, most of the putative genomes identified by binning belonged to *Prevotella* species, which comprised 8 separate bins, whereas the putative genomes of species belonging in the genera *Veillonella* and *Streptococcus* comprised 4 separate bins, respectively. The genomes of *Actinomyces* and *Rothia* species were detected in 2 distinct bins, each. The genomes of 9 other species as well as the family *Porphyromonadaceae* and the taxon Candidatus Saccharibacteria TM7x occupied a single distinct bin, respectively (Figure 6). All these genera were present in both supragingival biofilms and planktonic saliva samples.

The overall similarity of the read-based and genome-based microbiota validated each other, starting from the same saliva sequencing databases the two distinct bioinformatics approaches gave comparable pictures of the microbial communities.

4 | DISCUSSION

We applied next-generation sequencing to characterize the bacterial communities in individual supragingival biofilms of adolescent patients diagnosed with either induced or spontaneous gingivitis. Similar samples were taken from the teeth of age-matched healthy controls. Four distinct supragingival biofilm samples were collected from the surface of four different teeth of each study participant and were analyzed one by one. Furthermore, in order to reveal whether the characteristic features of the supragingival microbes were reflected in the salivary microbiota of the participants, we also identified the predominant bacterial taxa in the non-stimulated saliva samples of the same subjects.

Gingivitis is a non-destructive disease affecting both young and elderly worldwide (Clerehugh, 2008; Murakami et al., 2018). Typically, the causative agent is the dental plaque and removal of the supragingival biofilm from the surface can revert the inflammatory response elicited by the biofilm-forming bacterial community (Michelet et al., 1991; Offenbacher et al., 2010; Page & Schroeder, 1976; Theilade et al., 1966). When proper oral hygiene is not restored, gingival inflammation may persist, and in susceptible individuals, it can develop to periodontitis when the irreversible changes include loss of the tissues that attach the tooth to the alveolar structures accompanied with alveolar bone loss (Könönen et al., 2019; Lang et al., 2009; Schätzle et al., 2003). Metagenomic analysis of the bacterial taxa in dental plaque samples collected from gingivitis-affected teeth and non-affected teeth biofilms revealed differences in the composition and community structure (Huang et al., 2011, 2014, Huang et al., 2016; Kistler et al., 2013; Park et al., 2015; Shaw et al., 2016; Deng et al., 2017; Schincaglia et al., 2017). Most of the earlier sequencing studies analyzed pooled plaque samples of adult gingivitis patients or healthy adults (Belstrøm et al., 2018). These observations suggested that the relative abundance of various bacteria in supragingival as well as subgingival biofilms may play a role in the progress of gingival inflammation (Bartold & Van Dyke, 2019). A recent experimental gingivitis study pointed out substantial increase of the relative abundance of *Leptotrichia* in the supragingival plaques after oral hygiene discontinuation (Belstrøm et al., 2018). In other studies, the association of gingivitis with biofilm-forming members of the genus *Leptotrichia* was variable (Huang et al., 2011, 2014) versus (Huang et al., 2016; Kistler et al., 2013). In our current work, the relative abundance of *L. buccalis* was low and did not differ significantly between gingivitis and control subjects.

A significant fraction of adolescents and adults may undergo orthodontic treatment to correct crowded, rotated, buried, or prominent front teeth (Alhammadi et al., 2018). The application of braces may facilitate plaque accumulation by creating new retention sites, which are difficult to access during teeth cleaning (Koopman et al., 2015; Ren et al., 2014). The surface of orthodontic appliances may favor the build-up of bacterial communities distinct from the plaques of spontaneous gingivitis (Ren et al., 2014).

In our investigations, the most ubiquitous species belonged in the predominant phyla of the human oral microbiota, that is, *Firmicutes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* (Dewhirst et al., 2010). The overall composition and diversity of supragingival biofilm communities were similar in the three study groups (Figures 2a and 5a), although the ranking order of predominant bacterial species was unique for each group (Figures 2b and 5b). *V. parvula*, an early colonizer of the tooth surface, predominated the

supragingival biofilm microbiota in all three study groups (Figure 2a), whereas the diverse genus *Prevotella* was the most prevalent in the saliva samples (Figure 5a). *V. parvula* was abundant in supragingival plaques in an experimental gingivitis study, and it was suggested that *via* co-aggregation with other salivary bacteria, they played an important role in the formation and growth of multispecies microbial communities (Eberhard et al., 2013; Kolenbrander, 2011). In other studies, *Veillonella* was found to be one of the most abundant genera both in supragingival and subgingival plaques of healthy adults (Segata et al., 2012).

One may envisage gingivitis and periodontitis as successive stages of an inflammatory cascade (Kinane & Attstrom, 2005). In this respect, it is noteworthy that *Veillonella* species were implicated in both pathogenesis of periodontitis and commensal members of the so called microbial "purple complex" (Colombo & Tanner, 2019; Haffajee et al., 2008; Socransky et al., 1998) (Figure S1). The microbial complexes were denoted originally in subgingival plaque samples of periodontitis patients (Socransky et al., 1998), and a similar set of complexes has been recognized in the supragingival plaques (Haffajee et al., 2008), which are indicative of gingival health (Colombo & Tanner, 2019). Early colonizing bacteria on the supragingival tooth surfaces are members of the "yellow," "green," and "purple" complexes, whereas members of the "orange" complex integrate into the supragingival biofilm later (Carrouel et al., 2016; Colombo & Tanner, 2019; Socransky et al., 1998). Due to its elongated shape and diverse metabolic interactions, *F. nucleatum*, a member of the "orange" complex, contributes to the construction of supragingival biofilms (Brennan & Garrett, 2019). Our observations are compatible with the previously suggested role of *Fusobacterium*, *Campylobacter*, and *Treponema* in the pathogenesis of gingivitis, whereas the less abundant *Catonella*, *Lachonanaerobaculun*, *Schwartzia*, and *Akkermansia* genera remain to be characterized in this respect (Macuch & Tanner, 2000; Sharma et al., 2018). Some genera, including *Actinomyces*, *Kingella*, *Lautropia*, *Megasphaera*, *Neisseria*, *and Rothia*, were extensively more abundant in the control samples. These genera have been associated with gingival health (Al-Kamel et al., 2019; Colombo & Tanner, 2019; Grevich et al., 2019; Huang et al., 2016; Koopman et al., 2015; Mervish et al., 2019; Sanz et al., 2017). Comparison of relative abundances of bacterial species revealed a substantially higher abundance of the abundant *C. concisus* in the two gingivitis groups versus controls (Figure 3). Interestingly, the less abundant *C. curvus* did not follow the same pattern. *C. concisus*, a member of the "green" complex, is ubiquitous in the oral cavity. It has been found both in periodontal inflammation and periodontal health-associated microbiomes (Colombo & Tanner, 2019). It is noteworthy that *C. concisus*, a primary colonizer of the oral cavity, can be translocated into the gastrointestinal tract and recent observations revealed an association of *C. concisus* with inflammatory bowel disease (reviewed by Liu et al., 2018). Thus, one may speculate that this bacterium, which is capable to damage the intestinal epithelial barrier, may also contribute to the development of gingivitis as well where a similar defense line of the human host has to be defeated. Species belonging in the "orange" complex, such as *P. intermedia*, *F. nucleatum*. *P. micra*, and "purple" complex (*A. segnis*) as well as the new periodontopathogenic species *D. pneumosintes* (Ayala Herrera et al., 2019; Ferraro et al., 2007) (shown in the gray box in Figure S1), were more abundant in supragingival biofilms of the non-induced gingivitis group, but not in the induced gingivitis group (Figure 3) (Carrouel et al., 2016; Colombo & Tanner, 2019; Ferraro et al., 2007; Socransky et al., 1998).

Comparisons at species level did not expose conceivable differences in the relative abundances of the Gram-negative anaerobic periodontal pathogens belonging in the "red" complex (*P. gingivalis*, *T. denticola*, and *Tannerella forsythia*), although the relative abundance of the genus *Treponema* was higher in gingivitis samples (Figure 3).

In contrast, the bacterial species Candidatus Saccharibacteria oral taxon TM7x, *R. dentocariosa*, *R. mucilaginosa*, *L. mirabilis*, and *H. parainfluenzae* were detected in higher relative occurrence in supragingival plaques of the control group, relative to the gingivitis patients (Figure 3). Candidatus Saccharibacteria oral taxon TM7x was implicated in periodontal disease, while the increased abundance of the other species in control samples is compatible with their classification as "Associated with Periodontal Health" (Colombo & Tanner, 2019; Huang et al., 2016). It is noteworthy that *F. periodonticum* was identified in the control samples only although *F. nucleatum* occurred in all three study groups.

The development of gingivitis in patients undergoing orthodontic therapy has been linked to plaque accumulation at the new retention sites, which are difficult to access for oral hygiene (Koopman et al., 2015; Ren et al., 2014). The composition of the bacterial communities in induced gingivitis was also reported distinct from the microbiomes in naturally occurring gingivitis (Ren et al., 2014). Our results, based on more advanced bioinformatics analysis, did not corroborate these findings (Figure 4). In spite of the overall similarity, differences were detected at the species level resolution: Candidatus Saccharibacteria oral taxon TM7x, and health-associated species *R. dentocariosa* and *H. parainfluenzae* were markedly higher in patients with induced versus non-induced gingivitis (Figure 3b), while *F. nucleatum*, *P. intermedia*, *P. micra*, *C. concisus*, *C. curvus*, and *A. segnis* showed opposite tendencies (Figure 3b).

It is important to note that no correlation between the pathogenicity of the bacterial members of the supragingival plaques and the status of the subjects (gingivitis or health) could be recognized. Nevertheless, three separate microbial communities could be distinguished, two of them could be associated with gingivitis, that is, predominated by genera classified as members of "purple" and "orange" complex while the third one harbored mostly "yellow complex" bacteria. It is tempting to relate this finding to the two subpopulations of gingivitis patients defined on the basis of clinical signs (Tatakis & Trombelli, 2004; Trombelli et al., 2004), although this connection should be established in future studies. We have also investigated the potential relationship between oral health status and the subjects' gender, age, and orthodontic appliance wearing duration. Although there was no significant correlation between any of these parameters, a tendency indicating better status of the female patients relative to the male ones and shorter duration of wearing

braces versus long exposure was noted. These may suggest that there are no substantial differences among the study groups in their general oral health status; hence, gingivitis (induced or spontaneous) can be reversed with proper oral hygiene.

Mapping the salivary bacterial community may be an easy and helpful diagnostic tool of distinct pathological processes (Zarco et al., 2012; Zhang et al., 2016). Nevertheless, under the employed stringent evaluation conditions we did not observe major differences between the salivary microbiome of patients with gingivitis compared to controls. This finding may be related to the salivary microbiome comprising the easily detachable mixture of diverse bacterial communities inhabiting the surface of the tongue, tonsils, and throat as well as the microbes of the supragingival plaque (Segata et al., 2012). The predominance of the genus *Prevotella* in the saliva relative to the supragingival biofilms is remarkable. The genomes of Prevotella sp. are very dynamic and subject to frequent horizontal gene transfer (Zhang et al., 2017). This and methodological differences (Lu & Salzberg, 2020) may explain the differences between our results and some previous observations. It is noteworthy that pregnancy-associated gingivitis alters the microbial community substantially (Gursoy et al., 2009; Lin et al., 2018). Moreover, our study highlights the differences between the observations using classical microbiology approaches on isolated colonies (Alaluusua et al., 1996; Gursoy et al., 2009; Lie et al., 2001) and metagenomic methods. Previous studies emphasized the predominance of *P. nigrescens* in the gingivitis community, whereas we could detect only traces of this species. It is possible that this organism, present in low abundance, appeared as predominant because it grew better in the cultivation medium than *P. intermedia* did. We did not perform cultivation experiments; therefore, this issue remains to be clarified in future studies.

The results obtained with the RDP and RefSeq databases complemented each other fairly well although the correlation was not perfect. The genus *Actinomyces* represented one example of the inconsistencies. *Actinomyces* was identified as abundant taxon by RDP, but no *Actinomyces sp.* was detected using the RefSeq database. Similarly, the genus *Aggregatibacter* was not recognized using RDP, but *A. segnis* and *A. aphrophilus* showed up among the abundant species according to RefSeq (Table S3). These inconsistencies are due to the distinct content, that is, small ribosomal gene database (RDP) where the sequences are linked to genera and a large whole genome database (RefSeq) where the sequences are linked to species (Lu & Salzberg, 2020).

5 | CONCLUSIONS

Our study indicated that the relative abundance of distinct bacterial taxa in supragingival biofilms may differ noticeably, although not extensively, between induced and spontaneous gingivitis patients in spite of the global similarities.

The higher relative abundance of certain bacterial taxa in supragingival biofilms of gingivitis patients versus controls signals their involvement in the pathogenesis of the disease and may thus be of diagnostic value to prevent further escalation toward periodontitis.

Mapping salivary microbiome may obscure certain variations between healthy and gingivitis status. At any rate, a stringent and coherent bioinformatics workflow should be employed to detect the relatively small alterations in microbiome composition and taxonomic abundances.

ACKNOWLEDGEMENTS

This research was funded by the European Regional Development Fund to a project led by JM grant GINOP-2.3.2-15-2016-00011. Additional projects GINOP-2.2.1-15-2017-00081, GINOP-2.2.1-15-2017-00033, and EFOP-3.6.2-16-2017-00010 also contributed to this work. RW (PD132145) and GM (FK123899) received support from the National Research, Development and Innovation Office (NKFIH), Hungary. This work was also supported by the János Bolyai Research Scholarship (for GM) of the Hungarian Academy of Sciences. The authors thank the funding agencies for their support. We are indebted to Dr. Márió Gajdács and the unknown Reviewers for critical reading of the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Roland Wirth: Data curation; Supervision; Writing – review and editing. **Gergely Maróti:** Conceptualization; Methodology; Supervision; Writing – original draft. **Lídia Lipták:** Formal analysis; Investigation; Writing – original draft. **Monika Klára Mester:** Formal analysis; Investigation; Methodology; Writing – original draft. **Alaa Al Ayoubi:** Data curation; Investigation; Methodology; Writing – original draft. **Bernadett Pap:** Formal analysis; Investigation; Methodology; Writing – original draft. **Melinda Madléna:** Conceptualization; Project administration; Supervision; Writing – original draft. **János Minárovits:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing – original draft.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/odi.13883.

ORCID

Alaa Al Ayoubi https://orcid.org/0000-0002-2886-4615
Kornél L. Kovács https://orcid.org/0000-0002-3926-0497

REFERENCES

Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., Grüning, B., Guerler, A., Hillman-Jackson, J., Von Kuster, G., Rasche, E., Soranzo, N., Turaga, N., Taylor, J., Nekrutenko, A., & Goecks, J. (2016). The Galaxy platform for accessible, reproducible and

collaborative biomedical analyses: 2016 update. *Nucleic Acids Research*, *44*, W3–W10.

Agarwala, V., Khozin, S., Singal, G., Oconnell, C., Kuk, D., Li, G., Gossai, A., Miller, V., & Abernethy, A. P. (2018). Real-world evidence in support of precision medicine: Clinico-genomic cancer data as a case study. *Health Affairs*, *37*, 765–772.

Alaluusua, S., Mättö, J., Grönroos, L., Innilä, S., Torkko, H., Asikainen, S., Jousimies-Somer, H., & Saarela, M. (1996). Oral colonization by more than one clonal type of mutans streptococcus in children with nursing-bottle dental caries. *Archives of Oral Biology*, *41*, 167–173.

Al-Ghutaimel, H., Riba, H., Al-Kahtani, S., & Al-Duhaimi, S. (2014). Common periodontal diseases of children and adolescents. *International Journal of Dentistry*, *2014*, 1–7.

Alhammadi, M. S., Halboub, E., Fayed, M. S., Labib, A., & El-Saaidi, C. (2018). Global distribution of malocclusion traits: A systematic review. *Dental Press Journal of Orthodontics*, *23*, e1–e10.

Al-Kamel, A., Baraniya, D., Al-Hajj, W. A., Halboub, E., Abdulrab, S., Chen, T., & Al-Hebshi, N. N. (2019). Subgingival microbiome of experimental gingivitis: Shifts associated with the use of chlorhexidine and N-acetyl cysteine mouthwashes. *Journal of Oral Microbiology*, *11*, 1608141.

Alneberg, J., Bjarnason, B. S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U. Z., Loman, N. J., Andersson, A. F., & Quince, C. (2013). CONCOCT Clustering cONtigs on COverage and ComposiTion. *arXiv*, 1–28.

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology*, *4*, 1–6.

Ayala Herrera, J. L., Apreza Patrón, L., Martínez Martínez, R. E., Domínguez Pérez, R. A., Abud Mendoza, C., & Hernández Castro, B. (2019). Filifactor alocis and Dialister pneumosintes in a Mexican population affected by periodontitis and rheumatoid arthritis: An exploratory study. *Microbiology and Immunology*, *63*, 392–395.

Bartold, P. M., & Van Dyke, T. E. (2019). An appraisal of the role of specific bacteria in the initial pathogenesis of periodontitis. *Journal of Clinical Periodontology*, *46*, 6–11.

Bellagambi, F. G., Lomonaco, T., Salvo, P., Vivaldi, F., Hangouët, M., Ghimenti, S., Biagini, D., Di Francesco, F., Fuoco, R., & Errachid, A. (2020). Saliva sampling: Methods and devices. An overview. *TrAC Trends in Analytical Chemistry*, *124*, 115781.

Belstrøm, D., Sembler-Møller, M. L., Grande, M. A., Kirkby, N., Cotton, S. L., Paster, B. J., Twetman, S., & Holmstrup, P. (2018). Impact of oral hygiene discontinuation on supragingival and salivary microbiomes. *JDR Clinical and Translational Research*, *3*, 57–64.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*, 2114–2120.

Brennan, C. A., & Garrett, W. S. (2019). Fusobacterium nucleatum — symbiont, opportunist and oncobacterium. *Nature Reviews Microbiology*, *17*, 156–166.

Burt, B. (2005). Position paper: Epidemiology of periodontal diseases. *Journal of Periodontology*, *76*, 1406–1419.

Califano, J. V. (2003). Position Paper : Periodontal diseases of children and adolescents. *Journal of Periodontology*, *74*, 1696–1704.

Campbell, J. H., O'Donoghue, P., Campbell, A. G. et al (2013). UGA is an additional glycine codon in uncultured SR1 bacteria from the human microbiota. *Proceedings of the National Academy of Sciences*, *110*, 5540–5545.

Carrouel, F., Viennot, S., Santamaria, J., Veber, P., & Bourgeois, D. (2016). Quantitative molecular detection of 19 major pathogens in the interdental biofilm of periodontally healthy young adults. *Frontiers in Microbiology*, *7*, 1–16.

Clerehugh, V. (2008). Periodontal diseases in children and adolescents. *British Dental Journal*, *204*, 469–471.

Colombo, A. P. V., & Tanner, A. C. R. (2019). The role of bacterial biofilms in dental caries and periodontal and peri-implant diseases: A historical perspective. *Journal of Dental Research*, *98*, 373–385.

Connors, J., Krzywinski, M., Schein, J., Connors, J., Gascoyne, R., Horsman, D., Jones, S. J., & Marra, M. A. (2009). Circos: An information aesthetic for comparative genomics. *Genome Research*, *19*, 1639–1645.

Delmont, T. O., Quince, C., Shaiber, A., Esen, Ö. C., Lee, S. T., Rappé, M. S., McLellan, S. L., Lücker, S., & Eren, A. M. (2018). Nitrogen-fixing populations of Planctomycetes and Proteobacteria are abundant in surface ocean metagenomes. *Nature Microbiology*, *3*, 804–813.

Deng, K., Ouyang, X. Y., Chu, Y., & Zhang, Q. (2017). Subgingival microbiome of gingivitis in Chinese undergraduates. *The Chinese Journal of Dental Research: The Official Journal of the Scientific Section of the Chinese Stomatological Association (CSA)*, *20*, 145–152.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Wen-Han, Y., Lakshmanan, A., & Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, *192*, 5002–5017.

Dietrich, T., Bernimoulin, J.-P., & Glynn, R. J. (2004). The effect of cigarette smoking on gingival bleeding. *Journal of Periodontology*, *75*, 16–22.

Dietrich, T., Krall Kaye, E., Nunn, M. E., Van Dyke, T., & Garcia, R. I. (2006). Gingivitis susceptibility and its relation to periodontitis in men. *Journal of Dental Research*, *85*, 1134–1137.

Eberhard, J., Grote, K., Luchtefeld, M., Heuer, W., Schuett, H., Divchev, D., Scherer, R., Schmitz-Streit, R., Langfeldt, D., Stumpp, N., Staufenbiel, I., Schieffer, B., & Stiesch, M. (2013). Experimental gingivitis induces systemic inflammatory markers in young healthy individuals: A single-subject interventional study. *PLoS ONE*, *8*.–https://doi.org/10.1371/journal.pone.0055265

Eren, A. M., Esen, Ö. C., Quince, C., Vineis, J. H., Morrison, H. G., Sogin, M. L., & Delmont, T. O. (2015). Anvi'o: An advanced analysis and visualization platform for 'omics data. *PeerJ*, *3*, e1319.

Ferraro, C. T. L., Gornic, C., Barbosa, A. S., Peixoto, R. J. M., & Colombo, A. P. V. (2007). Detection of Dialister pneumosintes in the subgingival biofilm of subjects with periodontal disease. *Anaerobe*, *13*, 244–248.

Finn, D. R., Clements, J., & Eddy, R. S. (2011). HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Research*, *39*, W29–W37.

Finn, R. D., Bateman, A., Clements, J., Eddy, S. R., Luciani, A., Potter, S. C., Qureshi, M., Richardson, L. J., Salazar, G. A., Smart, A., Sonnhammer, E. L. L., Hirsh, L., Paladin, L., Piovesan, D., Tosatto, S. C. E., & Finn, R. D. (2014). Pfam: The protein families database. *Nucleic Acids Research*, *42*, 222–230.

Gong, Y., Lu, J., & Ding, X. (2011). Clinical, microbiologic, and immunologic factors of orthodontic treatment-induced gingival enlargement. *American Journal of Orthodontics and Dentofacial Orthopedics*, *140*, 58–64.

Grevich, S., Lee, P., Leroux, B., Ringold, S., Darveau, R., Henstorf, G., Berg, J., Kim, A., Velan, E., Kelly, J., Baltuck, C., Reeves, A., Leahey, H., Hager, K., Brittnacher, M., Hayden, H., Miller, S., McLean, J., & Stevens, A. (2019). Oral health and plaque microbial profile in juvenile idiopathic arthritis. *Pediatric Rheumatology Online Journal*, *17*, 1–10.

Guo, R., Lin, Y., Zheng, Y., & Li, W. (2017). The microbial changes in subgingival plaques of orthodontic patients: A systematic review and meta-analysis of clinical trials. *BMC Oral Health*, *17*, 1–10.

Gursoy, U. K., Kononen, E., & Uitto, V. J. (2009). Prevotella intermedia ATCC 25611 targets host cell lamellipodia in epithelial cell adhesion and invasion. *Oral Microbiology and Immunology*, *24*, 304–309.

Haffajee, A. D., Socransky, S. S., Patel, M. R., & Song, X. (2008). Microbial complexes in supragingival plaque. *Oral Microbiology and Immunology*, *23*, 196–205.

He, T., Qu, L., Chang, J., & Wang, J. (2018). Gingivitis models - Relevant approaches to assess oral hygiene products. *The Journal of Clinical Dentistry*, *29*, 45–51.

Huang, S., Li, R., Zeng, X., He, T., Zhao, H., Chang, A., Bo, C., Chen, J., Yang, F., Knight, R., Liu, J., Davis, C., & Xu, J. (2014). Predictive

modeling of gingivitis severity and susceptibility via oral microbiota. *ISME Journal*, *8*, 1768–1780.

Huang, S., Li, Z., He, T., Bo, C., Chang, J., Li, L., He, Y., Liu, J., Charbonneau, D., Li, R., & Xu, J. (2016). Microbiota-based signature of gingivitis treatments: A randomized study. *Scientific Reports*, *6*, 1–9.

Huang, S., Yang, F., Zeng, X., Chen, J., Li, R., Wen, T., Li, C., Wei, W., Liu, J., Chen, L., Davis, C., & Xu, J. (2011). Preliminary characterization of the oral microbiota of Chinese adults with and without gingivitis. *BMC Oral Health*, *11*, 33. https://doi.org/10.1186/1472-6831-11-33

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H. J., & Tappu, R. (2016). MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Computational Biology*, *12*, 1–12.

Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S. Y., Lopez, R., & Hunter, S. (2014). InterProScan 5: Genome-scale protein function classification. *Bioinformatics*, *30*, 1236–1240.

Kang, D. D., Froula, J., Egan, R., & Wang, Z. (2015). MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ*, *3*, e1165.

Kinane, D. F., & Attstrom, R. (2005). Advances in the pathogenesis of periodontitiss. Group B consensus report of the fifth European workshop in periodontology. *Journal of Clinical Periodontology*, *32*, 130–131.

Kistler, J. O., Booth, V., Bradshaw, D. J., & Wade, W. G. (2013). Bacterial community development in experimental gingivitis. *PLoS One*, *8*(8), e71227. https://doi.org/10.1371/journal.pone.0071227

Kolenbrander, P. E. (2011). Multispecies communities: Interspecies interactions influence growth on saliva as sole nutritional source. *International Journal of Oral Science*, *3*, 49–54.

Könönen, E., Gursoy, M., & Gursoy, U. K. (2019). Periodontitis: A multifaceted disease of tooth-supporting tissues. *Journal of Clinical Medicine*, *8*, 1135.

Koopman, J. E., Van Der Kaaij, N. C. W., Buijs, M. J., Elyassi, Y., van der Veen, M. H., Crielaard, W., Ten Cate, J. M., & Zaura, E. (2015). The effect of fixed orthodontic appliances and fluoride mouthwash on the oral microbiome of adolescents - A randomized controlled clinical trial. *PLoS ONE*, *10*, 1–17.

Lang, N. P., Schätzle, M. A., & Löe, H. (2009). Gingivitis as a risk factor in periodontal disease. *Journal of Clinical Periodontology*, *36*, 3–8.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*, 357–359.

Letunic, I., & Bork, P. (2019). Interactive Tree of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Research*, *47*, 256–259.

Li, D., Liu, C. M., Luo, R., Sadakane, K., & Lam, T. W. (2015). MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, *31*, 1674–1676.

Lie, M. A., Van Der Weijden, G. A., Timmerman, M. F., Loos, B. G., van Steenbergen, T. J., & van der Velden, U. (2001). Occurrence of Prevotella intermedia and Prevotella nigrescens in relation to gingivitis and gingival health. *Journal of Clinical Periodontology*, *28*, 189–193.

Lin, W., Jiang, W., Hu, X., Gao, L., Ai, D., Pan, H., Niu, C., Yuan, K., Zhou, X., Xu, C., & Huang, Z. (2018). Ecological shifts of supragingival microbiota in association with pregnancy. *Frontiers in Cellular and Infection Microbiology*, *8*, 1–11.

Lipták, L., Szabó, K., Nagy, G., Márton, S., & Madléna, M. (2018). Microbiological changes and Caries-Preventive effect of an innovative varnish containing chlorhexidine in orthodontic patients. *Caries Research*, *52*, 272–278.

Liu, F., Ma, R., Wang, Y., & Zhang, L. (2018). The clinical importance of Campylobacter concisus and other human hosted Campylobacter species. *Frontiers in Cellular and Infection Microbiology*, *8*, 243. https://doi.org/10.3389/fcimb.2018.00243

Lobene, R. R., Mankodi, S. M., Ciancio, S. G., Lamm, R. A., Charles, C. H., & Ross, N. M. (1989). Correlations among gingival indices: A methodology study. *Journal of Periodontology*, *60*, 159–162.

Lobene, R. R., Weatherford, T., Ross, N. M., Lamm, R. A., & Menaker, L. (1986). A modified gingival index for use in clinical trials. *Clinical Preventive Dentistry*, *8*, 3–6.

Lu, J., & Salzberg, S. L. (2020). Ultrafast and accurate 16S rRNA microbial community analysis using Kraken 2. *Microbiome*, *8*, 1–11.

Macuch, P. J., & Tanner, A. C. R. (2000). Campylobacter species in health, gingivitis, and periodontitis. *Journal of Dental Research*, *79*, 785–792.

Marsh, P. D. (2005). Dental plaque: Biological significance of a biofilm and community life-style. *Journal of Clinical Periodontology*, *32*, 7–15.

Matsson, L. (1978). Development of gingivitis in pre-school children and young adults. *Journal of Clinical Periodontology*, *5*, 24–34.

Menzel, P., Ng, K. L., & Krogh, A. (2016). Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nature Communications*, *7*, 1–9.

Mervish, N. A., Hu, J., Hagan, L. A., Arora, M., Frau, C., Choi, J., Attaie, A., Ahmed, M., Teitelbaum, S. L., & Wolff, M. S. (2019). Associations of the oral microbiota with obesity and menarche in inner city girls. *Journal of Childhood Obesity*, *4*, 1–20.

Michelet, H., Fontaniere, B., Magloire, H., Durand, B. M., & Tabone, E. (1991). Extracellular matrix and intermediate filaments in the first stages and repair of experimental gingivitis in man. *Journal de Biologie Buccale*, *19*, 329–341.

Morishita, M., Aoyama, H., Tokumoto, K., & Iwamoto, Y. (1988). The concentration of salivary steroid hormones and the prevalence of gingivitis at puberty. *Advances in Dental Research*, *2*, 397–400.

Murakami, S., Mealey, B. L., Mariotti, A., & Chapple, I. L. C. (2018). Dental plaque–induced gingival conditions. *Journal of Clinical Periodontology*, *45*, S17–S27.

Offenbacher, S., Barros, S., Mendoza, L., Mauriello, S., Preisser, J., Moss, K., de Jager, M., & Aspiras, M. (2010). Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans. *Journal of Clinical Periodontology*, *37*, 324–333.

Oh, T. J., Eber, R., & Wang, H. L. (2002). Periodontal diseases in the child and adolescent. *Journal of Clinical Periodontology*, *29*, 400–410.

Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics*, *385*, 1–9.

Page, R. C., & Schroeder, H. E. (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Laboratory Investigation*, *34*, 235–249.

Park, O. J., Yi, H., Jeon, J. H., Kang, S. S., Koo, K. T., Kum, K. Y., Chun, J., Yun, C. H., & Han, S. H. (2015). Pyrosequencing analysis of subgingival microbiota in distinct periodontal conditions. *Journal of Dental Research*, *94*, 921–927.

Parks, D. H., & Beiko, R. G. (2010). Identifying biologically relevant differences between metagenomic communities. *Bioinformatics*, *26*, 715–721.

Paulson, J. N., Stine, O. C., Bravo, H. C., & Pop, M. (2013). Robust methods for differential abundance analysis in marker gene surveys. *Neture Methods*, *10*, 1200–1202.

Ranjan, R., Rani, A., Metwally, A., McGee, H. S., & Perkins, D. L. (2016). Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. *Biochemical and Biophysical Research Communications*, *469*, 967–977.

Ren, Y., Jongsma, M. A., Mei, L., van der Mei, H. C., & Busscher, H. J. (2014). Orthodontic treatment with fixed appliances and biofilm formation–a potential public health threat? *Clinical Oral Investigations*, *18*, 1711–1718. https://doi.org/10.1007/s00784-014-1240-3

Rinke, J., Schäfer, V., Schmidt, M., Ziermann, J., Kohlmann, A., Hochhaus, A., & Ernst, T. (2013). Genotyping of 25 leukemia-associated genes in a single work flow by next-generation sequencing technology

with low amounts of input template DNA. *Clinical Chemistry*, *59*, 1238–1250.

Roller, B. R. K., Stoddard, S. F., & Schmidt, T. M. (2016). Exploiting rRNA operon copy number to investigate bacterial reproductive strategies. *Nature Microbiololgy*, *1*, 1–7.

Sanz, M., Beighton, D., Curtis, M. A., Cury, J. A., Dige, I., Dommisch, H., Ellwood, R., Giacaman, R. A., Herrera, D., Herzberg, M. C., Könönen, E., Marsh, P. D., Meyle, J., Mira, A., Molina, A., Mombelli, A., Quirynen, M., Reynolds, E. C., Shapira, L., & Zaura, E. (2017). Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. *Journal of Clinical Periodontology*, *44*, S5–S11.

Schätzle, M., Löe, H., Bürgin, W., Anerund, A., Boysen, H., & Lang, N. P. (2003). Clinical course of chronic periodontitis: I. role of gingivitis. *Journal of Clinical Periodontology*, *30*, 909–918.

Schincaglia, G. P., Hong, B. Y., Rosania, A., Barasz, J., Thompson, A., Sobue, T., Panagakos, F., Burleson, J. A., Dongari-Bagtzoglou, A., & Diaz, P. I. (2017). Clinical, immune, and microbiome traits of gingivitis and peri-implant mucositis. *Journal of Dental Research*, *96*, 47–55.

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics*, *27*, 863–864.

Segata, N., Haake, S., Mannon, P., Lemon, K. P., Waldron, L., Gevers, D., Huttenhower, C., & Izard, J. (2012). Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biology*, *13*(6), R42. https://doi.org/10.1186/gb-2012-13-6-r42

Sharma, N., Bhatia, S., Sodhi, A. S., & Barta, N. (2018). Oral microbiome and health. *Microbiology*, *4*, 42–66.

Shaw, L., Harjunmaa, U., Doyle, R., Mulewa, S., Charlie, D., Maleta, K., Callard, R., Walker, A. S., Balloux, F., Ashorn, P., & Klein, N. (2016). Distinguishing the signals of gingivitis and periodontitis in supragingival plaque: A cross-sectional cohort study in Malawi. *Applied and Environment Microbiology*, *82*, 6057–6067.

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*, 3210–3212.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology*, *25*, 134–144.

Tatakis, D. N., & Trombelli, L. (2004). Modulation of clinical expression of plaque-induced gingivitis: I. Background review and rationale. *Journal of Clinical Periodontology*, *31*, 229–238.

Theilade, E., Wright, W. H., Jensen, S. B., & Löe, H. (1966). Experimental gingivitis in man: II. A longitudinal clinical and bacteriological investigation. *Journal of Periodontal Research*, *1*, 1–13.

Trombelli, L., Farina, R., Silva, C. O., & Tatakis, D. N. (2018). Plaque-induced gingivitis: Case definition and diagnostic considerations. *Journal of Clinical Periodontology*, *45*, S44–S67.

Trombelli, L., Tatakis, D. N., Scapoli, C., Bottega, S., Orlandini, E., & Tosi, M. (2004). Modulation of clinical expression of plaque-induced gingivitis: II. Identification of 'high-responder' and 'low- Responder' subjects. *Journal of Clinical Periodontology*, *31*, 239–252.

Wang, Y., Xu, L., Gu, Y. Q., & Coleman-Derr, D. (2016). MetaCoMET: A web platform for discovery and visualization of the core microbiome. *Bioinformatics*, *32*, 3469–3470.

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*, 1–13.

Wu, Y. W., Simmons, B. A., & Singer, S. W. (2015). MaxBin 2.0: An automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics*, *32*, 605–607.

Zarco, M. F., Vess, T. J., & Ginsburg, G. S. (2012). The oral microbiome in health and disease and the potential impact on personalized dental medicine. *Oral Diseases*, *18*, 109–120.

Zhang, C. Z., Cheng, X. Q., Li, J. Y., Zhang, P., Yi, P., Xu, X., & Zhou, X. D. (2016). Saliva in the diagnosis of diseases. *International Journal of Oral Science*, *8*, 133–137.

Zhang, Y., Zhen, M., Zhan, Y., Song, Y., Zhang, Q., & Wang, J. (2017). Population-genomic insights into variation in Prevotella intermedia and Prevotella nigrescens isolates and its association with periodontal disease. *Frontiers in Cellular and Infection Microbiology*, *7*, 1–13.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wirth R, Maróti G, Lipták L, et al. Microbiomes in supragingival biofilms and saliva of adolescents with gingivitis and gingival health. *Oral Dis*. 2022;28:2000–2014. https://doi.org/10.1111/odi.13883