Ecological Perturbations Impact Species-Specific Gene Expression of the Oral Microbiota

Daniel Belstrøm  
University of Copenhagen Faculty of Health and Medical Sciences: Kobenhavns Universitet Sundhedsvidskabelige Fakultet

Florentin Constancias (florentinconstancias@gmail.com)  
SCELSE/NTU  https://orcid.org/0000-0002-8612-384X

Daniela I Drautz-Moses  
NTU: Nanyang Technological University

Stephan C Schuster  
NTU: Nanyang Technological University

Mark Veleba  
NTU: Nanyang Technological University

Frédéric Mahé  
Centre de Coopération Internationale en Recherche Agronomique pour le Développement: CIRAD

Michael Givskov  
University of Copenhagen: Kobenhavns Universitet

Research

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Abstract

Background: The purpose of the present investigation was to use the oral cavity as an in-vivo model to study the impact of internal and external perturbations on bacterial biofilm communities. We tested the hypotheses that bacterial gene expression of the healthy microbiota reflects habitation to site-specific ecological perturbations, and that the perturbation effect of chronic inflammation, i.e. periodontitis, impacts bacterial gene expression not only locally, but also at other sites of the oral cavity. Oral microbial samples were collected from three oral sites – plaque, tongue and saliva from patients with periodontitis and healthy controls. Paired metagenomics and metatranscriptomics were used to perform concomitant characterization of taxonomic composition and to determine species specific bacterial activity as expressed by the ratio of messenger RNA to the corresponding genomic DNA.

Results: Here we show the impact of two perturbations – oral site and periodontitis - on bacterial gene expression of the oral microbiota. The oral site was the main determinant of taxonomic composition as well as bacterial gene expression. However, bacterial activity at the three oral sites (plaque, tongue, and saliva) was significantly impacted by periodontitis, with a reduction of the carbohydrate metabolism.

Conclusions: Data from the present study characterize the impact of two perturbations – oral site and periodontitis - on bacterial gene expression of the oral microbiota. The oral site was the main determinant of taxonomic composition as well as bacterial gene expression. However, presence of periodontitis had impact on bacterial activity of both plaque but also on tongue and the salivary microbiota. Collectively, data suggest that periodontitis associates with impaired carbohydrate metabolism of the oral microbiota. Future longitudinal and interventional studies are warranted to evaluate the potential pathogenic role of impaired bacterial carbohydrate metabolism not only in periodontitis, but also in other diseases with low grade inflammation, such as type 2 diabetes mellitus.

Background

Of the 700 different bacterial species colonizing the human body, 200 are found in the oral cavity, with a baseline of appr. 50 different bacterial species at each oral site. The oral cavity harbours the second most diverse microbiota found in the human organism, which is comprised of more than 700 different bacterial species [1].

The oral microbiota continuously adapts to the host, which is why only a fraction of the total microbiota – some 200 different species – is identified in an oral cavity, with a threshold of approximately 50 different species being present in the polymicrobial biofilm colonizing each oral site 1. The oral cavity is an open-ended eco-system, which is why the oral microbiota is constantly stressed by internal and external perturbations [2]. The prime constant ecological determinant is O₂ availability, which is critically different across oral sites, with the buccal mucosa and the tongue surface as examples of areas with extreme anaerobic and aerobic conditions, respectively. As a consequence of microbial adaptation to the ecological conditions being present at different oral sites, considerable taxonomic variations are observed at various oral sites [3, 4]. As the taxonomic composition of the oral microbiota has been characterized in depth in oral health, the oral cavity is an ideal model system to use in studies on the effect of internal and external perturbations on polymicrobial biofilms thriving in areas with different ecological conditions.

Periodontitis, which is a chronic inflammatory disease of the tooth supporting tissue, is an example of an internal perturbation, while cigarette smoking and electronic cigarettes are potential external stressors on the oral microbiota, all with known effect on bacterial gene expression in the biofilm found adjacent to the teeth – plaque [5–7]. Recently, we have used contemporary paired metagenomic and metatranscriptomic approach to reveal that periodontitis as perturbation impacts bacterial activity in saliva [8], which suggest that periodontitis as perturbation, may impact other parts of the oral microbiota than the local biofilm, which is directly involved in the biological interactions caused by the perturbation itself. However, another explanation could be that parts of the oral microbiota are sometimes translocated from one site to another with saliva as the transport vehicle, as demonstrated by correlation of plaque and salivary presence of specific bacterial species in patients with periodontitis [9, 10]. On the other hand, the anaerobic crypts of the tongue offers ecological conditions comparable of those identified adjacent to the teeth, which is why anaerobic bacterial species associated with periodontitis are also part of the of the resident tongue microbiota [11–13]. Therefore, the tongue microbiota could in turn be the donator of such gram-negative anaerobic bacterial species belonging to saliva.

In the present investigation we used the oral cavity as an in-vivo model to study the impact of internal and external perturbations on bacterial biofilm communities. We chose the mouth as model system, since the oral cavity offers easy access to sampling sites with substantially different ecological conditions and microbial communities. Metagenomics and metatranscriptomics were employed to perform concomitant characterization of taxonomic composition as well as specific bacterial gene expression profiles. Furthermore, pairing of metagenomic and metatranscriptomic data enabled us to determine species specific bacterial activity as expressed by the ratio of messenger RNA to the corresponding genomic DNA. In other words, the resolution of our paired deep sequencing approaches enable us to discriminate between
members of the microbiota, with over-expression of important pathways (log10(RNA/DNA) > 0) as compared to members with less expression (log10(RNA/DNA) < 0). Samples from three different oral sites – plaque, tongue and saliva – were collected to test the hypothesis that bacterial gene expression of the healthy microbiota reflects habituation to site specific ecological perturbations. In addition, we tested the hypothesis that the perturbation effect of chronic inflammation i.e. periodontitis impacts bacterial gene expression not only locally (plaque), but also at other sites of the oral cavity (tongue and saliva).

Methods

Study population and sample collection

The study population included 11 patients with chronic periodontitis, and 11 orally healthy controls, which were enrolled in January 2018 at University of Copenhagen, Department of Odontology. Periodontitis was defined as bleeding on probing ≥ 25% of total sites + minimum two teeth with clinical attachment level ≥ 4 mm + minimum two teeth with probing depth ≥ 6 mm [14]. Exclusion criteria: age < 50 yrs., systemic diseases, use of any kind of medication including usage of antibiotics within the last three months.

The periodontitis group was comprised of 5 males and 6 females with a mean age of 64 yrs. (55–77 yrs.), whereas the control group included 4 males and 7 females with at mean age of 60 yrs. (50–71 yrs.). Four periodontitis were current daily smokers, as compared to three current smokers in the control group. All 22 participants signed informed consent. The study was approved by the regional ethical committee (H-16016368) and reported to the local data authorization of University of Copenhagen (SUND-2018-8).

A total of three microbial samples were collected from each participants, including plaque samples (n = 22), tongue scrapings (n = 22) and stimulated saliva samples (n = 22), which were collected between 8:00 AM and 11:00 AM. Microbial Samples were consequently collected in the same order: stimulated saliva, tongue coating, and plaque according to standardized protocols as previously described [15, 16]. Plaque and tongue coatings were suspended in 2 mL Sodium Chloride (9 mg/mL). Immediately, after collection each sample was divided in two aliquots of 1 mL each, one each for metagenomics and metatranscriptomics analysis. RNAlater (Life Technologies, Denmark) was added to the aliquot allocated for metatranscriptomics and all aliquots were immediately stored at -80 °C until further processing.

Metagenomic and metatranscriptomic library preparation and sequencing

Preparation of DNA and RNA library was performed according to Illumina's TruSeq Nano DNA Sample Preparation protocol and Stranded mRNA, respectively. DNA samples were sheared on a Covaris E220 to ~ 450 bp, following the manufacturer's recommendation, and uniquely tagged with one of Illumina's TruSeq HT DNA dual barcode combination to enable sample pooling for sequencing. The following modifications were applied to Illumina's TruSeq Stranded mRNA protocol. The oligo-dT mRNA purification step was omitted and instead, 200 ng of total RNA were directly added to the Elution2-Frag-Prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely tagged with one of Illumina's TruSeq HT RNA dual barcode combination to allow pooling of libraries for sequencing. Both DNA and RNA libraries were quantitated using Promega's QuantiFluor dsDNA assay and the average library size was determined on an Agilent Tapestation 4200. Library concentrations were then normalized to 4 nM and validated by qPCR on a QuantStudio-3 real-time PCR system (Applied Biosystems), using the Kapa library quantification kit for Illumina platforms (Kapa Biosystems).

The libraries were then pooled at equimolar concentrations and sequenced on the Illumina HiSeq2500 platform at a read-length of 250 bp paired-end and 100 pb paired-end for DNA and RNA libraries, respectively.

Read preprocessing

Illumina TruSeq adaptors, 5’ or 3’ bases with quality scores lower than 20, as well as read pairs having a mate with any ambiguous base (i.e., N) or shorter than 150 or 50 bp for DNA and RNA sequences, respectively, were trimmed using atropos [17] in paired-end mode (version 1.1.25, -max-n 0 -n 1-q 20,20 –quality-base 33 –minimum-length 150/50 -O 6).

RNA reads were then subjected to sortmerna [18] version 2.1, default parameters, 5S, 16S, 23S, 18S and 28S databases), using provided rRNA databases for in silico depletion of ribosomal RNA. Ribosomal RNA sequences represented an average of 83.9% of the quality-trimmed RNA reads (71.1 – 88.4%). Human reads were then removed from both metagenomic and metatranscriptomic datasets by aligning DNA and mRNA read pairs to the human genome (GRCh38, ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_28/GRCh38.primary_assembly.genome.fa.gz, downloaded on 08/08/2018). Alignments were performed using Bowtie2 [19] (version 2.3.4.1, -dovetail) for DNA reads and hisat2 (20) version 2.1.0, -dta) for RNA reads, respectively. Samtools ([21] version 1.3, view -b -f 12 -F 256) and bedtools ([22] version 2.24.0, bamToFastq) were then used...
to identify and extract read pairs which consistently did not map the human reference genome, those were considered for microbial and taxonomical and functional profiling.

**Taxonomic and functional community profiling**

Taxonomic composition of metagenomic reads was assessed using MetaPhlan (version 3.0.1, 25 Jun 2020), --min_ab 0.000001 using the latest available database i.e., mpa_v30_CHOCOPhiAn_201901). Briefly, MetaPhlan relies on read mapping against a built-in collection of clade-specific marker genes database which allows an unambiguous estimation of species relative abundance across samples with a low species resolution false positives rate as compared to read classifiers. This is especially true when working with human microbiome data (https://www.researchgate.net/publication/343635031_Tutorial_Assessing_metagenomics_software_with_the_CAMI_benchmarking_toolkit). As the tool is based on marker genes and is not designed to assign taxonomic information to all reads nor rRNA sequence as taxonomic marker, a direct taxonomic classification of metatranscriptomic reads is not possible.

Sample-specific taxonomic profiles generated using MetaPhlan on metagenomes were used to filter the HUMAnN2 ([23] built-in pangenomes database to the organism present in the sample. Metagenomic and metatranscriptomic reads were then mapped using Bowtie2 to sample-specific pangenomes. Metagenomic and metatranscriptomic reads failing to align to the pangenome databases were then blasted against UniRef90 using DIAMOND [24]. Hits were counted per gene family and normalized for length and alignment quality. Gene family abundances were then combined into pathways level using MetaCyc [25] and normalized to relative abundances. Functional gene family tables were regrouped to KEGG’s orthologs and Enzyme (Enzyme commission number, i.e., EC numbers) with the provided humann2 UniRef90_to_KO and UniRef90_to_EC numbers mapping files, respectively [23].

Since sample-specific HUMAnN2 pangenome database are filtered based on Metaphlan taxonomic profiles, a complementary taxonomic profiling analysis was conducted using mOTUs profiler ([26] version 2.5.0) which also includes MAGs (i.e., species without reference genomes in the standard databases) to confirm the observed patterns.

**Statistical analysis**

Taxonomic profiles generated using MetaPhlan as well as functional metagenomic and metatranscriptomic pathway and gene family tables generated using HUMAnN2 were imported in R and loaded into phyloseq objects for data handling [27].

Taxonomic tables as well as Pathway and Gene family tables were characterized using alpha-diversity indices (i.e., number of observed Species/Pathways/Gene families, Shannon diversity index and Pielou's evenness). Taxonomic and functional data were also characterized in terms of β-diversity (i.e., taxonomic community structure and metabolic profiles) using Atchinson distance computed on taxonomic count tables as well as weighted and binary Jaccard metrics for Pathways and Genes relative abundance normalized tables. Principle COordinate Analysis (i.e., PCoA) was used to depict microbial community structure using vegan R package (cmdscale()). Relationship with community distance and ordination space was assessed using Shepard Diagrams to ensure quality of the ordinations.

Differences between sites and health status were tested using two-sided non parametric Kruskal-Wallis and PERMANOVA statistical tests for alpha and beta-diversity, respectively. Multivariate Welch t-test was also run to confirm highlighted PERMANOVAS patterns [28] and multivariate homogeneity was assessed to ensure PERMANOVA’s assumptions.

Aldex2 was used in order to detect differentially abundant Species among Oral Site and Health Status on count normalized species tables [29] and MaAsLin2 (https://huttenhower.sph.harvard.edu/maaslin/) for pathway and gene families relative abundance normalized tables. Pathway and gene families table from metagenomes (DNA) and metatranscriptomes (RNA) and computed DNA/RNA ratio were log transformed and only features occurring in at least 20% of the samples were considered. UNMAPPED and UNITEGRATED pathways were removed from alpha/beta-diversity analysis but kept for linear modelling approach using MaAsLin2 in order to limit the potential for housekeeping functions to artificially inflate in less-well-characterized samples.

When testing for differences between Oral Sites, Subject identifier (Subject ID) was added as random factor and PERMANOVA’s permutations were restricted to take into account repetitive sampling within the same Subject.

In order to correct for multiple comparisons p.values generated using Kruskal-Wallis, Aldex2 Maaslin2 and PERMANOVAS tests were FDR corrected.

**Results**

**Sequence processing:**
A total of 7 samples failed quality controls, which means that 59 samples were included in downstream DNA and RNA analyses. From a total of 59 microbial samples (19 plaque samples, 18 tongue biofilm, 22 saliva samples) 1,215 M DNA sequences (per-sample mean 20.59 M; range 15.14 M – 35.05 M); and 3,093 RNA sequences (per-sample mean 52.43 M; range 39.97 M – 74.65 M) were generated.

In total, 612, 851 M (mean 10.38M; range 0.52 M – 21.67 M) DNA read pairs and 95, 943 M (mean 1.62 M; range 0.27 M – 2.9 M) RNA read pairs were used after quality filtering, rRNA removal and host (i.e., human) contamination for further analysis. A comparable average number of DNA and RNA sequences passing quality control were identified in samples from each site: plaque (DNA: 7.86M, RNA: 1.16M), tongue (DNA: 16.84M, RNA: 1.62M), and saliva (DNA: 7.29M, RNA: 2.03M), and as well as in patients with periodontitis (DNA: 9.94M, RNA: 1.49M) versus orally healthy controls (DNA: 10.84M, RNA: 1.76M).

**Microbial diversity and taxonomic composition is influenced by site and health status**

A comparison of α-diversity as measured by Shannon index, Species Richness and Evenness revealed significant differences based on site, with higher α-diversity in plaque and saliva, as compared to tongue biofilm. However, α-diversity at each site was not influenced by health status (Fig. 1A). Also, species community structure quantified by Atchinson distance was more influenced by site (PERMANOVA, R² = 0.33, p = 0.001) than health status (PERMANOVA, R² = 0.07, p = 0.001, Fig. 1B) and the interaction term between oral site and health status was not significant indicating that health status is consistent with species community structure. Figure 1C displays relative abundance of top 27 predominant bacterial species across the three sites and their health status. The microbiota of each site was characterized with a specific species combination being clearly different from the other sites. On the other hand, plaque was the only site, where the predominant species were influenced by health status, as exemplified by *Tannerella forsythia*, which was present in almost all samples from patients with periodontitis, and in only one sample from the healthy controls. A comparison of relative abundance of all species across all samples revealed 25 species, with significantly different relative abundances in samples from patients with periodontitis versus healthy controls, with higher relative abundances of periodontal pathogens such as *Filifactor alocis, Parvimonas micra, Prevotella intermedia, Treponema denticola* and *T. forsythia* identified in samples from patients with periodontitis, and higher relative abundances of *Actinomyces* species in samples from healthy controls (Fig S1A). In general, the highest abundances of periodontal pathogens were observed in plaque samples, followed by saliva, and with the lowest abundance in tongue biofilm (Fig. S1B).

**Global bacterial pathway expression is shaped mostly by site, but also by oral health status**

Figure 2A shows overall pathway species contribution richness for both metagenomic (DNA) and metatranscriptomic (RNA) datasets in all samples. As can be seen, all pathways were not expressed (RNA) by all taxa with a potential of expressing the corresponding pathways (DNA). Some pathways, such as *adenosine ribonucleotides de novo biosynthesis* and *guanosine ribonucleotides de novo biosynthesis*, which are critical to cell survival, could be expressed by almost all members of the oral microbiota (> 190 taxa). On the other hand, more specialized pathways involved in carbohydrate metabolism, including *starch degradation V* and *lactose and galatose degradation I* could be expressed by less than 75 different taxa.

Figure 2B displays the relationship between pathway abundance and contributional species diversity (Shannon diversity index). Each point represents a specific pathway and depicts its sample abundance as well as diversity of species with potential (DNA) and actual (RNA) contribution. Correlation in terms of pathway abundance and contributing species diversity was observed in metagenomes (DNA). While the same trend was also observed in the metatranscriptomes (RNA), some pathways were clearly identified with high expression abundances despite low bacterial numbers of the contributing bacteria. In total, no differences were seen in overall bacterial activity (log10(RNA/DNA)) at each site in relation to health status. However, a significantly higher overall bacterial activity was observed in plaque samples as compared to saliva and tongue (Fig S2).

Figure 2C shows per-pathway DNA versus RNA contributions of top 27 bacterial species as described in Fig. 1C. Each point represents the overall species transcriptional activity averaged within samples from the three different sites in all individual. Major differences were observed within each predominant genus, with some members exhibiting an overall tendency for high transcriptional activity (log10(RNA/DNA) > 0), as compared low (log10(RNA/DNA) < 0) by other members. In general, the highest RNA/DNA ratios were identified in *Streptococcus* and *Prevotella* species, while *Actinomyces* species in general were identified with (log10(RNA/DNA) < 0). As seen, high species-specific expression of certain pathways was observed, as demonstrated by *LACTOSECAT-PWY* and *ANAGLYCOLYSIS-PWY* by *Streptococcus salivarius* and *Streptococcus infantis*, respectively.

Figure 2D shows the pathway expression by bacterial transcriptional activities, of the 25 bacterial species identified with significantly different relative abundance in samples from patients with periodontitis versus healthy controls (Fig.S1A). Bacterial expression profiles were
Significantly different bacterial pathway expression in plaque and saliva in patients with periodontitis

While α-diversity metrics of potential and expressed pathways (DNA and RNA datasets, respectively) was not significantly different between site or health status (Fig. S3A), both site and health status had a significant impact on β-diversity of pathway RNA/DNA expression (Fig. 3A & 3B). In addition, bacterial activity of 22 pathways (as quantified by pathway level RNA/DNA ratio) was significantly different between periodontitis versus healthy controls, when comparing samples from all sites. Nineteen pathways were highly expressed in conditions of oral health, most of which were pathways contributing to carbohydrate metabolism. On the other hand, only three pathways were identified with higher RNA/DNA ratios in periodontitis, including pyrimidine deoxyribonucleotide phosphorylation and 6-hydroxymethyl dihydropterin diphosphate biosynthesis I (Fig. 3B).

Figure 3C shows bacterial species with the potential to express (DNA) the pathways identified with different expression in periodontitis versus health, including which bacterial species that were actually expressing the pathways (RNA). As seen, the bacterial signatures, in terms of DNA and RNA expression, were completely different based on site. Furthermore, it was evident that while some species despite high abundances of specific pathways within the metagenomes, were actually not contributing extensively to the total expression profiles, as evident from their low levels of concomitant pathway’s proportion in the RNA dataset. In addition, RNA expression of the 22 significant pathways was clearly different in plaque samples in health versus diseases, with much higher RNA expression by L. hofstadii in health. When comparing samples from each site based on health status, a total of 14 pathways were identified with significantly different RNA/DNA ratios in plaque, whereas a significantly higher RNA/DNA ratio of sucrose degradation III was observed in saliva of the healthy samples. No differences in RNA/DNA ratios were observed in tongue biofilm in health versus periodontitis (Fig. S3B-C).

Bacterial gene expression reveals impaired carbohydrate metabolism in periodontitis

Alpha-diversity of DNA and RNA gene expression as quantified by observed, evenness and shannon-index was significantly influenced by site, but not by health status (Fig. 4A, Fig. S4A). On the other hand, β-diversity of RNA/DNA ratios were significantly influenced by site, and also to a lesser but significant degree by health status (Fig. 4B, Fig. S4B).

Figure 5A shows the 48 bacterial genes (EC numbers), which were identified by a significantly different RNA/DNA ratio in periodontitis versus health (based on analysis of all samples). Specifically, 36 genes were observed as having higher RNA/DNA ratios in health as compared to 12 genes in periodontitis. When performing pairwise comparison of samples collected at each site, 84 genes in plaque (Fig. 5B, 61 health associated, 23 periodontitis associated), and 7 genes in tongue (Fig. 5C, 4 health associated, 3 periodontitis associated), were identified with a significantly different RNA/DNA ratio in periodontitis versus health. No genes were observed with significant different RNA/DNA ratio in saliva samples obtained from health and periodontitis. Comparable findings were attained considering gene families matching KEGG’s orthologs (Figs. S5A-S5D).

Nine out of the 10 genes with the highest RNA/DNA ratios were significantly more expressed in oral health. Six of these genes contribute to carbohydrate metabolism, specifically in different steps of glycolysis. On the other hand, the only top 10 gene identified with higher RNA/DNA ratio in periodontitis was Gingipain_K, which is solely expressed by the periodontal pathogen Porphyromonas gingivalis (Fig. 5A).

Discussion

The purpose of the present investigation was to study the impact of an internal perturbation, periodontitis, on bacterial gene expression profiles in three different compartments of the oral microbiota – plaque, tongue and saliva. We used contemporary metagenomics and metatranscriptomics analyses to test the hypothesis that periodontitis exerts an effect on bacterial gene expression not only locally in the plaque microbiota, but also in saliva and at distant sites such as the tongue. To the best of our knowledge, this is the first study to perform paired metagenomic and metatranscriptomic guided characterization of multiple compartments of the oral microbiota in oral health and disease.

As expected, major differences in taxonomic composition of predominant bacterial species identified in plaque, tongue biofilm and saliva were observed (Fig. 1C). In general, correlation was seen between tongue and saliva. Thus, data supports the present view of the tongue being the prime contributor shaping the salivary microbiota [4]. On the other hand, while predominant bacterial species identified in plaque were almost completely absent in tongue, small amounts were identified in concomitant saliva samples. These findings suggest that at least
a smaller proportion of the salivary microbiota is expelled from plaque, as previously suggested by concordance of specific bacterial species in subgingival plaque and saliva in patients with periodontitis [10, 30]. Notably saliva is sterile, when entering the oral cavity [31], and in the present study traces of the predominant plaque microbiota were observed in saliva not only from patients with periodontitis but also from healthy controls. Thus, our species-resolution data analysis confirms the salivary microbiota as a conglomerate of bacteria shed from various oral surfaces not only in periodontitis but also in healthy conditions.

In health, substantial site-specific variations in taxonomic composition were observed within some genera, as exampled by major differences in relative abundance of specific *Rotinia* and *Streptococcus* species identified in plaque versus tongue. Specifically, *Rotinia dentocariosa* and *Streptococcus oralis* were identified with high abundances in plaque, but almost absent in tongue, whereas *Rotinia mucilaginosa* and *Streptococcus salivarius* were predominant in tongue (Fig. 1C). While species within the same genus are taxonomically closely related, site-specific preferences support the idea of co-evolution of the resident microbiota together with the host, which results in gradual adaptation of specific bacterial species to the environment offered at the various oral sites [32].

Bacterial gene expression is shaped by oral site in health, as seen by significant differences in β-diversity at pathway (Fig. 3A) as well as gene level (Fig. 4B). Moreover, bacterial pathway expression as measured by their RNA/DNA ratios identified at each site in oral health, demonstrated site-specific variations in term of general bacterial activity. This is clearly visualized in Fig. 2D with species such as *L. hofstedii* being placed in the upper left corner in plaque, in contrast to saliva and tongue. Likewise, species contribution of pathways being expressed significantly different in periodontis and oral health were completely different at each site (Fig. 3C). Collectively, these findings confirm that taxonomic composition and bacterial gene expression of the resident microbiota in oral health is shaped by the prevailing ecological properties, which are present at each niche in the oral cavity [33].

As perturbation, periodontitis had a clear impact on the composition of the plaque microbiota, as seen by significant different β-diversity (Fig. 1B), together with significantly higher relative abundance of predominant species such as *T. forsythia* (Fig. 1C). Also, 25 bacterial species, including periodontal pathogens such *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *T. denticola* were observed with higher abundances in plaque from patients with periodontitis (Fig.S1A), which is in accordance with previous studies [30, 34]. Likewise, periodontitis had a significant effect on β-diversity of pathway and gene expression, as judged from their RNA/DNA ratios (Figs.S3A and 4A). Furthermore, pronounced differences were observed in specific bacterial gene expression in plaque at both pathway (Figs. 3A and S3B) and gene levels (Figs. 5A-B). Specifically, bacterial activity (RNA/DNA ratios) of pathways involved in carbohydrate metabolism, such as *lactose and galactose degradation I* and *glucose and glucose-1 phosphate degradation* were significantly lower in plaque from patients with periodontitis. Also, significant differences were observed with genes involved in carbohydrate metabolism, where *glyceraldehyde-3-phosphate dehydrogenase* was significantly higher expressed in periodontitis, as compared to *fructose biphosphate aldolase*, which was significantly less expressed. Collectively, these findings probably reflect that the microbiota compositionally and functionally adapts to the perturbation, *i.e.* ecological characteristics such as conditions of anaerobiosis and chronic inflammation being present in the local periodontal environment [34, 35]. Taken together with a recent theory suggesting that frequent carbohydrate consumption may induce inflammation in the periodontal tissues [36], our finding that periodontitis as perturbation impairs carbohydrate metabolism of the plaque microbiota, provides a possible explanation as to why excessive carbohydrate intake may contribute to the pathogenesis of periodontitis. Interestingly, periodontitis is linked with medical disorders such as type 2 diabetes, with conditions of systemic low-grade inflammation as the immediate communality [37]. Thus, it is an interesting hypothesis that impaired bacterial carbohydrate metabolism could be a factor aggravating systemic low-grade inflammation, in general.

Periodontitis also had an effect on bacterial activity in saliva and the tongue, as visualized by specific pathways and their gene expression profiles (Figs. 3C and 5C). In addition, higher activity of specific bacterial species such as *L. hofstadii* was evident in saliva from healthy individuals (Fig. 2B). Specifically, significantly lower bacterial activity of the *sucrose degradation III* pathway was observed in saliva from patients with periodontitis. Furthermore, bacterial activity of genes related to lipid metabolism (*glycerol-3-phosphate-acyltransferase* and carbohydrate metabolism (*beta fructofuranosidase*)) was significantly lower in tongue biofilm from patients with periodontitis. We have previously showed an impact of periodontitis on salivary bacterial activity [8]. However, this is the first study to perform simultaneous characterization of bacterial pathways and their gene expression profiles in plaque, tongue and saliva. It is therefore interesting that pathways and genes identified with significantly different RNA/DNA ratios in saliva and tongue were not the same as those identified in plaque. Therefore, data suggest that periodontitis as perturbation acts differently on bacterial gene expression in different oral compartments as visualized in the present study by plaque, tongue and saliva.

Another interesting finding which was evident from analysis of our high-resolution dataset was that expression of *Gingipain_K* was solely identified in samples from patients with periodontitis (Fig. 5A). *Gingipain_K* is an endopeptidase with strict specificity for lysyl bonds, which is only produced by the periodontal pathogen *P. gingivalis* [38]. Interestingly, previous *in-vitro* studies have shown that presence of *P. gingivalis* dramatically alters the transcriptomic profiles of oral commensals in an artificially grown biofilm [39], which is one of the...
reasons that *P. gingivalis* is the main act in the recently presented key stone hypothesis [40]. Our finding of bacterial expression of *Gingipain_K* exclusively in samples from patients with periodontitis is therefore intriguing. However, future studies are warranted to reveal if *P. gingivalis in vivo* also alters the transcriptome of the resident microbiota or alternatively is counteracted by reliance mechanism of the commensals.

Some limitations apply to the present investigation, including the relatively small sample size, which however is comparable to other metatranscriptomic-based studies on the oral microbiota [41–43]. Furthermore, even though very deep sequencing of DNA and RNA was performed, it was still not possible to portray total gene expression of specific species with an overall low abundance. Accordingly, we could not determine if the transcriptomic profile of for example *P. gingivalis* differentiated between sites and in health versus disease.

**Conclusions**

In conclusion, data from the present study characterize the impact of two perturbations – oral site and periodontitis - on bacterial gene expression of the oral microbiota. The oral site was the main determinant of taxonomic composition as well as bacterial gene expression. However, presence of periodontitis had impact on bacterial activity of both plaque but also on tongue and the salivary microbiota. Collectively, data suggest that periodontitis associates with impaired carbohydrate metabolism of the oral microbiota. Future longitudinal and interventional studies are warranted to evaluate the potential pathogenic role of impaired bacterial carbohydrate metabolism not only in periodontitis, but also in other diseases with low grade inflammation, such as type 2 diabetes mellitus.

**Declarations**

*Ethics approval and consent to participate*

All 22 participants signed informed consent. The study was approved by the regional ethical committee (H-16016368) and reported to the local data authorization of University of Copenhagen (SUND-2018-8).

*Availability of data and material*

Raw sequence data have been deposited at NCBI (Sequence Read Archive) and are available under the BioProject (submission process in progress). Bioinformatic open-source tools and parameters used in this present study are defined in the respective method section and scripts can be found in the following github repository (https://github.com/fconstancias/oral-microbiome/). More details regarding the code to reproduce the analyses is available upon request.

*Competing interests*

The authors declare no conflict of interest.

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*Author contributions*

Daniel Belstrøm (DB), Florentin Constancias (FC) and Michael Givskov (MG) planned the experiment. DB collected the samples. Daniela I. Drautz-Moses (DM) and Stephan C. Schuster (SCS) performed DNA and RNA sequencing. FC defined and performed bioinformatic and biostatistical analyses. Mark Veleba (MV) and Frédéric Mahé (FM) contributed to data handling and statistical analyses. DB wrote the first draft of the paper, which was critically revised by FC and MG.

All authors approved the final version of the manuscript.

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