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Original Article

The Complex Microbiome of Caries-Active and Caries-Free Supragingival Plaques in Permanent Dentition

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ABSTRACT

Background and Aim: Dental caries is one of the most common diseases seen in the oral cavity in all periods of deciduous, mixed, and permanent dentition. A comprehensive study of the oral microbiome is required to understand its polymicrobial etiology. The aim of this study was to reveal the plaque microbiome of caries-active and caries-free adults. **Materials and Methods:** A total of 52 samples were collected from 26 caries-active patients and 26 caries-free controls. Dental supragingival plaque samples were collected from each subject and the bacterial 16S rDNA, expanded V3–V4 region, was amplified using next generation sequencing. **Results:** The core microbiome was defined with 235 shared bacteria in genus level, and among all microbiome 14.8% of all bacteria showed significant difference ($P < 0.05$). The bacteria responsible of caries may be listed as *Anaeroglobus*, *Atopobium*, *Bifidobacterium*, *Centipeda*, *Cryptobacterium*, *Desulfobulbus*, *Filifactor*, *Howardella*, *Lactobacillus*, *Leptotrichiaceae* (unclassified), *Megasphaera*, *Mycoplasma*, *Olsenella*, *Phocaeicola*, *Propionibacterium*, *Pseudoramibacter*, *Scardovia*, *Schwartzia*, *Treponema*, *and Veillonellaceae* (unclassified). **Conclusion:** The present study provides comprehensive knowledge of the microbiological etiology of caries in permanent dentition.

KEYWORDS: *Caries, microbiome, next generation sequencing, permanent dentition*

INTRODUCTION

Biofilm is a layer of prokaryotic and eukaryotic cells that are firmly attached to a living or non-living surface and embedded in an organic matrix of biological origin.[1] The oral cavity of an adult person, which contains approximately 50–100 billion bacteria, is an extremely complex structure.[2] There are approximately 700 species of prokaryotes in the human oral cavity and these bacteria live in balance within the framework of certain connections and relationships.[3-5]

Dental caries is one of the most common diseases seen in the oral cavity in all periods of deciduous, mixed, and permanent dentition.[6] Caries formation is the result of multifactorial events such as plaque microflora, host immune system, dietary habits, and oral hygiene of an individual.[7]

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The detection of oral microorganisms by culture method, besides being time consuming, remains inadequate in most cases due to the unculturable nature of oral bacteria.[3,8] Molecular methods provide a great advantage in the diagnosis of bacteria that cannot be cultured and/or difficult to culture.[9,10]

Next generation sequencing (NGS) method can analyze a large number of sequences with high accuracy and ultra-fast speed.[11] In addition, it allows the genome to be sequenced without any host cell and provides rich and original information.[12,13]

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Therefore, the aim of our study is to define the bacteria responsible for carious and non-carious plaque in permanent dentition with the NGS technology and reveal the bacterial nature of carious lesions.

Materials and Methods

A total of 52 patients aged between 18 and 50 years were divided into two main groups: caries-active (n = 26) and caries-free (n = 26). All subjects were systemically healthy, having no hereditary, morphologically and drug-related structural changes (amelogenesis imperfecta, dentinogenesis imperfecta, fluorosis, molar-incisal hypomineralization, etc.) in their teeth, not received any antibiotic treatment in the last 6 weeks, and having oral hygiene habits were similar (brushing their teeth with toothpaste at least once a day) were included. Caries classification was made on the basis of ICDAS II criteria.[14]

All patients had signed the informed consent form, and before sample collection, they were instructed to avoid any dietary and oral hygiene practices after 20:00 pm. Plaque samples (12 h) were collected with a sharp periodontal curette between 08:00 and 09:00 am transferred to Eppendorf tubes with DNA/RNA shield solution. (Ethical approval of a study was obtained from the Marmara University Faculty of Dentistry Ethics Committee (06.06.2017; #2017-100).

DNA isolation was performed according to the manufacturer's instructions on the isolation kit.

Samples were processed and analyzed by ZymoBIOMICS™ Service – Targeted Metagenomic Sequencing (ZymoResearch, Irvine, CA, USA). 16S library preparation: Bacterial 16S ribosomal RNA gene-targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA, USA). The bacterial 16S primers used expanded the V3–V4 region of the 16S rRNA gene. These primers were specially designed by Zymo Research to provide the best coverage of the 16S gene with high sensitivity. They potentiate the V3–V4 region of the 16S rRNA gene. The sequencing library was prepared using an innovative library preparation process where PCR reactions were performed on real t-PCR machines to control the loops and thus prevent PCR chimera formation. The final PCR products were pooled based on equal molarity as measured by qPCR fluorescence readings. Finally, the common library was filtered with Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, USA), then measured with TapeStation® and Qubit®.

Sequencing: The final library was sequenced on Illumina® MiSeq™ with a V3 reagent kit (600 cycles). Sequencing was performed with >10% PhiX. Bioinformatics analysis of raw data Bioinformatics analysis was carried out by researchers (ZCC & AC) using NGS Mothur software on usegalaxy.org open-source address. The raw data of our study provided by ZymoResearch by following the Standard Operating Procedure presented by the Schloss laboratory within Galaxy, the creator of the Mothur software package.[15] These analyzes took place under the following headings and subheadings:

- Obtaining and preparing data
- Understanding the input data
- Transfer data to Galaxy program
- Quality control
- Sequencing and reduction of sequencing and PCR errors
- Evaluation of error rates according to sample population
- OTU analysis
- Calculating the diversity of species
- Visualization
  - Phinch
  - Krona-Pie-Chart
- Additional operations
- Determining the statistical significance of clusters

Data were analyzed descriptively using the software Stata v. 15.1 (StataCorp, College Station, TX, USA); with the level of significance set at $P < 0.05$ (excluding Bonferroni corrections). Pearson Chi-square and Fisher's exact tests were used to evaluate the relationships between categorical variables. Histogram, Kolmogorov–Smirnov, Q–Q Plot tests, Shapiro-Wilk, Skewness-Kurtosis tests were applied to evaluate the distribution in terms of normality. Since the data distributions were not parametric, Kruskal Wallis tests were used for the evaluation of more than two groups and Mann–Whitney U tests were used for intergroup post-hoc analysis. The P significance level for the Kruskal Wallis test was re-determined as 0.05/4 = 0.0125 according to the number of groups, and statistical significance was examined. Mann–Whitney U tests were used for comparisons between the two groups and $P < 0.05$ was considered statistically significant.

Results

Demographic information, tooth-brushing habits, caries information were shown in Table 1.

The caries status of the patients was diagnosed and recorded according to ICDAS II criteria shown in Table 2.

While 85.2% of the bacteria isolated from carious and non-carious plaque samples did not differ, 14.8% (n = 35) were found to be statistically significant. Descriptive data (median = p50) were listed in Table 3.

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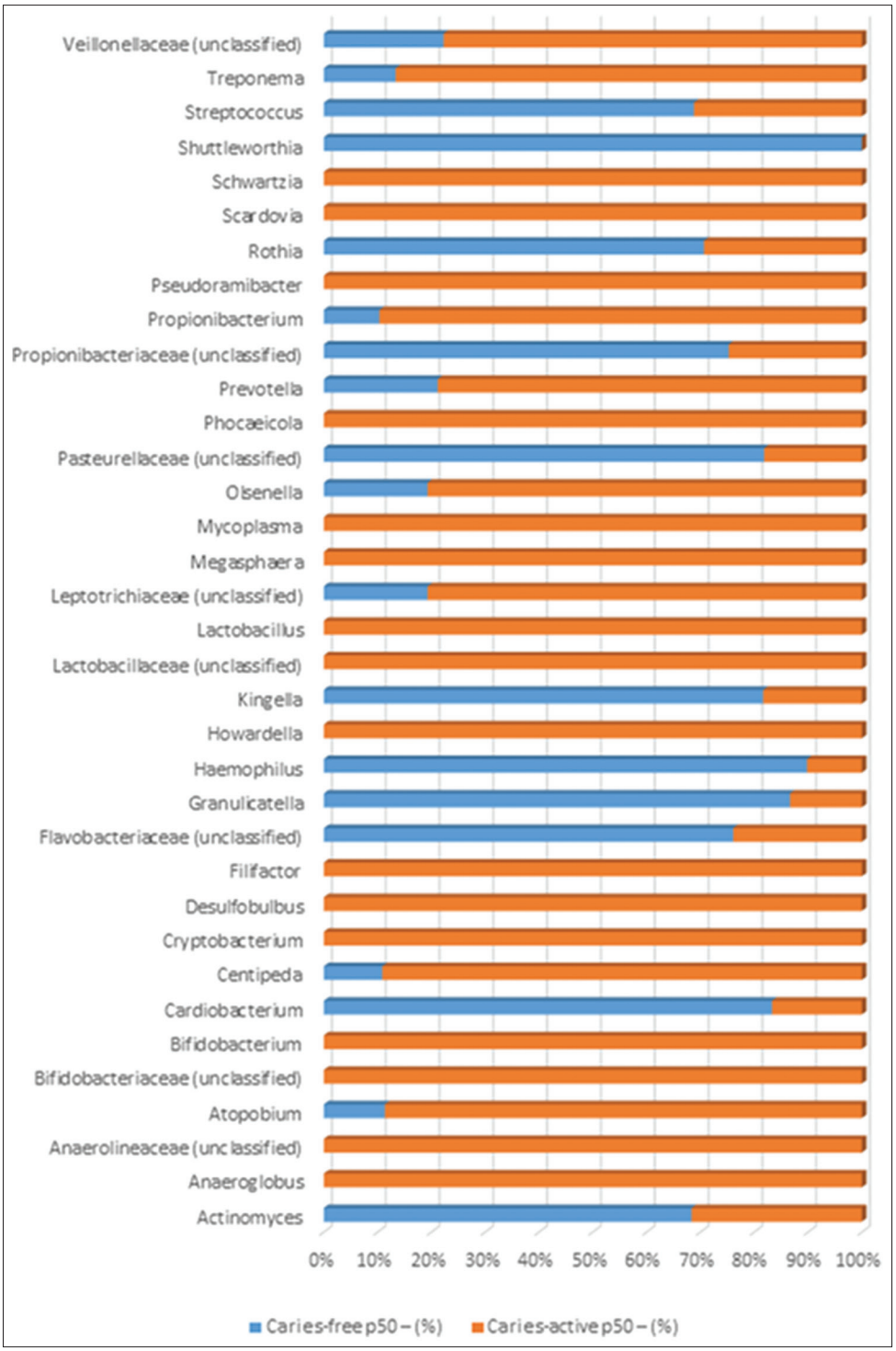

Figure 1: Distribution of oral bacteria in caries-free and caries-active plaques

Distribution of all bacteria in caries-free and caries-active plaques samples was shown in Figure 1.

Discussion

Contemporary concepts of dental caries etiology and pathogenesis emphasize a "mixed bacterial-ecological approach" as being responsible for lesion initiation and progression.[16] Rather than being considered an "infectious disease" caused by a specific organism, dental caries are now understood to be a biofilm-mediated disease.[17] Dental caries can thus be considered to be anendogenous infection, which may occur when members of resident flora obtain a selective ecological advantage over other species, disrupting the homeostatic balance of the biofilm, and thereby initiating the disease process.[18]

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Table 1: Demographic information, tooth-brushing habits, caries information of caries-acrive, and caries-free groups

	Caries-active			Caries-free		
	Female	Male	Total	Female	Male	Total
Age						
Mean±SD	26,2±6,9	30,8.4±7,8	28,7±7,1	24,9±5,8	29,1±8,2	26.7±8.3
Gender						
n (%)	12 (46)	14 (54)	26 (100)	15 (58)	11 (42)	26 (100)
Tooth-brushing habit						
n/day (%)						
None/rare	3 (25)	5 (36)	8 (30)	0 (0)	0 (0)	0 (0)
1	7 (58)	6 (43)	13 (50)	5 (33)	3 (27)	8 (31)
2	2 (17)	3 (21)	5 (20)	7 (47)	6 (55)	13 (50)
2+	0 (0)	0 (0)	0 (0)	3 (20)	2 (18)	5 (19)
Average caries						
n_{median} ICDAS II	8	11	10	0	0	0

Table 2: Caries status of caries-active and caries-free groups

	ICDAS 0	ICDAS 1	ICDAS 2	ICDAS 3	ICDAS 4	ICDAS 5	ICDAS 6	Total
Caries-active *n* (%)	53 (9)	23 (4)	35 (6)	187 (32)	129 (22)	88 (15)	70 (12)	585 (100)
Caries-free *n* (%)	366 (67)	115 (21)	65 (12)	0 (0)	0 (0)	0 (0)	0 (0)	546 (100)

In this study, microbial diversity of caries-free and caries-active plaques in permanent dentition were investigated by using NGS.

In a study performed by Cephas *et al*.,[19] high bacterial diversity was noted in saliva of adults and infants. *Haemophilus*, *Neisseria*, *Veillonella*, *Fusobacterium*, *Oribacterium*, *Rothia*, *Treponema*, and *Actinomyces* were predominant in adults. Another study using the ICDAS II classification in the diagnosis of caries showed that Shuttleworthia was significantly higher in caries-free compared to caries-affected children ($P = 0.031$).[20] In addition, microbiota the black discolored plaque, which was found to be associated with caries-free status, were listed as listed *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Tannerella*, and *Treponema*.[10,21] Consistent with these findings, in our study, *Actinomyces*, *Shuttleworthia*, *Rothia*, *Haemophilus*, and *Granulicatella* were found to be statistically higher in caries-free plaques.

On the other hand, it has been suggested that *Streptococcus mutans* play a role in the main etiology of dental caries in the history of dentistry.[22,23] However, consistent with our study, some other studies contradict and suggest other bacterial species such as *Lactobacillus*, *Actinomyces* or *Bifidobacterium*, *Veillonella* may be dominant in the carious plaque.[6,24,25] Recent molecular studies support these findings, expanding the list of potential cariogenic bacteria to other bacterial species such as *Veillonella*, *Propionibacterium*, *Selenomonas*, *Neisseria*, *Eikenella*, *Fusobacterium*, *Leptotrichia*, *Enterococcus*, and *Atopobium*.[26-29] Additionally, *Scardovia wiggsiae* was found to be significantly associated with severe-early caries in the presence and absence of *S. mutans*.[30] In our study, consistent with these findings, *Atopobium*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Scardovia*, *Veillonellaceae* bacteria were found to be statistically higher in carious plaque samples ($P < 0.05$). In addition to these bacteria, *Anaeroglobus*, *Bifidobacteriaceae* (unclassified), *Centipeda*, *Cryptobacterium*, *Desulfobulbus*, *Filifactor*, *Howardella*, *Lactobacillaceae* (unclassified), *Leptotrichiaceae* (unclassified), *Megasphaera*, *Mycoplasma*, *Olsenella*, *Phocaeicola*, *Pseudoramibacter*, *Schwartzia*, and *Treponema* were also found to be related to caries formation.

The NGS method makes it possible to detect previously undetected species, but it also brings with a more detailed microbial analysis. The NGS method allows the analysis of a vast number of bacteria, quickly and precisely.[13] In this method, the 16S rRNA gene used to detect bacterial diversity has several properties that make it suitable for our purpose. It increases the reliability of the method by being present in all prokaryotes, having highly protected and variable regions, and being the most widely used gene for the identification of bacteria. Using the NGS technology, the present study aids to understand complex microbial diversity in caries-free and caries-active plaques in permanent dentition.

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Table 3: Microbial differences of caries-free and caries-active plaque samples

Bacteria	Caries-free p50 -(%)	Caries-active p50 -(%)	*P*
Actinomyces	3,45	1,56	0.0019
Anaeroglobus	0	0,032	0.0108
Anaerolineaceae (unclassified)	0	0,0022	0.0011
Atopobium	0,0036	0,028	0.0002
Bifidobacteriaceae (unclassified)	0	0,016	0.0000
Bifidobacterium	0	0,023	0.0000
Cardiobacterium	1,2	0,24	0.0001
Centipeda	0,0012	0,0098	0.0236
Cryptobacterium	0	0,002	0.0004
Desulfobulbus	0	0,002	0.0132
Filifactor	0	0,26	0.0077
Flavobacteriaceae (unclassified)	0,58	0,182	0.0083
Granulicatella	0,13	0,02	0.0071
Haemophilus	0,23	0,026	0.0061
Howardella	0	0,0024	0.0303
Kingella	0,74	0,216	0.0032
Lactobacillaceae (unclassified)	0	0,0035	0.0011
Lactobacillus	0	0,036	0.0000
Leptotrichiaceae (unclassified)*)*	0,0072	0,0301	0.0061
Megasphaera	0	0,042	0.0041
Mycoplasma	0	0,0114	0.0037
Olsenella	0,055	0,23	0.0071
Pasteurellaceae (unclassified)	10,4	2,32	0.0023
Phocaeicola	0	0,004	0.0004
Prevotella	3,2	12,4	0.0071
Propionibacteriaceae (unclassified)	0,14	0,046	0.0005
Propionibacterium	0,0037	0,032	0.0060
Pseudoramibacter	0	0,0116	0.0068
Rothia	0,64	0,258	0.0483
Scardovia	0	0,008	0.0090
Schwartzia	0	0,0035	0.0269
Shuttleworthia	0,0093	0	0.0105
Streptococcus	5,6	2,54	0.0225
Treponema	0,26	1,68	0.0483
Veillonellaceae (unclassified)	0,38	1,28	0.0378

P50: median

Conflicts of interest

There are no conflicts of interest.

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