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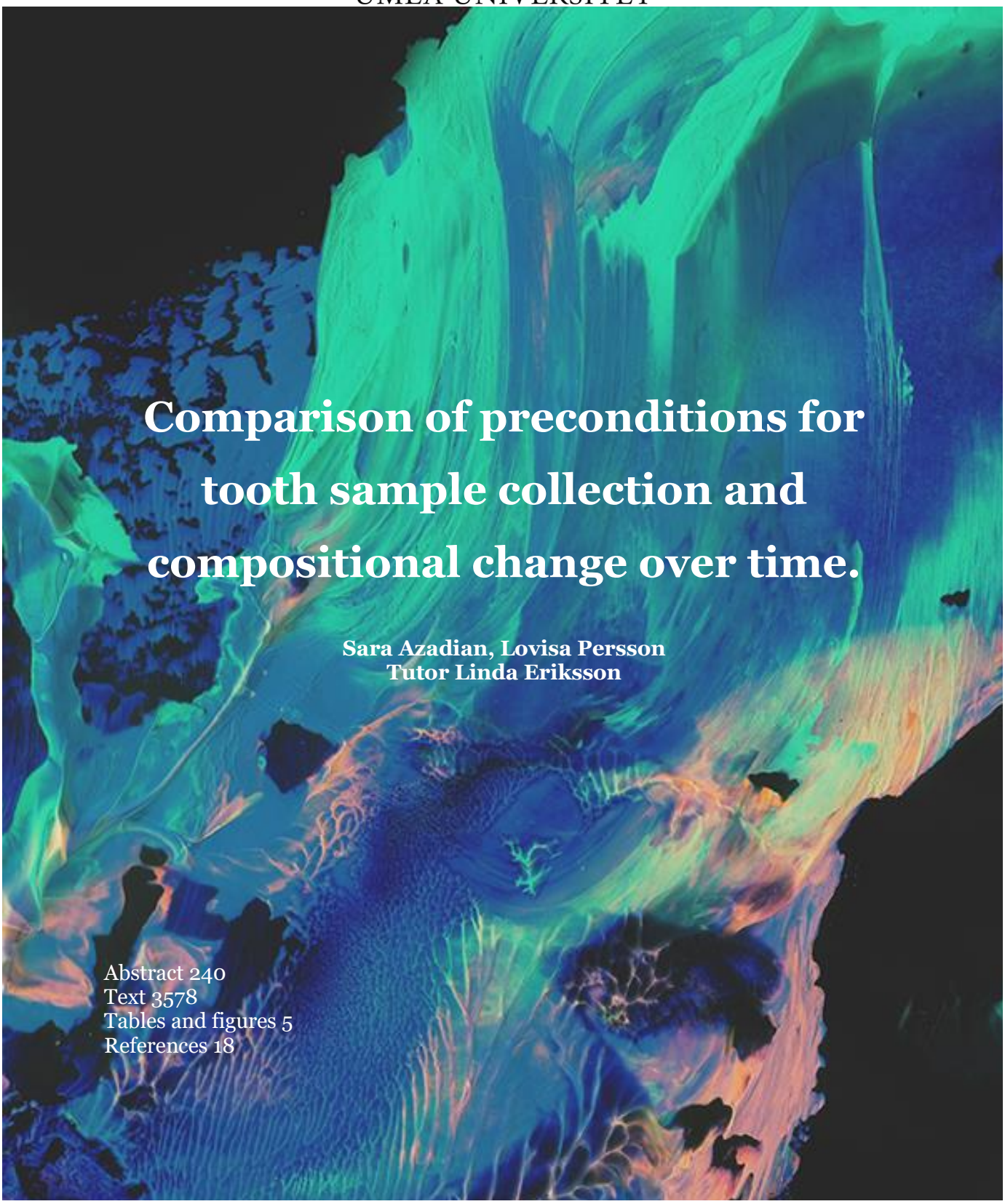

Comparison of preconditions for tooth sample collection and compositional change over time.

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ABSTRACT

Background

The mouth can be colonized by many bacterial species that are associated with both health and disease. Bacteria analyses are an important part of diagnosing and treating dental diseases, but there is no consensus regarding which sampling method to use for these analyses.

Aim

The aim of this study was to find out whether there are differences in bacterial composition in one, two and three days old dental plaque and if there are regional differences between anterior, posterior, right and left surfaces in the mouth over time.

Method

Plaque sample collection was performed at specific tooth sites on six healthy young adults. Sampling was made three times after dental plaque had been accumulated for one, two and three days respectively. During the accumulation periods the participants had to abstain from all forms of oral hygiene. Bacteria DNA extraction was performed followed by 16S sequencing. All gene sequences were matched against the Human Oral microbiome database. The results were analyzed in SPSS and SIMCA.

Result

When comparing the bacteria composition between day one, two and three a statistically significant difference was found. The analyzes also showed a difference in composition between the posterior and anterior regions of the mouth over time, but no significant difference between the right and the left side of the mouth over time.

Conclusion

The result suggests that the sampling method should be chosen carefully and determined by the specific aim of the dental plaque analysis.

BACKGROUND

The human organism contains approximately the same amount of bacteria as the number of human cells, which is about 3.8x10^13 (Sender et al. 2016). The microbiome differs between individuals but also varies between different parts of the human body. The oral microbiome includes all microorganisms that live in the mouth such as bacteria, fungi and viruses with bacteria being the absolute majority (Rogers, 2022). With both hard surfaces such as teeth and soft surfaces such as oral mucosa, the oral cavity is a heterogeneous environment that can be colonized by close to 1000 different species of microorganisms (Morse et al. 2018) and can be associated with both oral health and disease (Kilian et al. 2016).

Taxonomy is the systematic method of classifying microorganisms. The classification is done in a hierarchy order; domain, kingdom, phyla, class, order, family, genus and species. A binomial name consists of the genus name followed by the species name, where a genus consists of one or many different species with similar properties (Cain, 2022). Here the classification will be presented as genera and species. Further, bacterial species can also be divided into gram negative and gram positive. Gram negative species have an outer membrane with Lipopolysaccharides, LPS, and a thin peptidoglycan layer inside. LPS plays an important role in both the initiation of plaque formation and maturing. Gram positive bacteria have no outer membrane, but a thick peptidoglycan layer (Kim et al. 2015) (Ruhal et al. 2021).

To be able to attach and live on the hard tooth surfaces, the bacteria must form a dental plaque, through a complex process. Simplified, the dental plaque formation starts when the tooth surfaces become covered with a thin film of glycoproteins, called pellicle, from the saliva immediately after toothbrushing. The purpose of the pellicle is to protect the teeth from acid attacks, and it also contains receptors that some bacteria can attach to. The first bacteria that bind to the pellicle are called early colonizers, which is rapidly increasing in number. The early colonizers also have the ability to influence the environment in the dental plaque to make it favorable for growth and recruitment of late colonizers. Both commensal and pathogenic bacteria are found in the dental plaque, including both gram positive and gram negative bacteria, with the majority being facultative or obligate anaerobic (Fejerskov et al. 2015). When the dental plaque matures it becomes thicker and contains more anaerobe species (Rosan et al. 2000). Anaerobe species are located closer to the teeth, facultative anaerobes in the middle and aerobe on the outer layer of the dental plaque. The dental plaque matrix consists of polysaccharides produced by bacteria (Fejerskov et al. 2015).

In order to analyze oral bacteria, different methods have been used and evolved over the years. Some oral species are not possible to identify using traditional culture methods since they are strict anaerobes or for other reasons hard to grow on agar plates. Today one of the most common methods of identifying oral bacterial species is to extract, amplify and sequence the 16s rRNA gene from bacteria and then compare it to a database. This method has evolved, and the technique allows millions of sequences to be analyzed at once (Kilian et al. 2016). The 16s rRNA gene contains different regions that differ between species. The hypervariable regions v3 and v4 are the ones that differ most between oral bacteria species and are therefore often used when analyzing these bacteria. Unique primers including identifying codes are added to the start and end of each sequence to make sure they can be matched to its sample. These sequencing methods have led to databases such as Human Oral Microbial Database, (https://www.homd.org/) where gene sequences of 774 different species from the aerodigestive tract can be found. As the research progresses, more and more species are included in the database and thus it grows continuously.

Dental caries is a common, multifactorial disease that affects the teeth. Acid-producing bacteria in the dental plaque ferment carbohydrates which further possibly lead to demineralization of enamel and dentine. Caries decay occurs over time as a result of the acid exposure. Different host and lifestyle factors such as bacteria composition, dietary habits, use of fluoride products, saliva composition and quality can affect the development of dental caries. Bacteriological samples and analyses are a part of the diagnostics to be able to apply adequate treatment to caries and other dental diseases (Selwitz et al. 2007). Bacterial composition has been studied in samples from dental plaque, saliva, and soft tissue such as tongue and gingiva, but there is no consensus regarding which sampling method is better for specific analyses. Regarding dental plaque, it has been debated however samples should be pooled for all available surfaces in a subject or if the results could be skewed in relation to specific research questions by doing so. Further no consensus has been reached regarding accumulation time on dental plaque. Overall, this makes studies on dental plaque difficult to compare. In light of that, this study will focus on dental plaque as a first step in trying to unravel these questions.

The aim of this study is to investigate whether there are differences in bacterial composition in one, two and three days old dental plaque, and if there are regional differences between anterior, posterior, right and left surfaces in the mouth over time. The null hypothesis is that there are no differences in bacterial composition.

MATERIAL AND METHOD

Study participants

The study population consisted of six adults, recruited from the Dental School at Umeå University. The exclusion criteria were that a participant did not consent, had taken antibiotics during the last three months, had a chronic disease that varies in severity or was unable to communicate in Swedish or English.

Ethical reflections

All participants received vocal and written information about the project and then submitted a signed written consent. All data was pseudonymized and will not be published. The study was approved by the local ethical committee at the department of odontology and no violations have been committed.

Examination & Pre-sampling rules

Initially all participants were clinically examined and when indicated x-rays were taken and all findings were documented in a protocol (appendix 1). There were three accumulation periods, One day (24hr), two days (48hr) and three days (72hr) with one week between each period. Each accumulation period was followed by a dental plaque sample collection. The participants were instructed to keep their eating habits as usual and consistent during the three accumulation periods. There were strict rules to abstain from all forms of oral hygiene for 24, 48 and 72 hours respectively. They were not allowed to brush their teeth, floss, rinse, chew gum or use any product containing fluoride or chlorhexidine. Alcohol was not allowed during the accumulation period. Smoking and snuff were allowed if used in a standardized amount during all accumulation periods.

Recording of events that may affect the results

During a three-month period before the first sampling the participants had to note any special events such as sickness, medication, and use of antibiotics. During this time none of the participants experienced any of these.

Sample collection

The sampling was performed at the University Hospital in Umeå by six students at the dentistry program. It was made at the same time all three days. Supragingival dental plaque was carefully scraped off the teeth using sterile curettes. The collection was performed systematically with a new curette blade for each section of teeth. This was performed by dental students who all had been given the same instruction before. The collection was made in this order 17+16, 15+14, 13, 12+11, 21+22, 23, 24+25, 26+27, 37+36, 35+34, 33, 32+31, 42+42, 43,

44+45, 46+47. The teeth were divided based on types of teeth (molar, premolar, canine, incisors). Dental plaque was collected mainly from the buccal and palatal/lingual surfaces of the teeth and as much approximal as possible without touching the neighboring teeth. When dental plaque was collected from a tooth section, it was immediately transferred to a sterile Eppendorf tube containing 200 µL sterile 1xTE (10 mM Tris Ultrapure, pH 8,0 and 0,1 mM EDTA) solution. The curette was swirled in the solution for at least ten seconds or until the visible part of the accumulated dental plaque had come loose from the curette. Once one curette was dipped into the solution it was not allowed to go back and collect from that area again. All test tubes were marked with a unique number for each individual and tooth section. They were placed on ice until they reached the laboratory, where the samples were stored in a -80°C freezer until DNA extraction was made. After each sample collection was finished, the participants got a thorough cleaning and polishing (Top DENT 170 RDA) of all teeth followed by full mouth fluoride varnish (0.5ml Profluorid Varnish 2.26% F).

DNA extraction

One experienced person performed DNA-extraction using the GenElute™ bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). First, the samples were centrifuged, followed by lysis in a buffer with enzymes to break the cell wall in both gram negative and gram positive bacteria. After the samples were washed, the Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the amount of DNA. The whole DNA was isolated. The same procedure was done with a mixture consisting of 14 different bacterial species including both gram negative and positive to serve as a positive control. Sterile water was used as a negative control. The genes were then amplified with PCR (KAPA HiFi HotStart ReadyMix (2X), United States Wilmington, MA, USA) (Caporaso et al. 2012).

Miseq sequencing

The genes were amplicon sequenced using the Illumina Miseq platform and MiSeq reagent kit v3-v4 600 cycles (Illumina, Eindhoven, The Netherlands) at the Swedish Defense Research Agency research facility in Umeå, Sweden. All samples usually do not contain the same number of reads and in order to investigate the relative change of composition over time, the amount of each species is presented in percentage. This was made to make sure to measure the change in composition and not the ability to perform the sampling. When comparing the posterior and the anterior region the samples were divided into posterior = molars, and anterior = incisors and canines. The premolars were excluded from this analysis in order to compare tooth regions that are located further away from each other. For the comparison of right versus left side all samples were included.

Microbiota analysis

After sequencing, all sequences were compared against a database. The database used was the Human oral microbiome database, HOMD, since it includes most oral bacteria. If the genes matched with more than 98.5% to a specie it was considered a match. For a bacterium to be included in this study it had to be found in at least two samples. If it was found in only one sample, it was not included since it could be transient.

Data management and statistical analyses

The difference was analyzed between day one, two and three of the whole study group added together. In the continuous data the amount of a certain bacterium was proportional where all bacteria added from one sample equals one. This was made to make sure that we measured the change in composition and not the ability to perform the sampling

Multivariate models

Partial least squares regression, PLS, was used for multivariate analysis to find out if there was a systematic difference representing day one, two and three. Days were chosen for the X-axis. The samples were then analyzed in the program and the three components with the most variation between the days were added to the model. T1 was the component with the biggest variation on x. Since three components were used the result will be presented in a 3D-model. PSL was also used on the abundance data for each day when comparing posterior versus anterior regions and left versus right. For each component the Q2 and R2 values were used to determine if the result was significant or not. The explanatory value R2, is the ability of all bacteria to explain the difference in Y. Q2 measures the ability to predict the result if the analysis was to be reproduced. Variable importance for the projection, VIP, is a value that was added to each bacterium. The bacterium with the highest VIP was the most important for the result. A VIP >1 is considered to have an impact on the result and VIP>1.5 means that the bacterium has a high impact on the outcome. The VIP-value does not take into consideration if the bacterium is associated with e.g the posterior or anterior region. Correlation coefficient was therefore also used since it divides the samples into two groups, e.g anterior and posterior by giving one group positive values and the other negative. The p-value < 0.05 was considered statistically significant and all tests were two-sided. SPSS and SIMCA 17 were used for all analyzes.

Literature

Literature for this study was found in the medical library, on PubMed and on trusted web pages such as HOMD. Some articles were also recommended by the tutor. Free-term searching was

made on PubMed during 2021-2022 using search terms such as “dental plaque“, “dental plaque formation”, and “the oral microbiome”. Only articles that were written in English and were accessible in full text were included.

RESULTS

Participants and characteristics of the study group

The study group included six healthy young adults, four girls and two boys, in the age of 23-40, with a mean age of 26.7 as presented in table 1. Two participants had extracted two teeth each on orthodontic indication and some teeth had restorations or initial enamel caries lesions. All surfaces (S), including enamel (e), that are decayed (D), missing (M), or filled (F) can be summarized in an index; DeMFS. Mean DeMFS in the group was 7.5 and mean DeFS was 4.2. Each sequenced sample resulted in four reads, two from the actual sample and two from the primers to make sure it was possible to find which sample it was. A total of 288 (6x3x16) dental plaque samples and six controls were compared to the database. Each participant was colonized with approximately 120 species.

Difference between day one, two and three

A total of 260 bacterial species and 63 genera were found in all samples from all participants put together. 74.4% (196) of all species were found in samples from all three days (Figure 1). On day one a total of 192 bacteria species were found while day two and three contained 190 and 210 species respectively. The species that were specific for one of the three days are presented in table 2. Ten species were found only on day one, four species only on day two and 17 only on day three. The species found only on day one were a mixture of aerobe, facultative anaerobe and strict anaerobe. The species found only on day three were anaerobe, except for the *saccharibacteria* species that are sparsely explored and therefore it is still unknown if they are aerobe or anaerobe (Bor et al. 2019). Multivariate PLS-analyses of the dental plaque on day one, day and two showed that there was a systematic difference between all three days as shown in figure 2.

Difference in plaque composition over time between the frontal and posterior regions

Figure 3 shows the difference between posterior and anterior regions. For day one only component one (t[1]) was statistically significant ($R2 < 0.05$, $Q2 > 0.05$) while both component one (t[1]) and two (t[2]) were significant for day two ($R2 < 0.05$, $Q2 < 0.05$) and three ($R2 < 0.05$, $Q2 < 0.05$), meaning that the difference between anterior and posterior dental plaque was bigger on day two and three.

Difference in plaque composition over time on the left versus the right side

A PLS-model was used to compare the dental plaque on the left versus the right side of the mouth. It was used on all three days for all participants together. None of the components

were statistically significant (P>0.05) meaning that there was no difference in bacteria composition over time between the right and the left side.

DISCUSSION

A statistically significant difference in plaque composition between day one, two and three were found, and therefore the null hypothesis was rejected. When researching this area, no articles were found that compares the dental plaque composition between one, two and three days and therefore this study adds to this field. The fact that some species found in day one were not found in day three and vice versa can have many possible explanations. Over time the plaque gets thicker and changes from mostly aerobe to more anaerobe, which might favor some species and disfavor others (Fejerskov et al. 2015). This is consistent with our result as it was a mixture of both aerobe, facultative anaerobe, and anaerobe species found only on day one while all species that were only found on day three were anaerobe.

There are several studies showing that there are differences in bacteria composition between different areas in the mouth, but how the difference varies over time is still not fully known. Zaura. E et al. have shown differences in plaque composition between molars and incisors, but the study group only consisted of three people, why more studies are needed in this area (Zaura et al. 2009). Another previous study has shown that there are more anaerobic bacteria in the posterior regions than in the anterior part of the mouth (Sreenivasan et al. 2010). A change in composition between the posterior and anterior surfaces was also shown in this study and can have many possible explanations. It might be affected by acids produced by bacteria, prevalence of caries and deep gingival pockets, access to oxygen and anatomical differences. Other possible factors are the amount of saliva and its transportation from anterior to posterior regions due to the location of the salivary glands and muscle movements (Mark Welch et al. 2019).

The participants were examined concerning caries but not periodontal diseases. It is known that deep gingival pockets are associated with more anaerobe species and therefore it would have been interesting to also have access to periodontal data to study.

No articles were found comparing the plaque composition on tooth surfaces on the left versus the right side. Several studies have been made where one side is used as the test/intervention side and the other side is the control (Haydari et al. 2017). This assumes that the dental plaque composition without the intervention would be the same on the left versus the right side. This is supported by this study since there was no significant difference in dental plaque composition between the right and left side of the mouth over time.

All participants were not chosen randomly since two of the students are the ones performing the study. The study group was limited to six participants which can be considered a

weakness of this study. An advantage with the small study group was that all samplings could be made at the same place at the same time which made it easier to supervise the group. All participants were dental students that might be interested in the result of the study and therefore more likely to follow the pre-sampling rules. The participants can be assumed to have a non-cariogenic diet and microflora, which might have affected the bacteria composition. Participants with caries would probably have a more acidic composition and a different ratio between aerobe and anaerobe species (Takahashi et al. 2008).

The participants were instructed to keep their usual eating and drinking habits during the accumulation periods and to document everything. Knowing that you are not allowed to use any form of oral hygiene for a time might still instinctively affect the food choices, but nothing was reported from the participants that could have impacted the result during the accumulation periods.

The technique used when collecting the plaque samples is another factor that might have affected the result. There were six different students performing the sampling and each student performed all three collections on the same person. The method was standardized but the instruction could still be interpreted differently between individuals. A thorough polishing was made after each time in order to remove as much of the dental plaque as possible before the new accumulation period began.

Conclusion

When performing dental plaque collection, time and location are two factors to consider. This study showed that the dental plaque composition changes between one, two and three days. There was a difference in composition between the posterior and anterior regions on all three days, which can be relevant for further dental plaque studies. It shows no statistical difference in dental plaque composition between the right and the left side, which can be of interest when performing dental plaque collection since this suggests that it does not matter at which side the collection is made. There were differences in bacteria composition over time and between regions, but no general conclusion can be made in terms of which method to choose. As a final conclusion, the result suggests that the sampling method should be carefully chosen and determined by the specific aim of the dental plaque analysis.

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Participants

Characteristics	Mean (SD[1])
Women	67%
Age	26.7 (6.7)
DeMFS[2]	7.5 (5.8)
DeFS[2]	4.2 (6.0)

Table 1. Characteristics of the participant group.

1 = standard deviation

2 = third molars and fissure sealant not included.

	Day 1	Day 2	Day 3
Bacteria (species)	*Actinomyces sp.* HMT 896 *Kluyvera ascorbata* *Neisseria cinerea* *Saccharibacteria* (TM7) [G-1] bacterium HMT 488 *Saccharibacteria* (TM7) [G-1] bacterium HMT 869 *Schaalia meyeri* *Streptococcus constellatus* *Streptococcus cristatus clade* 886 *Streptococcus parasanguinis clade* 411 *Tannerella sp.* HMT 808	*Aggregatibacter actinomycetemcomitans* *Campylobacter curvus* *Fusobacterium sp.* HMT 248 *Schaalia sp.* HMT 172	*Anaeroglobus geminatus* *Atopobium rimae* *Megasphaera micronuciformis* *Oribacterium sinus* *Peptostreptococcaceae* [XI][G-1] [*Eubacterium*] *infirmum* *Prevotella shahii* *Prevotella sp.* HMT 305 *Prevotella sp.* HMT 314 *Saccharibacteria* (TM7) [G-1] *bacterium* HMT 349 *Saccharibacteria* (TM7) [G-5] *bacterium* HMT 356 *Saccharibacteria* (TM7) [G-8] *bacterium* HMT 955 *Scardovia wiggsiae* *Selenomonas sp.* HMT 134 *Selenomonas sp.* HMT 136 *Solobacterium moorei* *Treponema pectinovorum* *Treponema sp.* HMT 231

Table 2. Bacteria found on only day one (orange), two (green) or three (blue). This includes all tooth surfaces from all participants.

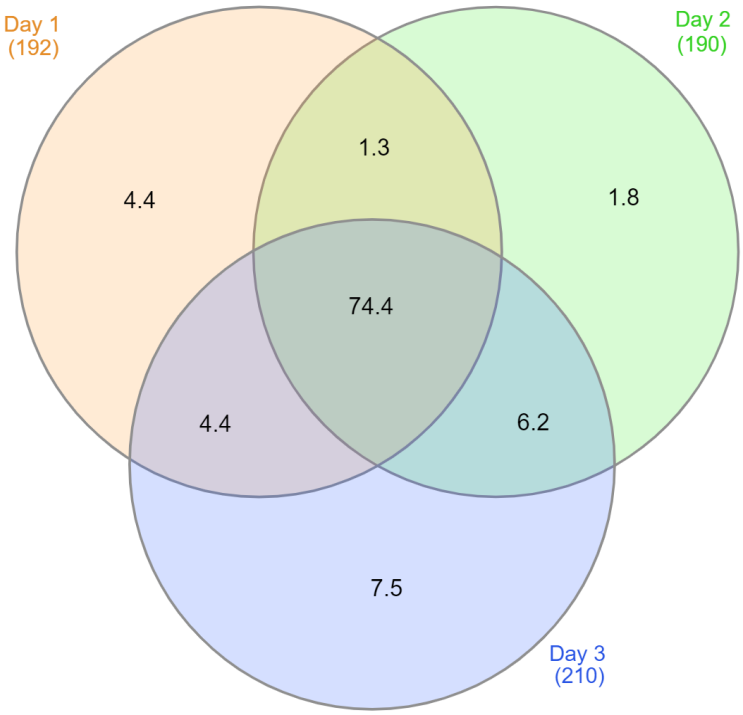

Figure 1. Venn diagram showing the percentage of all bacterial species found on day one (orange), two (green) and three (blue). This includes all tooth surfaces from all participants put together.

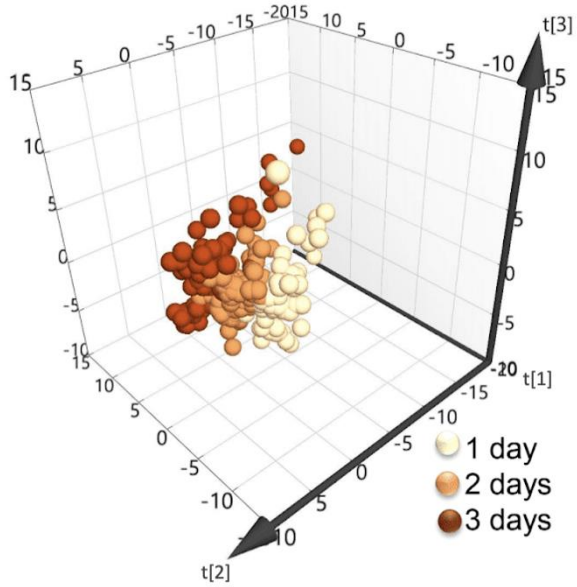

Figure 2. 3D-model based on multivariate PLS-analyses with three components (T1, T2, T3). The systematic difference in bacteria composition between day one, two and three is shown.

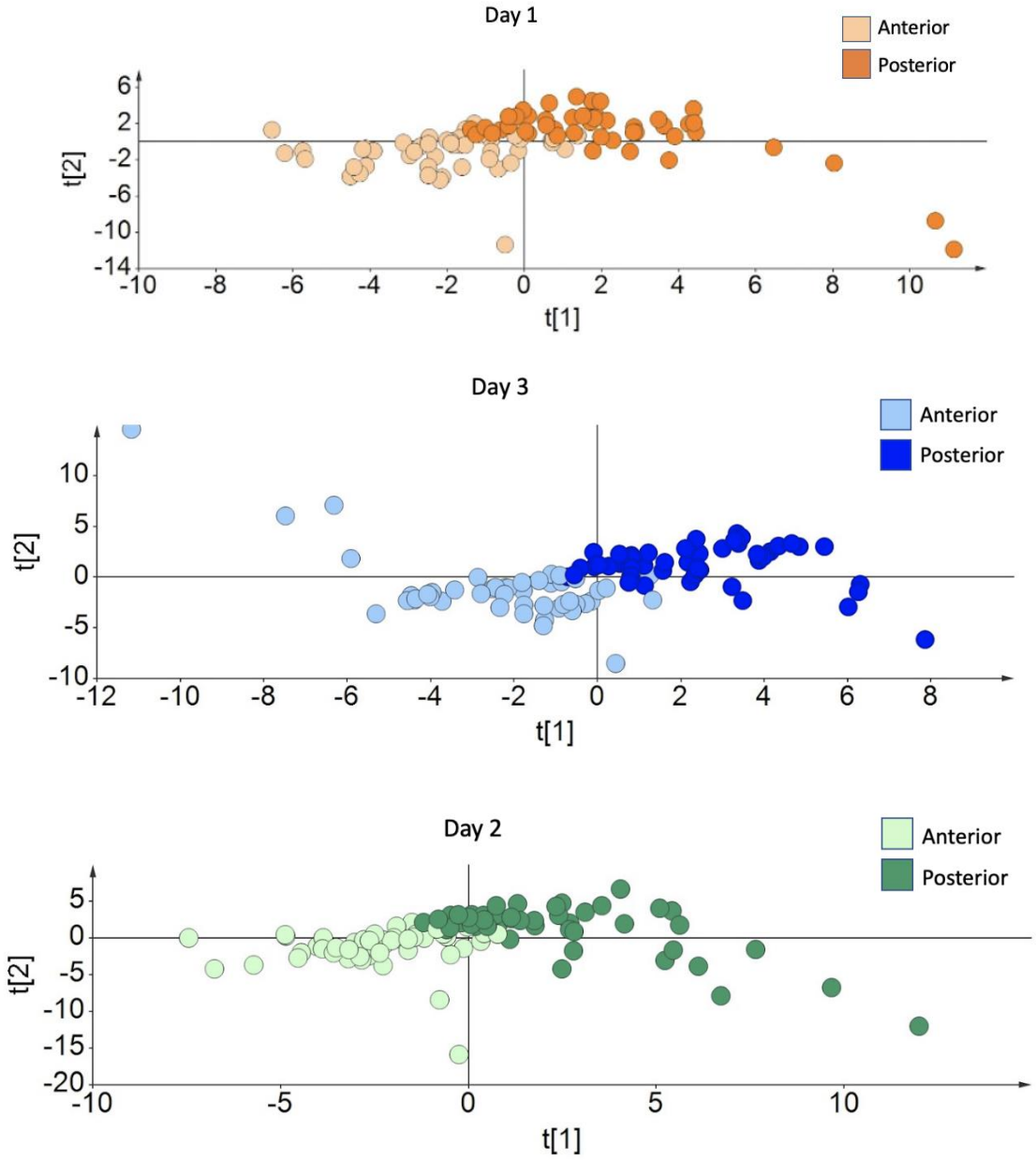

Figure 3. Score scatter plots based on a PLS-model with components T1 and T2. The difference in bacteria composition between the anterior (left) and posterior (right) regions is presented for day one (orange), day two (green) and day three (blue).

Appendix

Examination protocol

Upper Jaw

Date:	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
Previous dental work																
Defect																
Cavity																
Active / Inactive caries																
Other (rse,r, fi, c, f, md, o)*																

Lower Jaw:

Date:	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
Previous dental work																
Defect																
Cavity																
Active / Inactive caries																

Other (rse,r, fi, c, f, md, o)*																

Xerostomia, Saliva viscosity	

*

root surface exposure = rse
retainer = r
food impaction = fi
calculus = c
fluorosis = f
mineralisation deficiency = md
overcrowded = o