Long-Term Fluctuation of Oral Biofilm Microbiota following Different Dietary Phases

Anderson, Annette Carola ; Rothballer, Michael ; Altenburger, Markus Jörg ; Woelber, Johan Peter ; Karygianni, Lamprini ; Vach, Kirstin ; Hellwig, Elmar ; Al-Ahmad, Ali

DOI: https://doi.org/10.1128/AEM.01421-20

Long-term fluctuation of oral biofilm microbiota following different dietary phases

Running title: Oral biofilm fluctuations after dietary change

Annette Carola Anderson[a#+], Michael Rothballer[b+], Markus Jörg Altenburger[a], Johan Peter Woelber[a], Lamprini Karygianni[c], Kirstin Vach[d], Elmar Hellwig[a], Ali Al-Ahmad[a]

[a]Department of Operative Dentistry and Periodontology, Medical Center-University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

[b]Institute of Network Biology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

[c]Clinic for Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zürich, Switzerland

[d]Institute of Medical Biometry and Statistics, Faculty of Medicine and Medical Center - University of Freiburg, Freiburg, Germany

[+]ACA and MR contributed equally to this work

Address correspondence to:

Dr. Annette Anderson
Department of Operative Dentistry and Periodontology,
Hugstetter Straße 55
79106 Freiburg
Germany
annette.anderson@uniklinik-freiburg.de

Abstract

Caries development is associated with shifts in the oral biofilm microbiota and primarily linked to frequent simple carbohydrate consumption. Different nutritional ingredients can either promote or prevent caries development. To investigate their effect on the oral biofilm microbiota *in situ*, eleven study participants underwent 3-month-long dietary phases with regular diet (PI), additional frequent sucrose (PII), milk and yoghurt (PIII) and rich in dietary fiber (PIV) intake, then returning to their regular diet (PV). Oral biofilm was sampled and analyzed applying 16S rRNA Illumina Miseq sequencing. Additionally, the effect on the enamel was analyzed measuring enamel surface roughness with laser-scanning microscopy. The beta-diversity results showed that the microbiota in all the following phases differed significantly from PI and that the microbial community in PII was significantly different from all other phases. The abundance of the genus *Streptococcus* fluctuated over the course of the five phases with a significant increase in PII ($p=0.01$), decreasing in PIII and PIV (PIII and PIV vs. PII: $p<0.00001$) and increasing again towards PV. Other taxa showed varying fluctuations of their abundances, in PV returning approximately to the levels of PI. In conclusion, while elevated sucrose consumption favoured caries-promoting non-mutans streptococci, frequent milk and yoghurt intake caused a significant decrease in the abundance of these microbial taxa and in addition reduced enamel surface roughness. These results indicate that modulations of the oral biofilm microbiota can be attained even in adults through dietary changes and corresponding recommendations can be made for the prevention of caries development.

Importance

Caries affects a large proportion of the population worldwide resulting in high treatment costs. Its etiology can be ascribed to shifts of the microbiota in dental biofilms primarily driven by dietary factors. It is unclear, how diet affects the microbial community of plaque biofilm *in situ* and whether it can be modulated to help prevent caries development. To address this question we analyzed changes of the *in situ*-plaque microbiota following three-month long dietary changes, involving elevated sucrose, dairy and dietary fiber consumption over a long-term period of 15 months. Applying high-throughput sequencing we found non-mutans streptococci, a taxonomic group involved in the beginning stages towards microbial dysbiosis, in decreased abundance with elevated dairy and dietary fiber intake. Analyzing the enamel surface roughness, these effects were confirmed. Therefore correspondent dietary measures can be recommended for children as well as adults regarding caries prevention.

Introduction

The oral biofilm is considered an ecosystem at the micron scale comprising over 700 bacterial species building a spatial network of interacting entities(1). In a state of oral health, homeostasis prevails as a dynamic equilibrium providing mutual benefits for the host and the microbiota(1-3) . When environmental factors come into play and disturb the equilibrium the proliferation of species with pathogenic potential results in a dysbiotic state(4). Environmental influences, e.g. dietary factors such as a frequent availability of simple carbohydrates can lead to a higher proportion of acidogenic and acidiuric species resulting in the development of carious lesions as formulated in the extended ecological

plaque hypothesis(5). Caries affects about 2.4 billion people worldwide, constituting a major health concern implicating high treatment costs (6). Mutans streptococci and lactobacilli had long been considered the main cariogenic taxa responsible for acid production and demineralization of tooth structure. However, during the last decades, a more complex composition of the microbial community associated with caries, involving various additional bacterial species in the different stages of caries development, has been revealed(7, 8). Whereas non-mutans streptococci, such as *Streptococcus salivarius*, *Streptococcus parasanguinis* and the genus *Actinomyces* have been associated with the initial stages, *Veillonella* spp., *Lactobacillus* spp., *Atopobium* spp. and other taxa dominate the more advanced stages(9).

Apart from the well-known link between simple carbohydrate consumption and caries development, different specific dietary factors have been reported to exert caries-preventive effects. Particularly, frequent consumption of milk and dairy products as well as high-fiber and certain plant-based foods have been associated with a lower incidence of caries that is more pronounced in individuals with a relatively high daily sucrose-consumption frequency(10-12). Milk and dairy products have been presumed to protect oral health and possibly prevent caries due to different effects. The contained calcium and phosphate as well as caseinphosphopeptides (CPP) can counteract acidic demineralization and promote remineralization processes, specifically CPP forming nanocomplexes with amorphous calcium phosphate provide calcium and phosphate for remineralization(13-15). Furthermore, casein and other milk proteins have been shown to reduce bacterial adhesion and affect the growth of mutans streptococci(16).

Concerning plant-based foods, different parameters have been assumed to help prevent caries. Primarily, foods with a high fiber content have been considered to stimulate

salivary flow which buffers sudden pH drops and thereby protect the teeth. Also, phosphates in plant foods (mostly phytates) can reinforce remineralization(17). Furthermore, secondary plant substances, e.g. flavonoids and other polyphenols from cranberry juice and cruciferous vegetables have been shown to decrease the risk of caries by reducing bacterial adhesion, inhibiting growth or reducing capacity of biofilm formation of cariogenic microorganisms(10, 18, 19).

Nevertheless, so far there has been a lack of understanding about how the consumption of certain foods influences the complex composition of the oral biofilm in an *in vivo* situation, since most studies have either described *in-vitro* experimental approaches(13, 15, 20), animal experiments(16, 21) or epidemiological data(22, 23). Also, it was unclear whether it is possible to influence and modify the oral biofilm of adults, which is considered relatively stable, through a dietary intervention. Therefore, we investigated the influence of the frequent consumption of sucrose, dairy products and vegetables in addition to the regular diet on the supragingival biofilm microbiota using an *in-situ* splint system to collect 7-day dental plaque samples. Our previous study reported the influence of frequent sucrose consumption over the course of three months on the oral supragingival microbiome in greater detail(24). Here we present the compositional changes in oral biofilms caused by frequent additional milk and yoghurt, as well as dietary fiber consumption. To achieve the best possible overview over the cultivable as well as yet uncultivated bacterial taxa in the supragingival microbial community, high-throughput sequencing of the 16S rRNA gene was applied on the Illumina platform. In addition to the analysis of the supragingival microbiota, the effect of the phase-specific bacterial community on the enamel surface roughness was determined measuring surface roughness with 3D laser-scanning microscopy.

Results

Demographics and dietary intake

Eleven participants with an average age of 32 years were included in this study. They underwent five different three month-long dietary phases, first keeping their regular diet, then an elevated sucrose intake, followed by elevated dairy intake, elevated dietary fiber intake and finally returning to their regular diet (Figure 1). Supragingival plaque was sampled from bovine enamel slabs (BES) embedded in splint systems (Figure 2) worn for three x seven days towards the end of each dietary phase (three months), after an adaptation to the dietary phase for seven weeks (PI-PV). The regular diet of all participants corresponded to a high carbohydrate western diet, i.e. over 45% carbohydrate intake(25), the details of the main nutrient intake are shown in Table 1. None of the participants had active carious lesions and their mean DMFT value was 8.1. Detailed demographic and clinical data are reported in Anderson *et al*.(24). The dietary intake in the different phases did not reveal any statistically significant differences regarding the intake of main nutrients, except for simple carbohydrate intake in PI versus PIV, so that basically all changes in the biofilm composition in PII-PV can be ascribed to the phase-specific dietary changes.

Composition of the supragingival microbiota

A total number of 9.91 million high-quality reads was obtained from 151 samples from all five phases and 8.19 million sequences could be assigned to 346 species-level Operational Taxonomic Units (OTUs; 97% similarity). The sequence abundances of the three sampling time points per phase were averaged for the comparison of the five

phases and several samples did not yield a sequencing result (PIII, 3 samples; PIV, 6 samples; PV, 5 samples). Concerning relative bacterial phyla abundances (shown in Figure 2a), *Firmicutes* dominated in all phases (PI, 55.41%; PII, 59.37%; PIII, 42.05%; PIV, 41.37%; PV, 44.01%), followed by *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and *Saccharibacteria*. The OTUs represented 59 taxa on the genus level, the most abundant being *Streptococcus* (PI, 33.52%; PII, 40.28%; PIII, 24.34%; PIV, 24.22%; PV, 28.83%), followed by *Neisseria*, *Granulicatella*, *Gemella*, *Veillonella*, *Capnocytophaga* and *Porphyromonas* (Figure 3a). All these genera were detected in all five phases in all study participants. The full list of detected bacterial taxa and assigned OTUs with their relative abundances is given in Supplementary Tables S1 and S2. The manual species-level analysis of the genus *Streptococcus* revealed the proportional distribution of the oral streptococci (Figure 3b). The highest relative abundance was found for *Streptococcus mitis* (PI, 16.92%; PII, 20.83%; PIII, 13.34%; PIV, 13.17%; PV, 18.91%), followed by ambiguously assigned *Streptococcus* spp. (PI, 5.45%; PII, 5.19%; PIII, 3.48%; PIV, 4.39%; PV, 5.48%), *Streptococcus infantis* (PI, 5.70%; PII, 5.56%; PIII, 3.0%; PIV, 3.33%; PV, 2.43%), *Streptococcus sanguinis* (PI, 3.03%; PII, 4.87%; PIII, 1.37%; PIV, 1.42%; PV, 1.06%), *Streptococcus gordonii*, *Streptococcus parasanguinis* and *Streptococcus salivarius/vestibularis* (the latter species could not be discriminated based on the 16S rDNA). As for *Streptococcus mutans*, this was the *Streptococcus* species with the lowest relative abundance, only detectable in single study participants and at very low abundances of < 0.01%, except in PII (PI, 0.0006%; PII, 0.0115%; PIII, 0.0076%; PIV, 0.0011%; PV, 0.0005%). The relative abundances of the different oral *Streptococcus* species are shown in Supplementary Table S3.

Species richness and alpha-diversity changes

Over the course of the five phases, the species richness decreased significantly in the sucrose phase (PII; p=0.008) and then increased again in PIII (p=0.005) and PIV (PIV vs. PII, p=0.002) followed by a renewed slight decrease in PV. The alpha-diversity measures, Shannon effective and Simpson effective were significantly increased in PIII compared to PII (Shannon eff., Simpson eff., $p<0.0001$) and also in PIV compared to PII (Shannon eff., p=0.001; Simpson eff., p=0.007) whereas in PV both parameters were significantly decreased compared to PIII (Shannon eff., p=0.025; Simpson eff., p=0.011). The details of the alpha-diversity measures are shown in Table 2.

Beta-Diversity reveals significantly different microbiota in different dietary phases

In order to assess the differences between the microbial communities in PI-PV, the beta-diversity was analyzed based on UniFrac. Comparing PI with all following phases, the Permanova analysis revealed significant differences of the microbial communities between PI and PII (p=0.004), PIII (p=0.0016), PIV (p=0.0016) as well as PV (p=0.00016). Also, significant differences of the microbial communities were found in PII compared to PIII (p=0.0016), PIV (p=0.0016) and PV (p=0.0016) (Figure 4). There were no significant differences between PIII and PIV, PIII and PV and PIV and PV.

Fluctuations of bacterial taxa abundances in the course of PI-PV

Serial group comparisons were done in Rhea to compare relative abundances of microbial taxa in the different phases. This revealed both characteristic fluctuations and significant differences in abundances in PI-PV for several bacterial taxa (Figure 5a). The abundance of the phylum *Firmicutes*, with the genus *Streptococcus* being its main

representative in the supragingival biofilm, increased in PII and showed a significantly decreased abundance in PIII, PIV and PV compared to PI (PI-PIII, p=0.0002; PI-PIV, p<0.00001; PI-PV, p=0.007). The abundance of the genus *Streptococcus* showed a similar course, revealing a significantly decreased abundance in PIII and PIV in comparison to PI (PI-PIII, p=0.003; PI-PIV, p=0.002). Other taxa showed fluctuations of their relative abundance in different directions. The abundance of the family *Pasteurellaceae* (represented mainly by the oral genus *Haemophilus*) was decreased significantly in PII, yet increased in PIII when compared to PI (PI-PII, p=0.006; PII-PIII, p=0.004). Similarly, the class *Bacteroidia,* which was represented in the supragingival microbiota mainly by the genera *Porphyromonas* and *Prevotella*, showed a decreased abundance in PII and an increased abundance in PIII (PII-PIII: p<0.00001). *Rothia* spp., *Leptotrichiaceae* spp. and *Granulicatella* spp. showed other fluctuations: *Granulicatella* spp. showed a decreased abundance in PII-PV compared to PI (PI-PIV, p=0.03; PI-PV, p=0.001), whereas the genus *Rothia* had a higher abundance in PIII versus PI, PII and PIV (p=0.007; p=0.007 and p=0.03 resp.) and *Leptotrichiaceae* also had a higher abundance in PIV and PV versus PI (p<0.00001).

The analysis of the abundance of the genus *Streptococcus* on the species level revealed certain abundance fluctuations of different *Steptococcus* species that are depicted in Figure 5b. *S. sanguinis*, *S. gordonii*, *S. parasanguinis*, *S. mitis* and *S. infantis* showed decreased abundance levels in PIII, most of them significantly. In PIV, these species showed a similarly low abundance as in PIII or a slight increase, yet not significant. Lastly, in PV most *Streptococcus* species showed a similar abundance as in PI, with *S. sanguinis* and *S. infantis* showing a significantly lower abundance compared

to PI. Only *S. salivarius* showed increased relative abundance levels in PIII, but was only detected in a few individuals (6/30) and decreased again in PIV and PV.

Changes in surface area roughness in PI-PV

The mean enamel surface roughness (Ra) measured throughout PI-PV varied between 0.035 ± 0.037 (STD) µm (median: 0.032 µm) in PV and 0.062 ± 0.022 (STD) µm (median: 0.054 µm) in PII. Neither the increase in surface roughness in PII, nor the decrease in PIII (p=0.056) were significant. However, the values in PV were significantly lower compared to the sucrose phase (PII, p=0.005), and compared with PI, both the surface roughness in PIV as well as PV was significantly lower (p=0.018 and p=0.001 resp.). The mean values of the surface roughness in PI-PV are depicted in Figure 5.

Discussion

Caries is associated with shifts in the bacterial composition of the oral biofilm microbiota, when acidogenic and aciduric taxa proliferate, mostly due to frequent simple carbohydrate consumption, which results in cariogenic plaque. Other foods, e.g. dairy and plant-based whole-foods presumably exert a more positive influence on the oral microbiota, possibly having a protective effect regarding caries. To investigate the effect of long-term (3 months) dietary changes on the oral biofilm microbiota *in situ*, we analyzed supragingival plaque from eleven participants with high-throughput sequencing. The applied splint system with included bovine enamel slabs for the collection of oral biofilm is a well-established *in situ* model that has been proven to allow the formation of supragingival biofilm in the natural environment (26, 27). The long-term timespan of three month duration for each dietary phase facilitated the observation of

potential shifts in the oral microbiota and changes in the enamel surface roughness (28, 29). As the results of the frequent sucrose consumption have been discussed earlier, therefore our focus here is on the dairy and the dietary fiber phases(24).

Altogether, one of the most notable findings consisted in the significant change of the beta-diversity for all four phases PII-PV compared with the baseline phase (PI), and in the significant difference of the beta-diversity in the sucrose phase (PII) compared to all other phases. Thus, the microbial community showed fluctuations according to the additional phase-specific food items, with the microbiota in PII deviating most from the other phases. Even after returning to the regular diet for seven weeks in PV, the microbial community had not reverted to its initial composition.

Another prominent observation is the significantly lower abundance of the genus *Streptococcus* in the dairy as well as the dietary fiber phases (PIII and PIV). After a significant increase of streptococci in PII their abundance in PIII even decreased significantly in comparison to PI. Hence, following an increase in different acidogenic non-mutans streptococci in PII(24), the elevated milk and yoghurt intake and dietary fiber intake in PIII and PIV respectively resulted in a significant decrease of these taxa, presumably rendering the oral biofilm less cariogenic.

So far, it has been shown that compared to other human habitats the oral microbiota constitutes a rather stable and highly diverse microbial community that – given that there are no environmental factors causing perturbations – only varies in a very small percentage of low-abundant species over time in an individual(30-32). Zaura *et al*. demonstrated that although the treatment of patients with four different antibiotics caused considerable perturbations in the fecal microbiome, their salivary microbiome was fairly resilient and did not undergo any major shifts(33). In another study, the oral

biofilm metatranscriptome of five individuals thirty minutes before and after a carbohydrate-rich meal was analyzed, showing short-term changes for some of the participants, but not consistently and not for all(34).

In our study, however, the influence of diet as a major environmental factor was examined over the course of 4 x 3 months involving three specific food items. These longer-term dietary changes provoked persistent changes in the composition of the supragingival microbiota that had not reverted even after the participants went back to the original regular diet for three months, although in PV the abundance of some taxa, e.g. *Streptococcus* spp., *Pasteurellaceae*, *Bacteroidia*, and *Rothia* spp. showed a tendency to approximate the initial levels of PI again. This result indicates that certain foods, in our case sucrose, dairy products and dietary fibers, if consumed frequently, have the ability to induce changes in the composition of the oral biofilm microbiota, even in adults, that persist for several months. This finding has not been demonstrated *in vivo* so far. An extension of the last dietary phase with sampling of the oral biofilm at a later point in time would be necessary to show if the changes will prove long-term or if the microbiota will revert to the original composition after a certain period of time.

Concerning alpha-diversity and species richness, both Shannon effective and Simpson effective values as well as the species richness, after a decrease in PII, were significantly increased in PIII and in PIV compared to PII. In earlier studies of the oral biofilm microbiota in caries, alpha-diversity and richness were usually decreased in carious teeth and a higher diversity was found in healthy teeth(24). Although we analyzed oral biofilm from healthy individuals without active carious lesions, the diet-induced changes of the microbiota had an effect on richness and diversity parameters. The observed diversity measures confirm the notion that a higher sucrose consumption

is supposed to create a shift of the microbiota towards a cariogenic composition and higher dairy or dietary fiber intake presumably favors a healthy oral biofilm composition.

The effect of the consumption of milk and dairy products on caries development has been debated in the past. Some studies did not find any anti-cariogenic effect and only found less demineralization of enamel specimens with whole milk, but not skimmed milk compared to a sucrose solution(35, 36). However, these experiments were performed *in vitro* on a single species biofilm model with *Streptococcus mutans* which is not able to reasonably reflect the actual complexity of the oral biofilm *in situ* with a multitude of interacting species and more than one species producing acids. Animal studies reported findings of bovine milk not being cariogenic and slightly cariostatic in rodents, especially when consumed together with cariogenic challenges(37-39). However several epidemiological studies mainly revealed correlations of high milk or yoghurt consumption with lower caries incidence in children and adults, especially in individuals with a high sucrose intake, although some studies reported a neutral outcome, particularly in areas with an already low caries prevalence in the population(12, 17, 23, 40). For example, Lempert *et al.* found that Danish children and adolescents that had a milk and dairy intake above the mean showed a significantly lower caries incidence after three years(41). Tanaka *et al*. showed that young Japanese children had significantly lower caries prevalence when consuming yoghurt more than four times a week(23).

However, so far only one recent study explored the oral microbiota in detail in relation to self-reported milk intake using high-throughput sequencing(42), the same methodology that was used in the present study. Johansson *et al*. sequenced 139 supragingival plaque samples from adolescents whose milk intake had been recorded and categorized into low, medium and high intake (high = more than 3.7 servings per day). Interestingly

they found that certain taxa differed significantly between the individuals who reported high or low milk intake, e.g. *Streptococcus mutans* showed a significantly lower abundance with high milk intake. The caries prevalence however, did not differ between the groups, presumably because the higher milk intake was paralleled by a higher intake of sweets.

In the present study, as the participants changed to a high dairy intake, we observed a significant decrease in abundance of the whole genus *Streptococcus*, as well as the phylum *Firmicutes*, of which streptococci are the main representatives. This significant decrease was also shown on the species level for the oral streptococci *S. sanguinis*, *S. gordonii* and *S. mitis*. It is known that these non-mutans streptococci include so called 'low pH streptococci', i.e. strains that are able to reduce the pH of a glucose broth to less than 4.4(43). Albeit, it must be noted, that the species with the highest relative abundance was *S. mitis* (PII, 20.83%; PIII, 13.34%), a species that contains acid-tolerant isolates(44), however has not been reported to show a high acidogenic potential(43).

The prevalence and the abundance of *S. mutans*, the major acidogenic species was only found in two study participants in very low abundances (below 0.01%; except for PII, 0.115 %). Most probably, the reason for this is that we analyzed oral biofilm from healthy individuals with no active carious lesions(24). Nevertheless, the fluctuations of the abundance of *S. mutans*, although very low, follow the same pattern as other oral streptococci, which increased in PII and then substantially decreased in PIII and PIV, going back to almost their original abundance in PV.

Some other taxa showed a significantly increased abundance in PIII, including *Pasteurellaceae*, with *Haemophilus* its main representative in the oral biofilm, *Bacteroidia*, with the genera *Porphyromonas*, *Prevotella* and *Alloprevotella* as main

representatives and the genus *Rothia*. *Haemophilus* spp. as well as *Rothia* spp. have been found in high abundance as a normal part of the oral microbiota of caries-free individuals and can be regarded as health-associated(24, 45, 46). Regarding *Porphyromonas* spp. and *Prevotella* spp., Johansson *et al.* also observed a higher relative abundance for the genus *Porphyromonas* and some *Prevotella* species, although periodontitis-associated *P. intermedia* and *P. melaninogenica* showed a lower abundance in the group with high milk intake(42). In our study we were not able to classify the genus *Prevotella* on the species level by sequencing, yet the cultivation of corresponding samples revealed mainly *Prevotella nigrescens*, *Prevotella histicola*, *Prevotella loeschei* and *Prevotella salivae* (data not shown). It can be assumed that the abundance of these taxa increased as pH increased and also due to the higher availability of proteins and peptides stemming from the milk and yoghurt that can be utilized by these taxa.

In view of the ecological plaque hypothesis, the decrease of the genus *Streptococcus* in PIII is indicative of a reduction of aciduric and acidogenic bacterial taxa in the supragingival biofilm as a consequence of the elevated milk and yoghurt intake, hereby creating an environment that is less prone to the development of carious lesions. As we studied the oral biofilm microbiota of healthy participants and not longer than three months per dietary phase, it is not possible to predict the outcome regarding the long-term development of carious lesions on the enamel slab surfaces had the different dietary phases been prolonged. Nevertheless, the measurements of the enamel surface roughness confirmed our conclusion of an oral biofilm with low cariogenicity regarding its effect on the enamel surface. The roughness showed a decrease in PIII, with a clear trend towards significance ($p=0.056$). In PIV and PV the surface roughness was

significantly lower than in PI, pointing to a lasting smoothing effect of the dietary phases III and IV.

Different aspects concerning plant-based foods have been assumed to exert an effect on oral health. A diet high in plant-based, whole-foods has been linked to a lower prevalence of caries, where phosphates, polyphenolic compounds as well as dietary fibers that increase salivary flow are assumed to be protective factors(17). Polyphenols possess powerful antioxidant activity demonstrated in many *in vitro* studies and have shown antibacterial activity against several periodontal pathogens(19). Regarding dental caries, studies investigating specific polyphenols demonstrated inhibition of glucan synthesis, adherence or acid production of mutans streptococci. Moreover, whole plant extracts, including other components in addition to polyphenols, have been shown to result in a decrease in the growth and virulence of mutans streptococci *in vitro*(19). These results were also confirmed in animal experiments, examining rats infected with mutans streptococci(47).

However, to the best of our knowledge, the influence of plant-based food on the oral biofilm microbiota has not yet been investigated *in situ* in humans. In the present study, the effect of fibrous plant foods on the mechanical stimulation of salivary flow could not have been detected, since the participants consumed vegetable puree which did not require a lot of chewing. Therefore the influence of the frequent dietary fiber intake on the composition of the oral biofilm was thought to arise directly from plant compounds. However the constraint that in PIV the simple carbohydrate intake was significantly lower than in PI needs to be taken into account, which could also have had an effect.

Even though we did not select any specific foods containing very high amounts of certain polyphenols, all the vegetables used in the study contain a variety of phenolic compounds, e.g. phenolic acids or flavonoids(48). Frequent consumption of a combination of several vegetables was deemed more reasonable than concentrating on a certain food product, since it is more feasible for individuals to focus on a diet that is rich in a variety of vegetables which is a recommendation for general health already. In PIV we observed a microbial community that was not significantly different from PIII regarding the beta-diversity, i.e. it was characterized by a low abundance of the genus *Streptococcus* and a slightly decreasing abundance of *Pasteurellaceae*, *Bacteroidia* and *Granulicatella* spp.. The genera *Rothia* and *Granulicatella* showed a significantly lower abundance in PIV compared to PIII and PI, respectively, whereas *Leptotrichiaceae* had a significantly higher abundance compared to PI and PII, respectively. There is contradicting information found in literature concerning the genus *Leptotrichia*. It is reported to be one of the dominant genera in the resident oral flora and many studies have associated it with caries-free individuals(49, 50) but at the same time it has also been associated with caries in other reports(49, 51, 52). Altogether this phase reflects a composition of the oral biofilm microbiota with a low abundance of potentially acidogenic streptococci and simultaneously a high abundance of taxa that are mostly considered part of a healthy oral flora.

In conclusion, the dietary phases with elevated sucrose, dairy and dietary fiber intake induced fluctuations of the oral biofilm microbiota that were still detectable three months after returning to the original baseline diet. Increased sucrose consumption favoured caries-promoting non-mutans streptococci while frequent milk and yoghurt consumption lowered the abundance of these taxa. A high dietary fiber intake revealed a high

abundance of mostly representatives of the normal oral microbiota. The observed changes were reflected in the developments of the enamel surface roughness, which was lowered after elevated dairy and dietary fiber intake. These results support and confirm the ecological plaque hypothesis. They point towards the significance of possible modulations of the microbiota through dietary changes even in adults, and hence call for a multifactorial approach to help prevent caries as a multifactorial disease, one factor being diet. This also seems to be a suitable therapeutic approach against periodontal diseases(53). Thus, the proportion of potentially pathogenic species could be influenced while the homeostasis of the commensal microflora can be preserved.

Material and methods

Study group and study design

The study group consisted of eleven healthy adults between 21 and 56 years (5 male, 6 female), that had given their written informed consent. Ethical approval was obtained from the Ethics Committee of the University of Freiburg (No. 237/14) and all experimental procedures were performed in accordance with relevant guidelines and regulations. Exclusion criteria for the study comprised: severe systemic diseases or diseases involving salivary glands and oral mucosa; acute or chronic oral diseases; current dental treatment, use of antibiotics within the last 30 days, eating disorders, food allergies or intolerances, allergies to dental materials, pregnancy or lactation. At the beginning of each study phase, oral examinations were performed, including measurements of salivary flow rates and buffering capacity of saliva as well as the DMFT values. The study design, shown in Figure 1, consisted of five different dietary phases (PI-PV), in which supragingival plaque was sampled using splint systems worn

by the participants for three x seven days (with seven-day intermissions) towards the end of each three-month-long phase. In PI, the regular diet was kept, in PII to IV specific foods were added to the regular diet and in PV the participants returned to their regular diet. During each phase, the diet was monitored using a validated food frequency questionnaire(54) and analyzed statistically using paired t-test. In PII, participants consumed an additional 10 g rock candy per day (Weisser Kandis, Südzucker AG, Mannheim, Germany), sucking small pieces of 2 g five times in between meals. PIII included the additional consumption of 150 g plain yoghurt 3 times daily and 100 ml long-life milk twice a day (both 1.5 % fat, Schwarzwaldmilch GmbH, Freiburg, Germany). In PIV, the study participants consumed 500 g vegetable puree per day of the following types: white carrot, parsnip, carrot, 'jardinière' (carrot, potato, cauliflower, pea), pumpkin and 'garden vegetables' (carrot, potato, spinach, parsnip, leeks) (Reine Weiße Karotte, Reine Pastinake, Reine Frühkarotte and Gemüse-Allerlei, Hipp GmbH Pfaffenhofen, Germany; Kürbis pur, Gartengemüse, Alnatura, Darmstadt, Germany). The splint system was taken out and stored in saline solution (0.9% NaCl) for regular meals and during oral hygiene only, but worn while consuming the additional foods specific for the different phases; these foods were eaten slowly, exposing them to the oral cavity for several minutes. Participants brushed their teeth with standardized tooth brushes and toothpaste (both Friscodent, Aldi Süd, Germany, toothpaste with a sodium fluoride content of 1.450ppm).

Splint systems containing bovine enamel slabs

Lower jaw acrylic appliances with bovine enamel slabs (BES) for the collection of supragingival biofilm were manufactured as described in earlier studies (24). Each of

these splint systems was equipped with six BES which were placed in the area between upper premolars and molars facing the teeth for the biofilm to grow undisturbed by movements of the tongue (Figure 2). The BES were prepared as described earlier as cylindrical slabs with a diameter of 5 mm that were polished by wet grinding, disinfected and loaded on the splint systems. For each phase the *in situ* grown biofilm was harvested from the slabs and used for high-throughput sequencing analysis, while the BES itself was cleansed using cotton pellets and saline solution and used for the analysis of the surface roughness. The splint system was disinfected (70% ethanol for 5 min), equipped with fresh BES and stored in saline solution until it was worn again.

16S r DNA Illumina MiSeq sequencing

DNA of the supragingival biofilm obtained from the BES was extracted using the DNeasy blood & tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for bacteria with an additional step for Gram-positive bacteria. For enzymatic lysis in addition to lysozyme (20 mg/ml), mutanolysin (1.500 U/ml; Sigma Aldrich, Taufkirchen, Germany) was applied and incubated for 1.5 h at 37°C. Microbial DNA was eluted twice in 100 µl AE buffer and then used for amplicon library preparation with the primers S-D-Bact-0008-a-S-16 (5'- AGA GTT TGA TCM TGG C -3') and S-D-Bact 0343-a-A-15 (5'-CTG CTG CCT YCC GTA -3') amplifying a 335 bp fragment of the variable regions v1-v2 and including the recommended adaptors for Illumina sequencing. Illumina paired-end sequencing according to the Illumina MiSeq protocol for amplicon sequencing and data analysis was performed as described in earlier studies(24). From this study the sequencing data for PI and PII was taken and analyzed together with PIII-PV. Post-processing was done with the IMNGS platform and the assembled OTUs for PI-V were

deposited in GenBank(55, 56). Additional analysis of the genus *Streptococcus* on the species level was performed with a phylogenetic analysis in ARB after manual extraction of all OTU sequence assigned to the genus *Streptococcus*(57). All sequences with a similarity of 97% or higher with the oral streptococci, i.e. *S. infantis*, *S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis*, *S. parasanguinis*, *S. salivarius*/*vestibularis* and *S. mutans* were identified. If only ambiguous affiliations were achieved, these OTUs were grouped as *Streptococcus* spp.

Statistical analysis

Statistical analysis of the obtained OTUs was performed with the Rhea pipeline for R for the analysis of sequence abundances, as well as alpha- and beta-diversity with visualization using non-metric multi-dimensional scaling (NMDS)(55). All calculations were done with normalized data. Generalized Uni Frac was used to calculate beta-diversity. Subsequently, taxonomic binning and serial group comparisons were performed for which the details can be found in the download package of Rhea.

For the analysis of the alpha-diversity linear mixed models (with random intercept for each probing and study participant as clusters) were used to compare subgroups. The method of Scheffe was applied to correct for the multiple testing problem (adjustment of p-values). The intraclass correlation coefficient (ICC) was used to quantify the reliability of the different methods. All computations were done with STATA 16.l.

Surface roughness of the enamel

To measure surface roughness a Keyence 3D Laserscanning Microscope VK-X210 (Keyence Deutschland GmbH, Neu-Isenburg, Germany) was used. First, the enamel

slabs were thoroughly cleansed and visually checked for any residues with the camera unit. Then, a polygonal measuring field was selected and the complete surface was measured with a resolution of 1000/mm, λS=2.5 and λc=0.25, excluding areas where the specimens had been mechanically damaged.

Data availability

The datasets supporting the conclusion of this article are available through GenBank (file: SUB7330939; accession numbers: MT435139 - MT435484).

Acknowledgements:

The authors thank Bettina Spitzmüller, Lisa Römling, Kristina Kollmar and Arsin Sabunchi for their excellent technical assistance. This study was supported by the German Research Foundation (DFG, AL-1179/2–1).

AA and EH conceptualized the study. MJA, LK and JPW analyzed the results. ACA and LK collected the samples. MR and KV performed statistical analyzes. ACA and MR performed the experiments, analyzed the results, drafted the manuscript and prepared the figures. All authors edited the manuscript and approved the final article.

The authors declare that they have no competing interests.

References

1. Mark Welch JL, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. 2016. Biogeography of a human oral microbiome at the micron scale. Proc Natl Acad Sci U S A 113:E791-800.
2. Lamont RJ, Koo H, Hajishengallis G. 2018. The oral microbiota: dynamic communities and host interactions. 16:745-759.
3. Diaz PI, Valm AM. 2020. Microbial Interactions in Oral Communities Mediate Emergent Biofilm Properties. 99:18-25.
4. Sultan AS, Kong EF. 2018. The oral microbiome: A Lesson in coexistence. 14:e1006719.

526 5. Takahashi N, Nyvad B. 2008. Caries ecology revisited: microbial dynamics and the caries process.
527 Caries Res 42:409-18.
528 6. Meier T, Deumelandt P, Christen O, Stangl GI, Riedel K, Langer M. 2017. Global Burden of Sugar-
529 Related Dental Diseases in 168 Countries and Corresponding Health Care Costs. J Dent Res
530 96:845-854.
531 7. Marsh PD. 2018. In Sickness and in Health-What Does the Oral Microbiome Mean to Us? An
532 Ecological Perspective. Adv Dent Res 29:60-65.
533 8. Mira A. 2018. Oral Microbiome Studies: Potential Diagnostic and Therapeutic Implications. Adv
534 Dent Res 29:71-77.
535 9. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ. 2008. Bacteria of
536 dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol
537 46:1407-17.
538 10. Bradshaw DJ, Lynch RJ. 2013. Diet and the microbial aetiology of dental caries: new paradigms.
539 Int Dent J 63 Suppl 2:64-72.
540 11. Moynihan P, Petersen PE. 2004. Diet, nutrition and the prevention of dental diseases. Public
541 Health Nutr 7:201-26.
542 12. Petti S, Simonetti R, Simonetti D'Arca A. 1997. The effect of milk and sucrose consumption on
543 caries in 6-to-11-year-old Italian schoolchildren. Eur J Epidemiol 13:659-64.
544 13. Aimutis WR. 2004. Bioactive properties of milk proteins with particular focus on anticariogenesis.
545 J Nutr 134:989s-95s.
546 14. Reynolds EC, Cai F, Shen P, Walker GD. 2003. Retention in plaque and remineralization of enamel
547 lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. J Dent Res
548 82:206-11.
549 15. Schlafer S, Ibsen CJ, Birkedal H, Nyvad B. 2017. Calcium-Phosphate-Osteopontin Particles Reduce
550 Biofilm Formation and pH Drops in in situ Grown Dental Biofilms. Caries Res 51:26-33.
551 16. Guggenheim B, Schmid R, Aeschlimann JM, Berrocal R, Neeser JR. 1999. Powdered milk micellar
552 casein prevents oral colonization by Streptococcus sobrinus and dental caries in rats: a basis for
553 the caries-protective effect of dairy products. Caries Res 33:446-54.
554 17. Moynihan P. 2007. Foods and dietary factors that prevent dental caries. Quintessence Int
555 38:320-4.
556 18. Ko MO, Kim MB, Lim SB. 2016. Relationship between Chemical Structure and Antimicrobial
557 Activities of Isothiocyanates from Cruciferous Vegetables against Oral Pathogens. J Microbiol
558 Biotechnol 26:2036-2042.
559 19. Petti S, Scully C. 2009. Polyphenols, oral health and disease: A review. J Dent 37:413-23.
560 20. Grenby TH, Andrews AT, Mistry M, Williams RJ. 2001. Dental caries-protective agents in milk and
561 milk products: investigations in vitro. J Dent 29:83-92.
562 21. Bowen WH, Pearson SK, VanWuyckhuyse BC, Tabak LA. 1991. Influence of milk, lactose-reduced
563 milk, and lactose on caries in desalivated rats. Caries Res 25:283-6.
564 22. Rugg-Gunn AJ, Hackett AF, Appleton DR, Jenkins GN, Eastoe JE. 1984. Relationship between
565 dietary habits and caries increment assessed over two years in 405 English adolescent school
566 children. Arch Oral Biol 29:983-92.
567 23. Tanaka K, Miyake Y, Sasaki S. 2010. Intake of dairy products and the prevalence of dental caries
568 in young children. J Dent 38:579-83.
569 24. Anderson AC, Rothballer M, Altenburger MJ, Woelber JP, Karygianni L, Lagkouvardos I, Hellwig E,
570 Al-Ahmad A. 2018. In-vivo shift of the microbiota in oral biofilm in response to frequent sucrose
571 consumption. Scientific reports 8:14202-14202.
572 25. Feinman RD, Pogozelski WK, Astrup A, Bernstein RK, Fine EJ, Westman EC, Accurso A, Frassetto L,
573 Gower BA, McFarlane SI, Nielsen JV, Krarup T, Saslow L, Roth KS, Vernon MC, Volek JS, Wilshire
574 GB, Dahlqvist A, Sundberg R, Childers A, Morrison K, Manninen AH, Dashti HM, Wood RJ,

575 Wortman J, Worm N. 2015. Dietary carbohydrate restriction as the first approach in diabetes
576 management: critical review and evidence base. Nutrition 31:1-13.
577 26. Karygianni L, Follo M, Hellwig E, Burghardt D, Wolkewitz M, Anderson A, Al-Ahmad A. 2012.
578 Microscope-based imaging platform for large-scale analysis of oral biofilms. Appl Environ
579 Microbiol 78:8703-11.
580 27. Karygianni L, Ruf S, Follo M, Hellwig E, Bucher M, Anderson AC, Vach K, Al-Ahmad A. 2014. Novel
581 Broad-Spectrum Antimicrobial Photoinactivation of In Situ Oral Biofilms by Visible Light plus
582 Water-Filtered Infrared A. Appl Environ Microbiol 80:7324-36.
583 28. Xu H, Tian J, Hao W, Zhang Q, Zhou Q, Shi W, Qin M, He X, Chen F. 2018. Oral Microbiome Shifts
584 From Caries-Free to Caries-Affected Status in 3-Year-Old Chinese Children: A Longitudinal Study.
585 Frontiers in Microbiology 9.
586 29. Zero DT. 1995. In situ caries models. Adv Dent Res 9:214-30; discussion 231-4.
587 30. Rosier BT, Marsh PD, Mira A. 2018. Resilience of the Oral Microbiota in Health: Mechanisms That
588 Prevent Dysbiosis. J Dent Res 97:371-380.
589 31. Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, Podar M, Warner
590 B, Tarr PI, Nelson DE, Fortenberry JD, Holland MJ, Burr SE, Shannon WD, Sodergren E, Weinstock
591 GM. 2013. Biogeography of the ecosystems of the healthy human body. Genome Biol 14:R1.
592 32. Hall MW, Singh N, Ng KF, Lam DK, Goldberg MB, Tenenbaum HC, Neufeld JD, R GB, Senadheera
593 DB. 2017. Inter-personal diversity and temporal dynamics of dental, tongue, and salivary
594 microbiota in the healthy oral cavity. NPJ Biofilms Microbiomes 3:2.
595 33. Zaura E, Brandt BW, Teixeira de Mattos MJ, Buijs MJ, Caspers MP, Rashid MU, Weintraub A, Nord
596 CE, Savell A, Hu Y, Coates AR, Hubank M, Spratt DA, Wilson M, Keijser BJ, Crielaard W. 2015.
597 Same Exposure but Two Radically Different Responses to Antibiotics: Resilience of the Salivary
598 Microbiome versus Long-Term Microbial Shifts in Feces. mBio 6:e01693-15.
599 34. Benitez-Paez A, Belda-Ferre P, Simon-Soro A, Mira A. 2014. Microbiota diversity and gene
600 expression dynamics in human oral biofilms. BMC Genomics 15:311.
601 35. Giacaman RA, Munoz-Sandoval C. 2014. Cariogenicity of different commercially available bovine
602 milk types in a biofilm caries model. Pediatr Dent 36:1e-6e.
603 36. Munoz-Sandoval C, Munoz-Cifuentes MJ, Giacaman RA, Ccahuana-Vasquez RA, Cury JA. 2012.
604 Effect of bovine milk on Streptococcus mutans biofilm cariogenic properties and enamel and
605 dentin demineralization. Pediatr Dent 34:e197-201.
606 37. Bowen WH, Lawrence RA. 2005. Comparison of the cariogenicity of cola, honey, cow milk,
607 human milk, and sucrose. Pediatrics 116:921-6.
608 38. Bowen WH, Pearson SK. 1993. Effect of milk on cariogenesis. Caries Res 27:461-6.
609 39. Giacaman RA. 2018. Sugars and beyond. The role of sugars and the other nutrients and their
610 potential impact on caries. Oral Dis 24:1185-1197.
611 40. Johansson I, Holgerson PL, Kressin NR, Nunn ME, Tanner AC. 2010. Snacking habits and caries in
612 young children. Caries Res 44:421-30.
613 41. Lempert SM, Christensen LB, Froberg K, Raymond K, Heitmann BL. 2015. Association between
614 dairy intake and caries among children and adolescents. results from the Danish EYHS follow-up
615 study. Caries Res 49:251-8.
616 42. Johansson I, Esberg A, Eriksson L, Haworth S, Lif Holgerson P. 2018. Self-reported bovine milk
617 intake is associated with oral microbiota composition. PLoS One 13:e0193504.
618 43. Banas JA, Zhu M, Dawson DV, Blanchette DR, Drake DR, Gu H, Frost R, McCaulley G, Levy SM.
619 2016. Acidogenicity and acid tolerance of Streptococcus oralis and Streptococcus mitis isolated
620 from plaque of healthy and incipient caries teeth. J Oral Microbiol 8:32940.
621 44. Svensater G, Borgstrom M, Bowden GH, Edwardsson S. 2003. The acid-tolerant microbiota
622 associated with plaque from initial caries and healthy tooth surfaces. Caries Res 37:395-403.

45. Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, Nelson KE, Gill SR, Fraser-Liggett CM, Relman DA. 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. Isme j 4:962-74.
46. Peterson SN, Snesrud E, Liu J, Ong AC, Kilian M, Schork NJ, Bretz W. 2013. The dental plaque microbiome in health and disease. PLoS One 8:e58487.
47. Ooshima T, Osaka Y, Sasaki H, Osawa K, Yasuda H, Matsumura M, Sobue S, Matsumoto M. 2000. Caries inhibitory activity of cacao bean husk extract in in-vitro and animal experiments. Arch Oral Biol 45:639-45.
48. Rothwell JA, Perez-Jimenez J, Neveu V, Medina-Remón A, M'Hiri N, García-Lobato P, Manach C, Knox C, Eisner R, Wishart DS, Scalbert A. 2013. Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database 2013.
49. Eribe ERK, Olsen I. 2017. Leptotrichia species in human infections II. J Oral Microbiol 9:1368848.
50. Schoilew K, Ueffing H, Dalpke A, Wolff B, Frese C, Wolff D, Boutin S. 2019. Bacterial biofilm composition in healthy subjects with and without caries experience. J Oral Microbiol 11:1633194.
51. Eriksson L, Lif Holgerson P, Johansson I. 2017. Saliva and tooth biofilm bacterial microbiota in adolescents in a low caries community. Sci Rep 7:5861.
52. Johansson I, Witkowska E, Kaveh B, Lif Holgerson P, Tanner AC. 2016. The Microbiome in Populations with a Low and High Prevalence of Caries. J Dent Res 95:80-6.
53. Woelber JP, Tennert C. 2020. Chapter 13: Diet and Periodontal Diseases. Monogr Oral Sci 28:125-133.
54. Haftenberger M, Heuer T, Heidemann C, Kube F, Krems C, Mensink GB. 2010. Relative validation of a food frequency questionnaire for national health and nutrition monitoring. Nutr J 9:36.
55. Lagkouvardos I, Fischer S, Kumar N, Clavel T. 2017. Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ 5:e2836.
56. Lagkouvardos I, Joseph D, Kapfhammer M, Giritli S, Horn M, Haller D, Clavel T. 2016. IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep 6:33721.
57. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH. 2004. ARB: a software environment for sequence data. Nucleic Acids Res 32:1363-71.

Figure Legends

Figure 1

Study design. Supragingival biofilm samples grown on bovine enamel slabs on splint systems worn by 11 study participants were collected in five three month-long phases at three sampling times each (PIa,b,c – PVa,b,c). Regular diet (grey), dietary phases with

specific food intake added to the regular diet (pink), weeks in which splint system was worn (blue), weeks in which splint system was not worn (burgundy).

Figure 2

Splint system. Lower-jaw splint system with bovine enamel slabs (a-e) for the collection of supragingival oral biofilm

Figure 3

Bacterial composition of supragingival biofilm of 11 study participants in different dietary phases. Relative abundance of bacterial phyla and most abundant genera (> 2%) (a) and oral *Streptococcus* species (b) detected in dietary phases I-V (including zero values). In b) *Streptococcus* spp. refers to OTUs that could not be unambiguously assigned to one of the oral *Streptococcus* species.

Figure 3

Beta-diversity of the microbial communities in supragingival biofilm of 11 study participants in different dietary phases based on UniFrac. NMDS plots show significant differences comparing beta-diversity of PI-PV. PI-PV are indicated in the plot by numbers 1-5.

Figure 4

Box plots demonstrating significant differences in relative species abundance in supragingival biofilm in different dietary phases. a) Box plots showing the relative abundance (%) of different bacterial taxa that showed significant differences between

687 different dietary phases. b) Box plots showing the relative abundance (%) of oral
688 *Streptococcus* species that showed significant differences between different dietary
689 phases.

690

691 **Figure 5**

692 **Enamel surface roughness in different dietary phases**. Mean values [µm] of the
693 surface roughness profile (Ra) of the enamel samples for the different dietary phases PI-
694 PV.

695

696 **Tables**

697

698 **Table 1**

699 Mean values [%] of main nutrient intake by the 11 study participants in phase I-V.

700

Participant	Phase	% Simple CHO	% Complex CHO	% total CHO	% Proteins	% Fat
1	I	0.46	0.21	0.68	0.16	0.17
	II	0.23	0.40	0.63	0.20	0.16
	III	0.30	0.36	0.66	0.17	0.17
	IV	0.25	0.45	0.70	0.16	0.14
	V	0.26	0.41	0.67	0.18	0.15
2	I	0.22	0.42	0.64	0.15	0.21
	II	0.29	0.40	0.69	0.19	0.12
	III	0.37	0.22	0.60	0.23	0.18
	IV	0.15	0.38	0.53	0.31	0.16
	V	0.30	0.35	0.65	0.22	0.13
3	I	0.23	0.34	0.57	0.22	0.20
	II	0.32	0.29	0.61	0.20	0.19
	III	0.23	0.30	0.53	0.25	0.23
	IV	0.20	0.45	0.65	0.20	0.15
	V	0.15	0.45	0.60	0.21	0.18
4	I	0.24	0.22	0.46	0.22	0.32
	II	0.44	0.17	0.62	0.17	0.21
	III	0.27	0.36	0.63	0.17	0.20

	IV	0.30	0.36	0.66	0.18	0.17
	V	0.27	0.34	0.62	0.17	0.21
5	I	0.24	0.36	0.60	0.17	0.24
	II	0.13	0.47	0.60	0.18	0.21
	III	0.33	0.28	0.60	0.17	0.23
	IV	0.30	0.37	0.67	0.15	0.17
	V	0.43	0.25	0.68	0.15	0.17
6	I	0.23	0.27	0.51	0.25	0.24
	II	0.20	0.43	0.63	0.17	0.20
	III	0.28	0.35	0.63	0.18	0.19
	IV	0.11	0.33	0.44	0.21	0.35
	V	0.26	0.23	0.49	0.24	0.27
7	I	0.23	0.30	0.54	0.23	0.23
	II	0.23	0.26	0.49	0.23	0.28
	III	0.23	0.26	0.49	0.22	0.28
	IV	0.09	0.38	0.47	0.25	0.28
	V	0.11	0.48	0.59	0.20	0.20
8	I	0.36	0.28	0.64	0.18	0.18
	II	0.38	0.27	0.65	0.17	0.18
	III	0.30	0.24	0.54	0.16	0.30
	IV	0.37	0.21	0.58	0.18	0.24
	V	0.33	0.30	0.63	0.17	0.20
9	I	0.47	0.21	0.68	0.12	0.20
	II	0.37	0.33	0.69	0.14	0.16
	III	0.35	0.26	0.61	0.19	0.20
	IV	0.46	0.26	0.72	0.14	0.14
	V	0.38	0.22	0.60	0.19	0.21
10	I	0.21	0.35	0.56	0.18	0.26
	II	0.25	0.38	0.63	0.17	0.19
	III	0.36	0.34	0.70	0.14	0.16
	IV	0.27	0.34	0.60	0.21	0.19
	V	0.31	0.31	0.63	0.19	0.18
11	I	0.36	0.28	0.64	0.20	0.16
	II	0.31	0.10	0.41	0.21	0.38
	III	0.18	0.24	0.42	0.20	0.39
	IV	0.27	0.29	0.56	0.20	0.24
	V	0.28	0.25	0.53	0.23	0.25
Mean	I	0.28	0.30	0.58	0.20	0.22
	II	0.28	0.33	0.62	0.18	0.20
	III	0.28	0.29	0.57	0.18	0.24
	IV	0.25	0.34	0.59	0.20	0.21
	V	0.28	0.32	0.60	0.20	0.21

701

Table 2

Mean values of richness and alpha-diversity measures of the supragingival microbiota of the 11 study participants in phase I-V

Phase	Richness	Shannon effective	Simpson effective
I	193.48 ± 29.9	37.36 ± 13.2	20.07 ± 7.8
II	171.27 ± 31.5	30.58 ± 13.2	16.13 ± 8.0
III	194.30 ± 38.5	43.47 ± 19.1	24.70 ± 12.8
IV	194.56 ± 37.5	42.03 ± 16.3	22.39 ± 9.1
V	183.14 ± 37.9	34.55 ± 14.9	18.40 ± 8.7

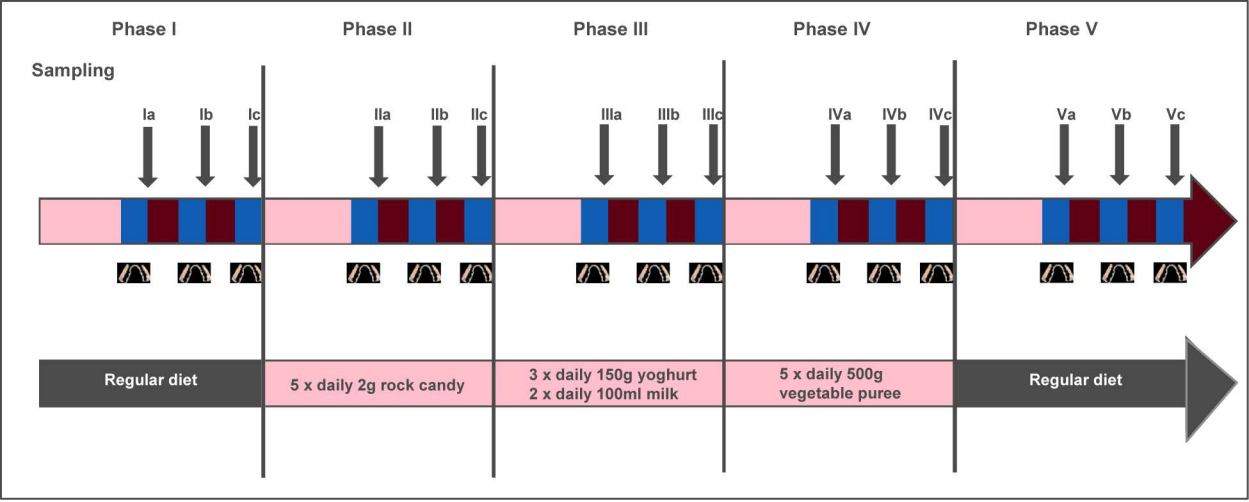
Additional Information:

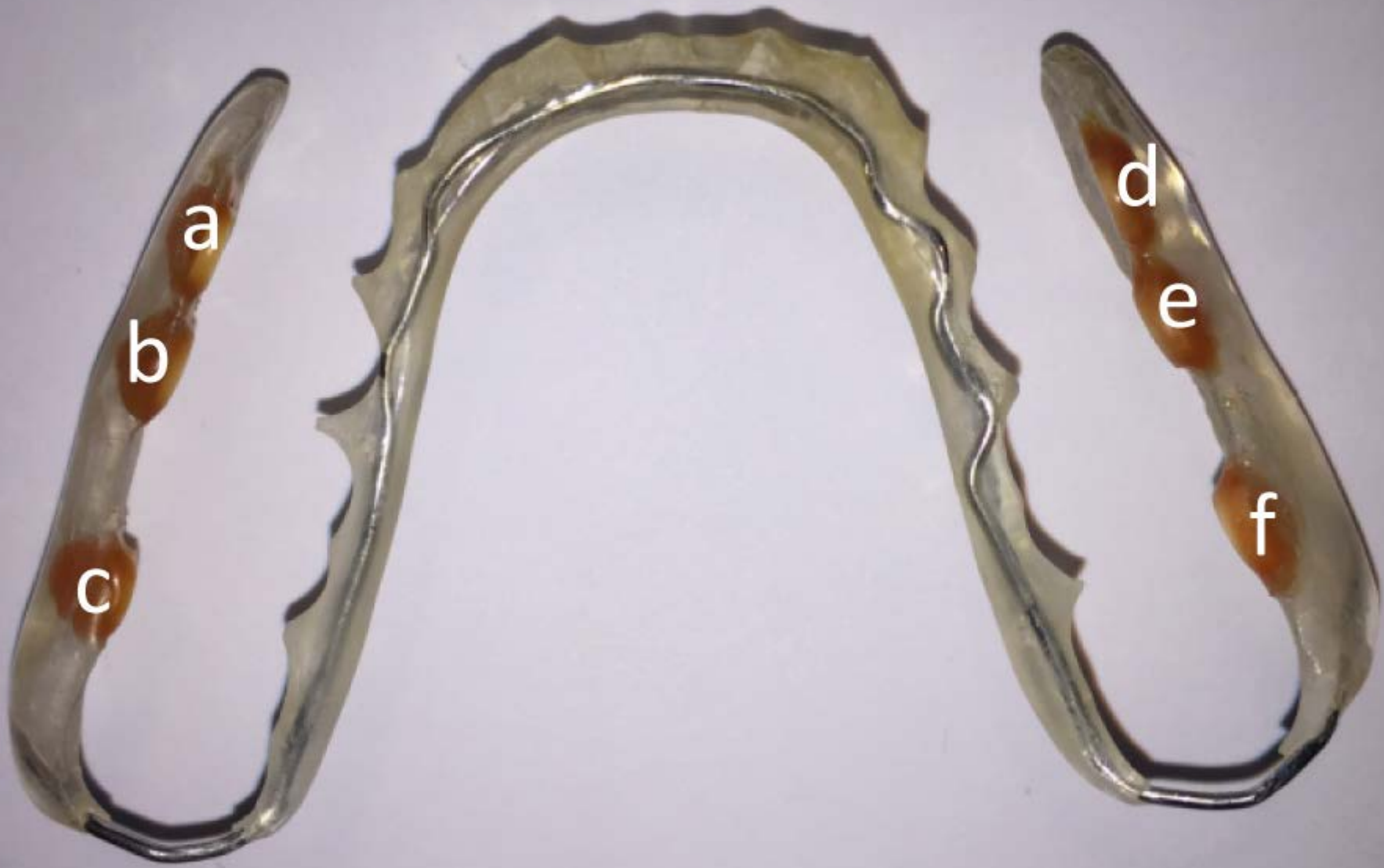
Supplementary Dataset:

Table S1: Bacterial taxa found in the supragingival biofilm samples of 11 study participants in phase I-V with their relative abundances.

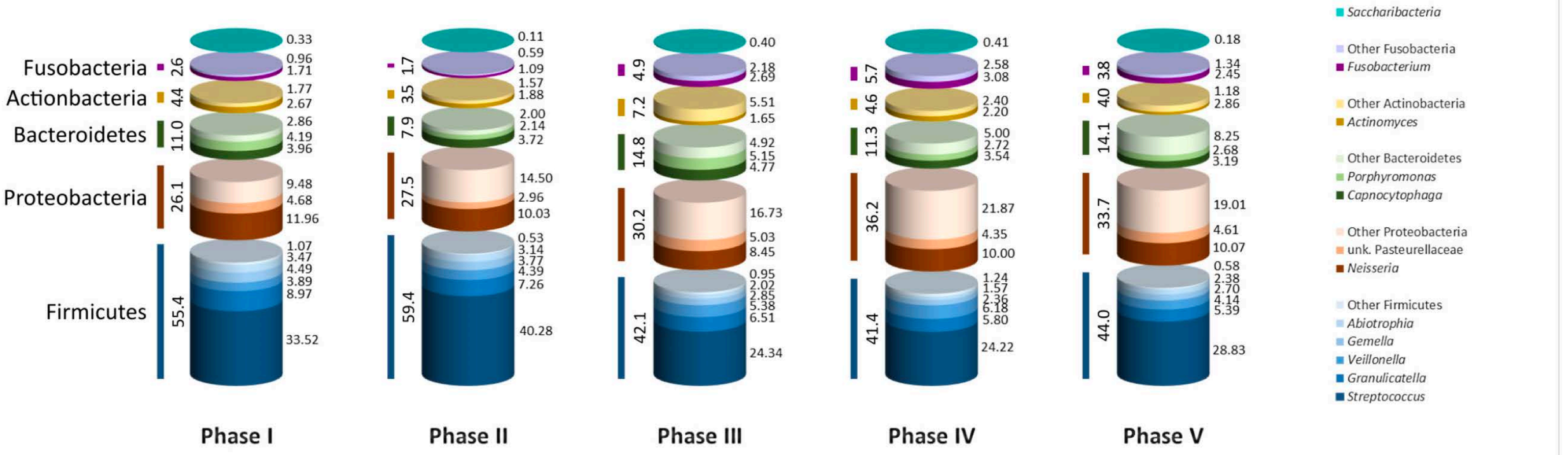
Table S2: OTUs assigned to the supragingival biofilm samples of 11 study participants in phase I-V with their relative abundances.

Table S3: Relative abundances of the different oral streptococci found in the supragingival biofilm of 11 study participants in phase I-V.

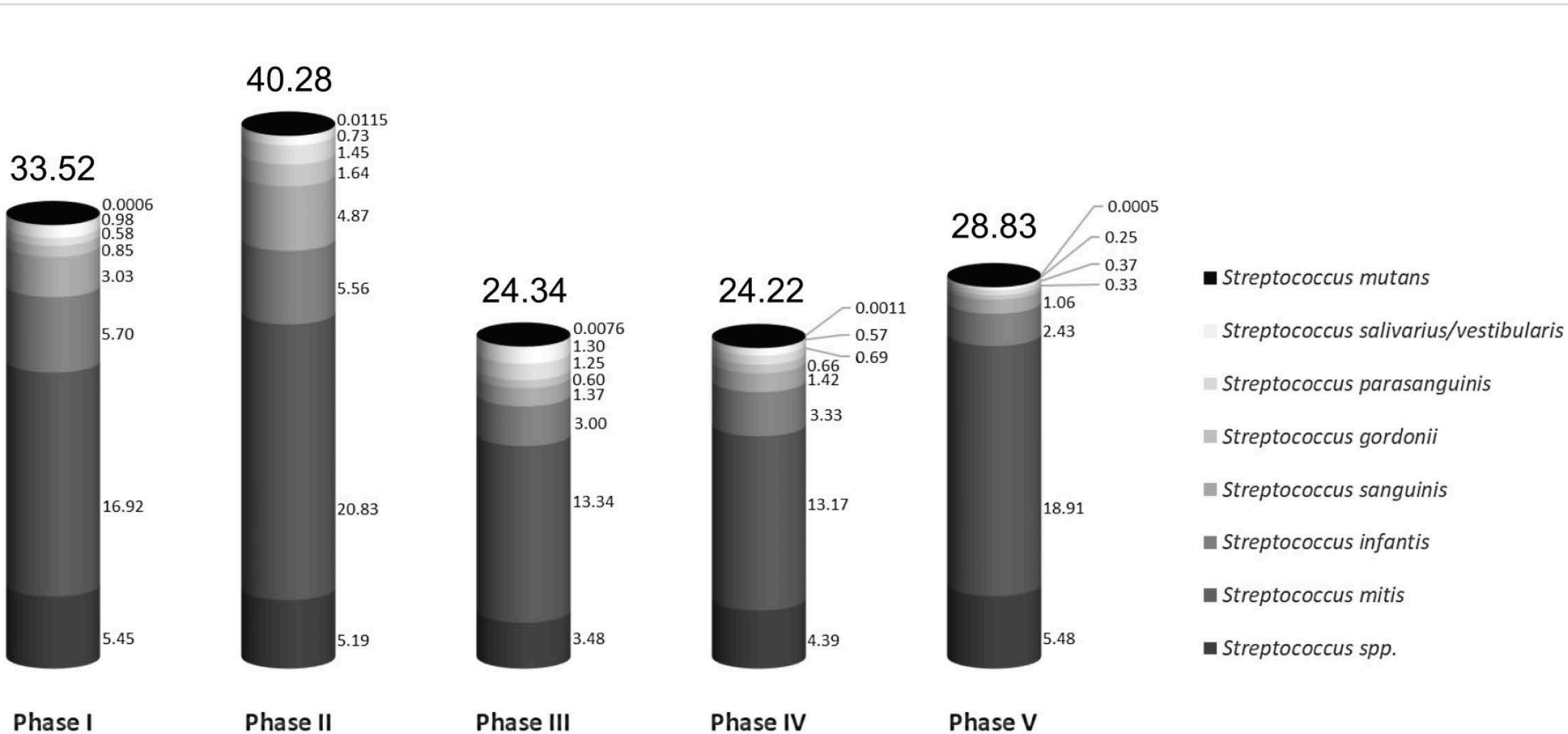

Phase I
Phase II
Phase III
Phase IV
Phase V
Sampling
Ia
Ib
Ic
IIa
IIb
IIc
IIIa
IIIb
IIIc
IVa
IVb
IVc
Va
Vb
Vc
Regular diet
5 x daily 2g rock candy
3 x daily 150g yoghurt
2 x daily 100ml milk
5 x daily 500g
vegetable puree
Regular diet
= 7 weeks no splint
= 7 days with splint
= 7 days without splint

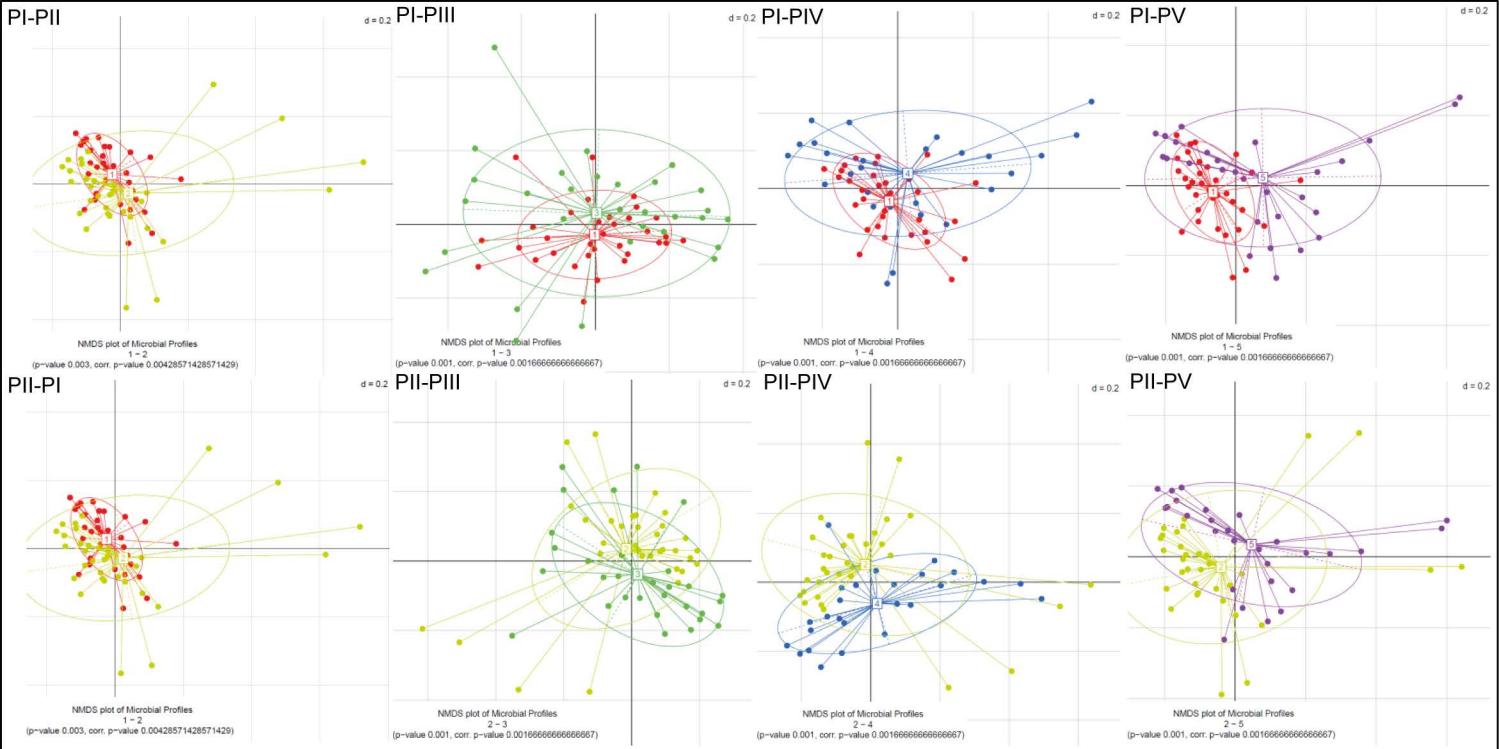

a
b
c
d
e
f

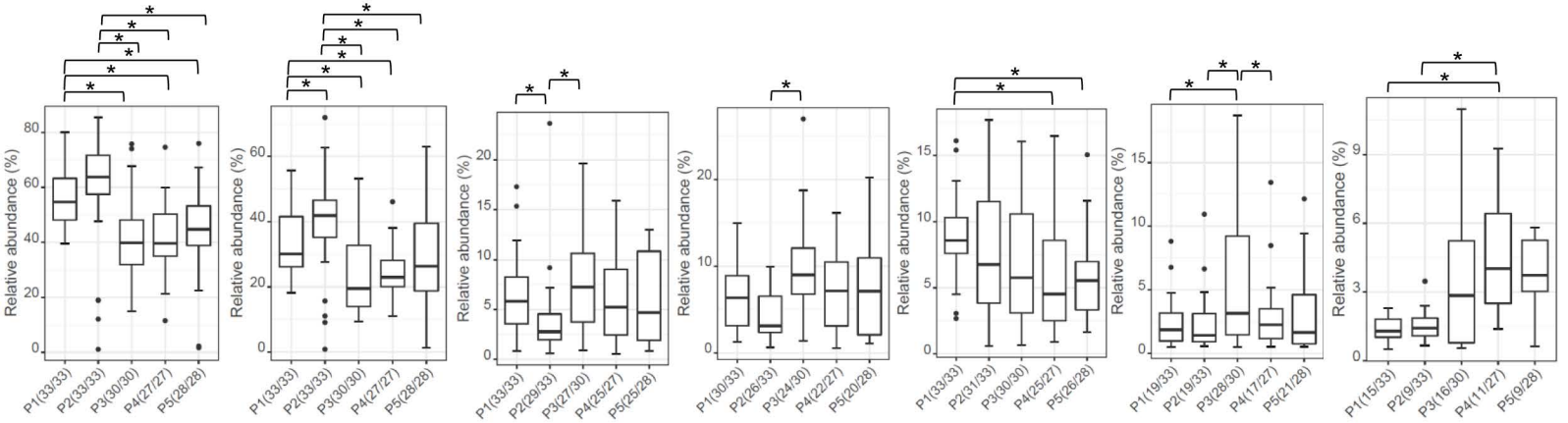
a

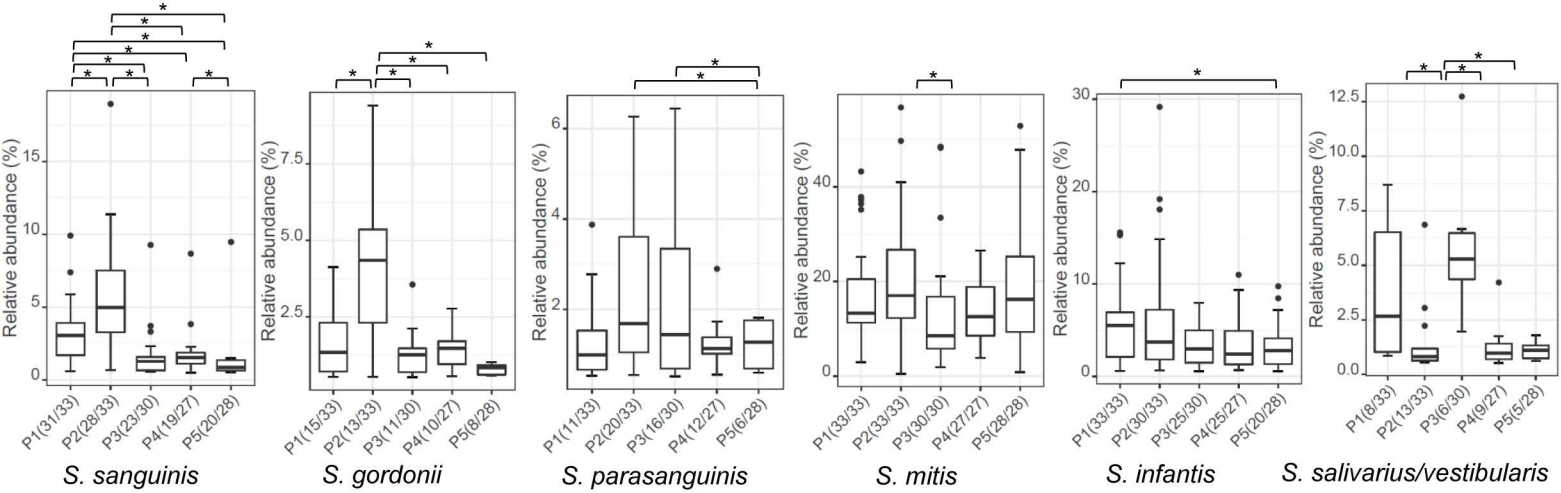

Fusobacteria
Actionbacteria
Bacteroidetes
Proteobacteria
Firmicutes
Phase I
Phase II
Phase III
Phase IV
Phase V
Saccharibacteria
Other Fusobacteria
Fusobacterium
Other Actinobacteria
Actinomyces
Other Bacteroidetes
Porphyromonas
Capnocytophaga
Other Proteobacteria
unk. Pasteurellaceae
Neisseria
Other Firmicutes
Abiotrophia
Gemella
Veillonella
Granulicatella
Streptococcus

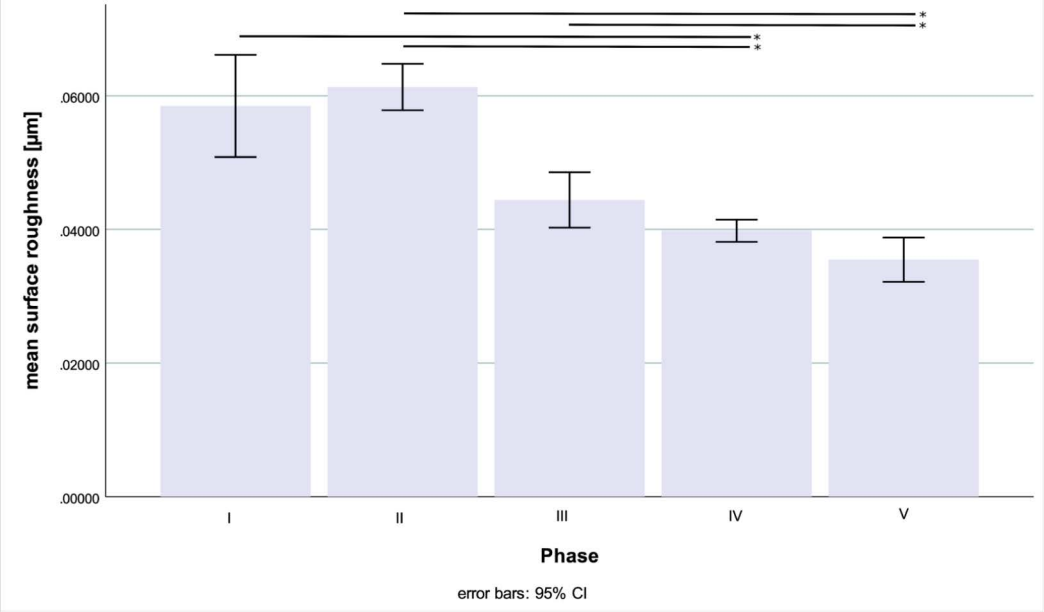
b


Phase I
Phase II
Phase III
Phase IV
Phase V
Streptococcus mutans
Streptococcus salivarius/vestibularis
Streptococcus parasanguinis
Streptococcus gordonii
Streptococcus sanguinis
Streptococcus infantis
Streptococcus mitis
Streptococcus spp.


PI-PII
d = 0.2
NMDS plot of Microbial Profiles
1 ~ 2
(p-value 0.003, corr. p-value 0.0042857142857429)
PI-PIII
d = 0.2
NMDS plot of Microbial Profiles
1 ~ 3
(p-value 0.001, corr. p-value 0.0016666666666667)
PI-PIV
d = 0.2
NMDS plot of Microbial Profiles
1 ~ 4
(p-value 0.001, corr. p-value 0.0016666666666667)
PI-PV
d = 0.2
NMDS plot of Microbial Profiles
1 ~ 5
(p-value 0.001, corr. p-value 0.0016666666666667)
PII-PI
d = 0.2
NMDS plot of Microbial Profiles
1 ~ 2
(p-value 0.003, corr. p-value 0.0042857142857429)
PII-PIII
d = 0.2
NMDS plot of Microbial Profiles
2 ~ 3
(p-value 0.001, corr. p-value 0.0016666666666667)
PII-PIV
d = 0.2
NMDS plot of Microbial Profiles
2 ~ 4
(p-value 0.001, corr. p-value 0.0016666666666667)
PII-PV
d = 0.2
NMDS plot of Microbial Profiles
2 ~ 5
(p-value 0.001, corr. p-value 0.0016666666666667)


Relative abundance (%)
P1(33/33)
P2(33/33)
P3(30/30)
P4(27/27)
P5(28/28)
Firmicutes
Streptococcus
Pasteurellaceae
Bacteroidia
Granulicatella
Rothia
Leptotrichiaceae


Relative abundance (%)
P1(31/33)
P2(28/33)
P3(23/30)
P4(19/27)
P5(20/28)
S. sanguinis
P1(15/33)
P2(13/33)
P3(11/30)
P4(10/27)
P5(8/28)
S. gordonii
P1(11/33)
P2(20/33)
P3(16/30)
P4(12/27)
P5(6/28)
S. parasanguinis
P1(33/33)
P2(33/33)
P3(30/30)
P4(27/27)
P5(28/28)
S. mitis
P1(33/33)
P2(30/33)
P3(25/30)
P4(25/27)
P5(20/28)
S. infantis
P1(8/33)
P2(13/33)
P3(6/30)
P4(9/27)
P5(5/28)
S. salivarius/vestibularis


mean surface roughness [µm]
.06000
.04000
.02000
.00000
I
II
III
IV
V
Phase
error bars: 95% CI