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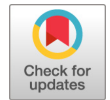

Long-Term Fluctuation of Oral Biofilm Microbiota following Different Dietary Phases

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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 the effects of selected ingredients on the oral biofilm microbiota *in situ*, 11 study participants underwent 3-month-long dietary phases with intake of a regular diet (PI), additional frequent sucrose (PII), milk and yoghurt (PIII), and a diet rich in dietary fiber (PIV) and then returned 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 by 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 versus PII: $P < 0.00001$) and increasing again toward PV. Other taxa showed various fluctuations of their abundances, with PV returning approximately to the levels of PI. In conclusion, while elevated sucrose consumption favored 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 these issues, we analyzed changes of the *in situ* plaque microbiota following 3-month-long dietary changes involving elevated sucrose, dairy, and dietary fiber consumption over a period of 15 months. Applying high-throughput sequencing, we found non-mutans streptococci, a taxonomic group involved in the beginning stages toward microbial dysbiosis, in decreased abundance with elevated dairy and dietary fiber intake. Through analysis of the enamel surface roughness, these effects were confirmed. Therefore, correspondent dietary measures can be recommended for children as well as adults for caries prevention.

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The oral biofilm is considered an ecosystem at the micrometer scale comprising more than 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 aciduric 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 with 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 has 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 casein phosphopeptides (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 reports have described either *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 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 3 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

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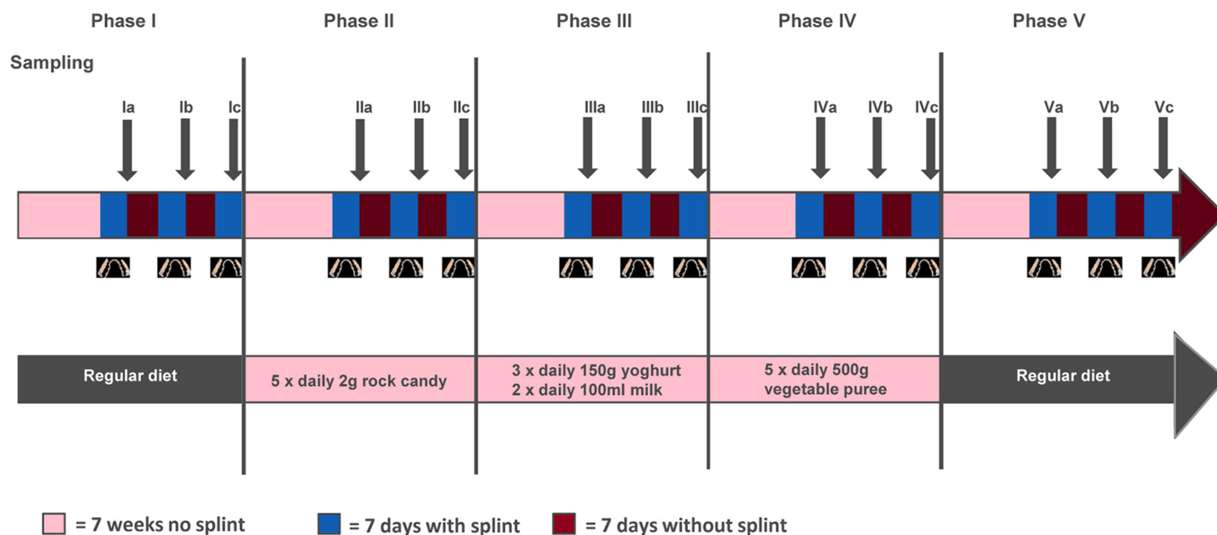

FIG 1 Study design. Supragingival biofilm samples grown on bovine enamel slabs on splint systems worn by 11 study participants were collected in five 3-month-long phases at three sampling times each (Ia, Ib, and Ic to Va, Vb, and Vc).

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 by measuring surface roughness with three-dimensional (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 3-month-long dietary phases (PI to PV), first keeping their regular diet, then having an elevated sucrose intake, followed by an elevated dairy intake and then an elevated dietary fiber intake, and finally returning to their regular diet (Fig. 1). Supragingival plaque was sampled from bovine enamel slabs (BES) embedded in splint systems (Fig. 2) worn for three times 7 days (with 7-day intermissions without a splint) toward the end of each 3-month-long phase. 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 decayed/missing/filled teeth (DMFT) value was 8.1. Detailed demographic and clinical data are reported by

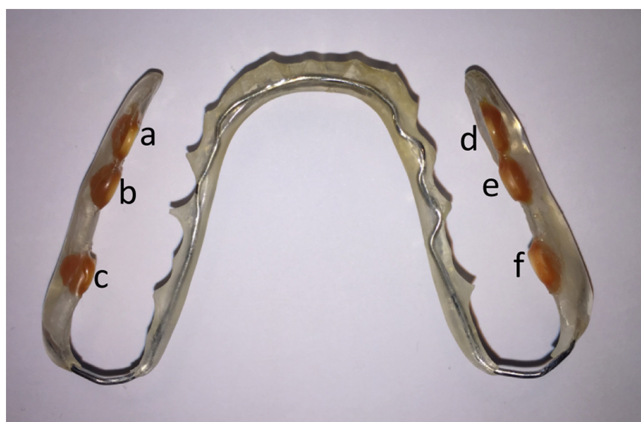

FIG 2 Splint system. Lower-jaw splint system with bovine enamel slabs (a to f) for the collection of supragingival oral biofilm.

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TABLE 1 Mean values of main nutrient intake by the 11 study participants in phases I to V

Participant	Phase	Relative fraction[a]				
		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

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TABLE 1 (Continued)

Participant	Phase	Relative fraction[a]				
		Simple CHO	Complex CHO	Total CHO	Proteins	Fat
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

[a]Relative fractions (where 1.0 equals 100%) are shown. CHO, carbohydrates.

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 basically all changes in the biofilm composition in PII to PV can be ascribed to the phase-specific dietary changes.

Composition of the supragingival microbiota. A total of 9.91 million high-quality reads were 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; and PV, 5 samples). Concerning relative bacterial phyla abundances (shown in Fig. 3a), *Firmicutes* dominated in all phases (PI, 55.41%; PII, 59.37%; PIII, 42.05%; PIV, 41.37%; and 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%; and PV, 28.83%), followed by *Neisseria*, *Granulicatella*, *Gemella*, *Veillonella*, *Capnocytophaga*, and *Porphyromonas* (Fig. 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 Tables S1 and S2 in the supplemental material. The manual species-level analysis of the genus *Streptococcus* revealed the proportional distribution of the oral streptococci (Fig. 3b). The highest relative abundance was found for *Streptococcus mitis* (PI, 16.92%; PII, 20.83%; PIII, 13.34%; PIV, 13.17%; and PV, 18.91%), followed by ambiguously assigned *Streptococcus* spp. (PI, 5.45%; PII, 5.19%; PIII, 3.48%; PIV, 4.39%; and PV, 5.48%), *Streptococcus infantis* (PI, 5.70%; PII, 5.56%; PIII, 3.0%; PIV, 3.33%; and PV, 2.43%), *Streptococcus sanguinis* (PI, 3.03%; PII, 4.87%; PIII, 1.37%; PIV, 1.42%; and PV, 1.06%), *Streptococcus gordonii*, *Streptococcus parasanguinis*, and *Streptococcus salivarius/vestibularis* (the last species could not be discriminated based on the 16S rRNA gene). As for *Streptococcus mutans*, this was the *Streptococcus* species with the lowest relative abundance, detectable only in single study participants and at very low abundances ($<$0.01%), except in PII (PI, 0.0006%; PII, 0.0115%; PIII, 0.0076%; PIV, 0.0011%; and PV, 0.0005%). The relative abundances of the different oral *Streptococcus* species are shown in 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 versus PII, $P = 0.002$), followed by a renewed slight decrease in PV. The alpha-diversity measures, Shannon effective value and Simpson effective value, were significantly increased in PIII compared to PII (Shannon effective value and Simpson effective value, $P < 0.0001$) and also in PIV compared to PII (Shannon effective value, $P = 0.001$; Simpson effective value, $P = 0.007$), whereas in PV, both parameters were significantly decreased compared to PIII (Shannon effective value, $P = 0.025$; Simpson effective value, $P = 0.011$). 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 to PV, the beta-diversity was analyzed based on UniFrac. Comparing PI with all following

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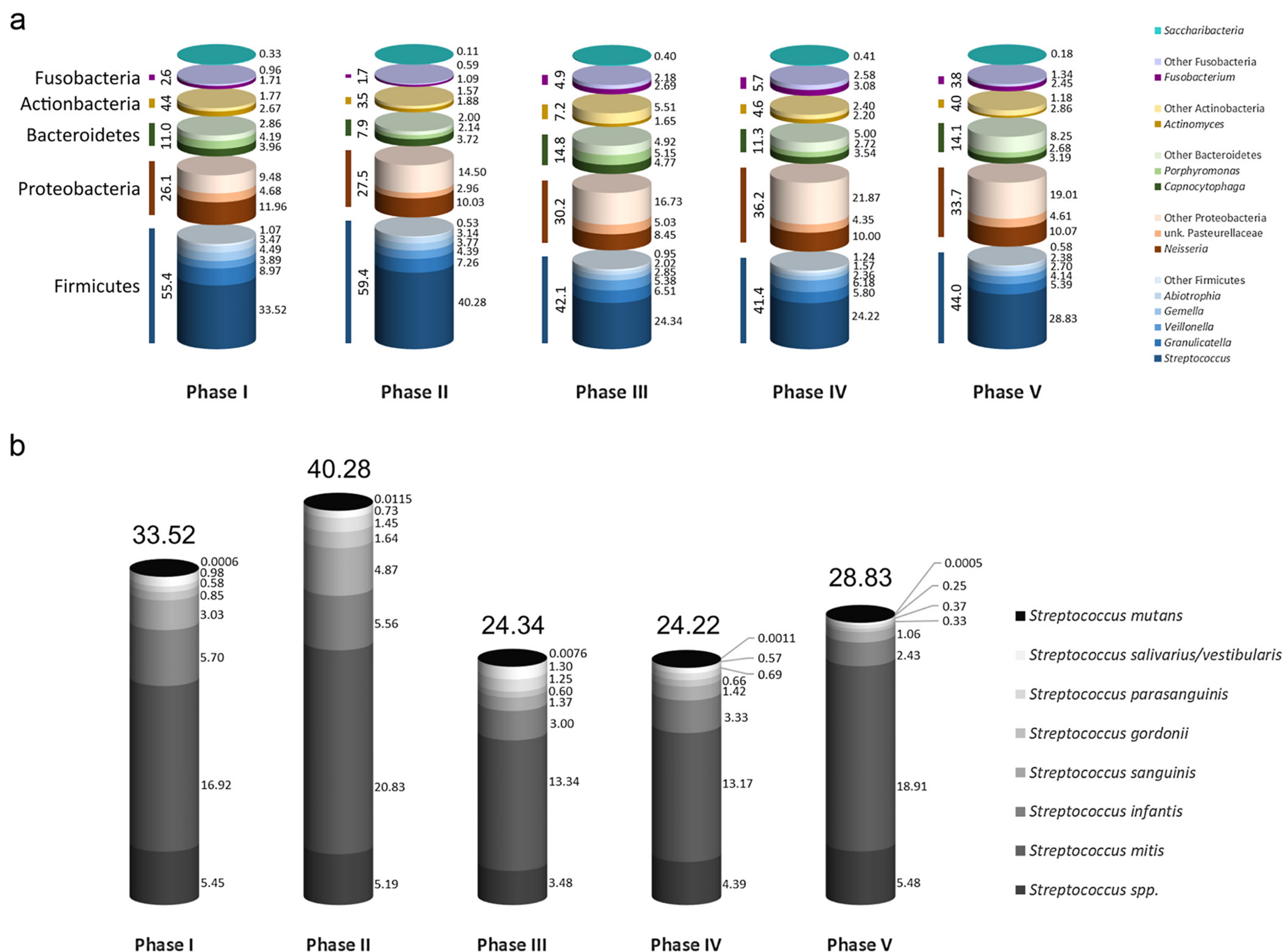

FIG 3 Bacterial composition of supragingival biofilm of 11 study participants in different dietary phases. Shown are relative abundances of bacterial phyla and the most abundant genera (>2%) (a) and oral *Streptococcus* species (b) detected in dietary phases I to V (including zero values). In panel b, "*Streptococcus* spp." refers to OTUs that could not be unambiguously assigned to one of the oral *Streptococcus* species.

phases, permutational multivariate analysis of variance (PERMANOVA) revealed significant differences of the microbial communities between PI and PII ($P = 0.004$), PIII ($P = 0.0016$), PIV ($P = 0.0016$), and 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 ($P = 0.0016$) (Fig. 4). There were no significant differences between PIII and PIV, PIII and PV, or PIV and PV.

Fluctuations of bacterial taxon abundances in the course of PI to 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 to PV for several bacterial taxa (Fig. 5a). The abundance of the phylum *Firmicutes*, with the genus *Streptococcus* being its main

TABLE 2 Values (means ± SD) of richness and alpha-diversity measures of the supragingival microbiota of the 11 study participants in phases I to V

Phase	Richness	Shannon effective value	Simpson effective value
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

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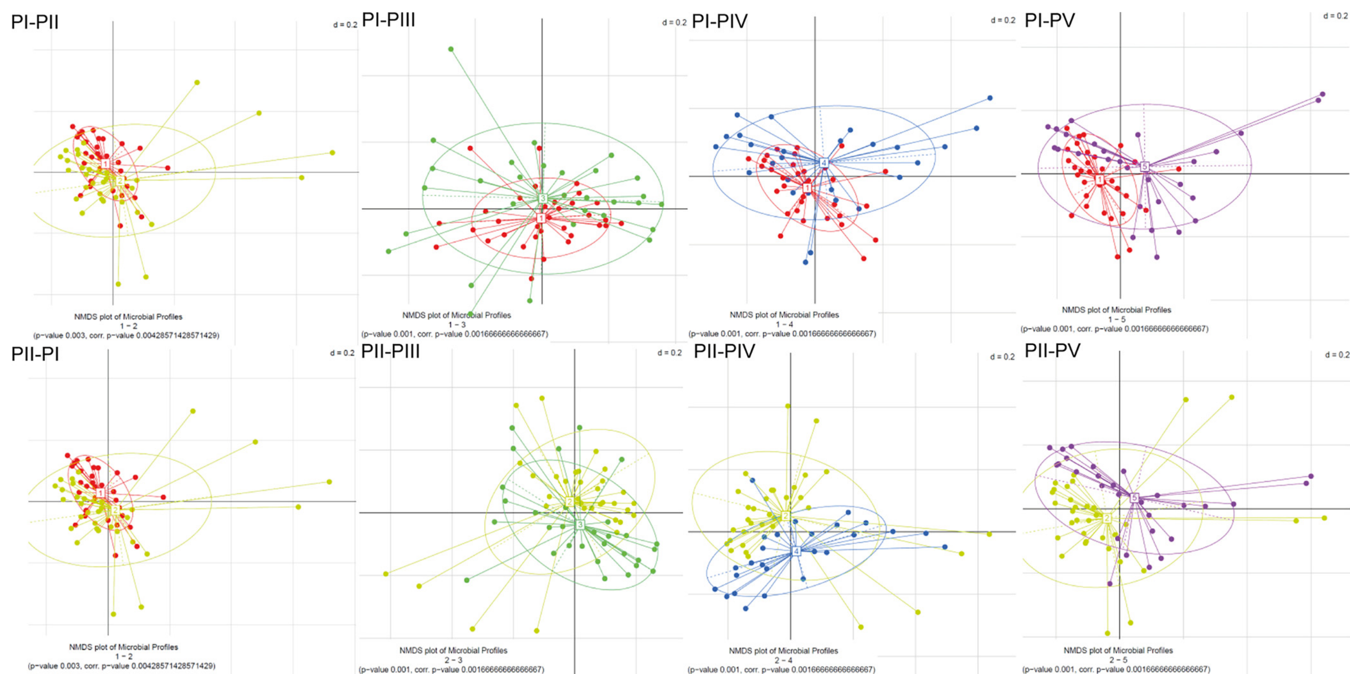

FIG 4 Beta-diversity of the microbial communities in supragingival biofilm of 11 study participants in different dietary phases based on UniFrac. NMDS plots show significant differences comparing beta-diversities of PI to PV. PI to PV are indicated in the plot by numbers 1 to 5.

representative in the supragingival biofilm, increased in PII and showed significantly decreased abundances in PIII, PIV, and PV compared to PI (PI to PIII, $P = 0.0002$; PI to PIV, $P < 0.00001$; and PI to PV, $P = 0.007$). The abundance of the genus *Streptococcus* showed a similar course, revealing significantly decreased abundances in PIII and PIV in comparison to PI (PI to PIII, $P = 0.003$, and PI to PIV, $P = 0.002$). Other taxa showed fluctuations of their relative abundances 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 compared to PI (PI to PII, $P = 0.006$, and PII to 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 to PIII: $P < 0.00001$). *Rothia* spp., *Leptotrichiaceae* spp., and *Granulicatella* spp. showed other fluctuations: *Granulicatella* spp. showed decreased abundances in PII to PV compared to PI (PI to PIV, $P = 0.03$, and PI to 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 $P = 0.03$, respectively), 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 Fig. 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 low abundances similar to that in PIII or a slight but not significant increase. Lastly, in PV most *Streptococcus* species showed an abundance similar to that in PI, with *S. sanguinis* and *S. infantis* showing a significantly lower abundance than in PI. Only *S. salivarius* showed increased relative abundance levels in PIII, but it was detected in only a few individuals (6/30) and decreased again in PIV and PV.

Changes in surface area roughness in PI to PV. The mean enamel surface roughness (Ra) measured throughout PI to PV varied between 0.035 ± 0.037 (standard deviation [SD]) μm (median: 0.032 μm) in PV and 0.062 ± 0.022 (SD) μm (median: 0.054 μm) in PII. Neither the increase in surface roughness in PII nor the decrease in PIII ($P = 0.056$) was significant. However, the values in PV were significantly lower than in

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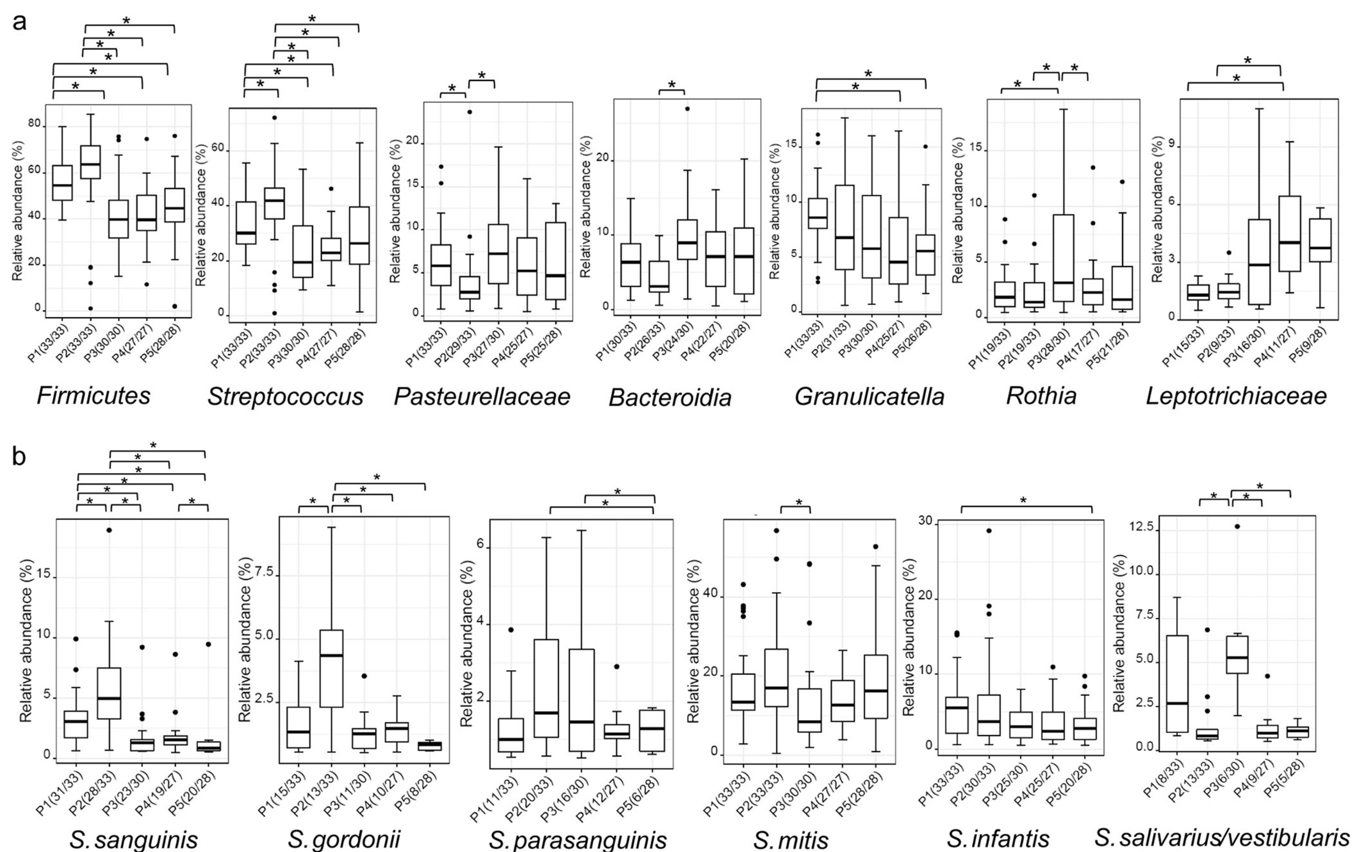

FIG 5 Box plots demonstrating significant differences in relative species abundance in supragingival biofilm in different dietary phases. (a) Box plots showing the relative abundances of different bacterial taxa that showed significant differences between different dietary phases. (b) Box plots showing the relative abundances of oral *Streptococcus* species that showed significant differences between different dietary phases. *, significant difference; $P < 0.05$.

the sucrose phase (PII, $P = 0.005$), and the surface roughness in PIV as well as PV was significantly lower than that in PI ($P = 0.018$ and $P = 0.001$, respectively). The mean values of the surface roughness in PI to PV are depicted in Fig. 6.

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 11 participants with high-throughput sequencing. The applied splint system with 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 time span, 3 months, 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 frequent sucrose consumption have been discussed earlier (24), our focus in this study was on the dairy and the dietary fiber phases.

Among the most notable findings consisted of the significant change of the beta-diversity for all four phases PII to PV compared with the baseline phase (PI) and 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

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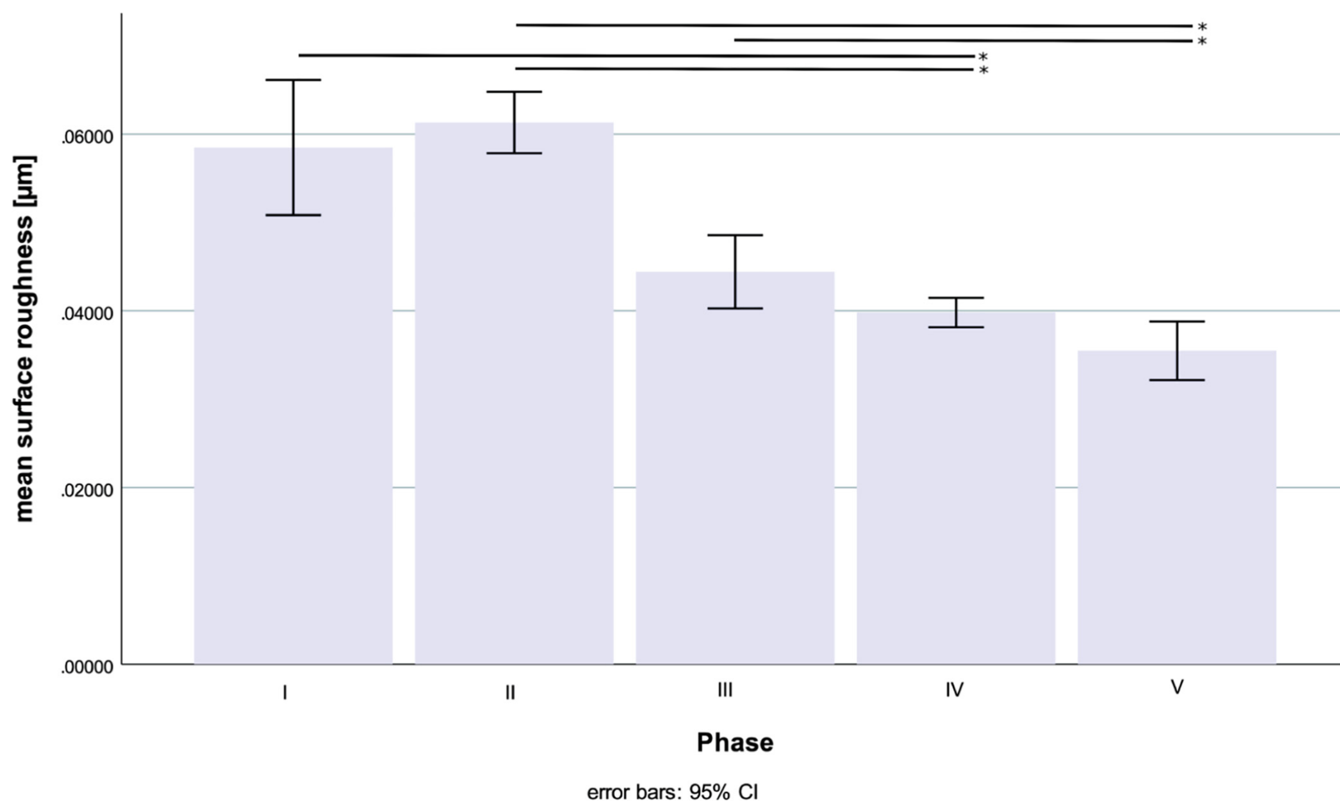

FIG 6 Mean values of the surface roughness profile (Ra) of the enamel samples for the different dietary phases PI-PV. *, significant difference; $P < 0.05$.

other phases. Even after returning to the regular diet for 7 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 and 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—varies only in a very small percentage of low-abundance 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, the salivary microbiome was fairly resilient and did not undergo any major shifts (33). In another study, the oral biofilm metatranscriptome of five individuals 30 min before and after a carbohydrate-rich meal was analyzed and showed 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 four 3-month phases 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 3 months, although in PV the abundances of some taxa, e.g., *Streptococcus* spp., *Pasteurellaceae*, *Bacteroidia*, and *Rothia* spp., showed a tendency to approximate the initial levels of PI. 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

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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.

Concerning alpha-diversity and species richness, both Shannon effective and Simpson effective values and 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 toward 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 anticariogenic effect and found only less demineralization of enamel specimens with whole milk, but not skimmed milk, compared to that with 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 being 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 3 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 (high = more than 3.7 servings per day). Interestingly, they found that certain taxa differed significantly between the individuals who reported high and low milk intakes; 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). It must be noted that the species with the highest relative abundance was *S. mitis* (PII, 20.83%, and PIII, 13.34%), a species that contains acid-tolerant isolates (44) but has not been reported to show a high acidogenic potential (43).

S. mutans, the major acidogenic species, was found in only 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

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low, follow the same pattern as for other oral streptococci, which increased in PII and then substantially decreased in PIII and PIV, going back to almost their original abundances 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 abundances 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 higher relative abundances for the genus *Porphyromonas* and some *Prevotella* species, although periodontitis-associated *Prevotella intermedia* and *P. melaninogenica* showed lower abundances in the group with high milk intake (42). In this 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 loescheii* and *Prevotella salivae* (data not shown). It can be assumed that the abundances 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 organisms.

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, 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 3 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 toward significance ($P = 0.056$). In PIV and PV, the surface roughness was significantly lower than in PI, pointing to a lasting smoothing effect of 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, and 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, and 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, which could also have had an effect, needs to be taken into account.

Even though we did not select any specific foods containing very large 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

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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 slightly decreasing abundances of *Pasteurellaceae*, *Bacteroidia*, and *Granulicatella* spp. The genera *Rothia* and *Granulicatella* showed significantly lower abundances in PIV than in PIII and PI, respectively, whereas *Leptotrichiaceae* had a significantly higher abundance than in 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 3 months after returning to the original baseline diet. Increased sucrose consumption favored caries-promoting non-mutans streptococci, while frequent milk and yoghurt consumption lowered the abundances 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 toward 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 could be preserved.

MATERIALS AND METHODS

Study group and study design. The study group consisted of 11 healthy adults between 21 and 56 years old (5 male and 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, and 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 Fig. 1, consisted of five different dietary phases (PI to PV), in which supragingival plaque was sampled using splint systems worn by the participants for three times 7 days (with 7-day intermissions without a splint) after an adaptation to the dietary phase for 7 weeks. 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 of 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 of plain yoghurt 3 times daily and 100 ml of long-life milk twice a day (both 1.5% fat; Schwarzwaldmilch GmbH, Freiburg, Germany). In PIV, the study participants consumed 500 g of vegetable puree per day of the following types: white carrot, parsnip, carrot, "jardinière" (carrot, potato, cauliflower, and pea), pumpkin, and garden vegetables (carrot, potato, spinach, parsnip, and leeks) (Reine Weiße Karotte, Reine Pastinake, Reine Frühkarotte, and Gemüse-Allerlei [Hipp GmbH, Pfaffenhofen, Germany] and Kürbis pur and 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 was 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 1.450 ppm).

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 previously (24). Each of these splint systems was equipped with six BES which were placed in the area between upper

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premolars and molars facing the teeth for the biofilm to grow undisturbed by movements of the tongue (Fig. 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 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 rRNA gene Illumina MiSeq sequencing. DNA of the supragingival biofilm obtained from the BES was extracted using the DNeasy blood and 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 of AE buffer (provided in the kit) and then used for amplicon library preparation with 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 previously (24). From this study, the sequencing data for PI and PII were taken and analyzed together with those for PIII to PV. Postprocessing was done with the IMNGS platform and the assembled OTUs for PI to PV were deposited in GenBank (see below) (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 sequences 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 nonmetric multidimensional scaling (NMDS) (55). All calculations were done with normalized data. Generalized UniFrac 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 laser scanning microscope (VK-X210; Keyence Deutschland GmbH, Neu-Isenburg, Germany) was used. First, the enamel slabs were thoroughly cleansed and visually checked for any residue with the camera unit. Then, a polygonal measuring field was selected and the complete surface was measured with a resolution of 1,000/mm, λS of 2.5, and λc of 0.25, excluding areas where the specimens had been mechanically damaged.

Data availability. The data sets supporting the conclusions of this article are available through GenBank (accession numbers MT435139 to MT435484).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 1.5 MB.

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A.A-A. and E.H. conceptualized the study. M.J.A., L.K., and J.P.W. analyzed the results. A.C.A. and L.K. collected the samples. M.R. and K.V. performed statistical analyses. A.C.A. and M.R. performed the experiments, analyzed the results, drafted the manuscript, and prepared the figures. All authors edited the manuscript and approved the final article.

We declare that we have no competing interests.

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