

Composition of Microbial Oral Biofilms during Maturation in Young Healthy Adults

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Abstract

In the present study we aimed to analyze the bacterial community structure of oral biofilms at different maturation stages in young healthy adults. Oral biofilms established on membrane filters were collected from 32 human subjects after 5 different maturation intervals (1, 3, 5, 9 and 14 days) and the respective phylogenetic diversity was analyzed by 16S rDNA amplicon sequencing. Our analyses revealed highly diverse entire colonization profiles, spread into 8 phyla/candidate divisions and in 15 different bacterial classes. A large inter-individual difference in the subjects' microbiota was observed, comprising 35% of the total variance, but lacking conspicuous general temporal trends in both alpha and beta diversity. We further obtained strong evidence that subjects can be categorized into three clusters based on three differently occurring and mutually exclusive species clusters.

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Introduction

High-throughput sequencing approaches have revolutionized our prospects and knowledge on microbial communities. For instance the human microbiome with its high variability between host individuals and distinct species assemblies on different parts of the human body was extensively examined [1–3]. Studies of these communities may elucidate interactions among microbes, as well as host-microbe interactions of ecological and clinical relevance. This may aid, e.g., the prevention of detrimental biofilms in prosthetics and dentistry. The oral microbiome is one of the most diverse of the human body. More than 700 species are reported to colonize multiple niches [4–6], including the soft tissue surfaces and the sub- and supragingival surfaces of the teeth, as a consequence of different environmental conditions (e.g., nutrients or pH) [7,8]. While the majority of the oral community plays an important role in preserving the oral and systemic health [6,9–12], pathogens are also included at a low percentage. As a consequence of environmental changes, host response and disturbance of microbial homeostasis, an increase of the pathogenic community might promote the development of oral diseases by leading to inflammation and infection [8,13]. Even though periodontal diseases are initiated by polymicrobial infection, several bacterial species are commonly linked to particular oral diseases. The oral microbiome is also associated with systemic diseases [14–17], including for instance cardiovascular disease and atherosclerosis [14,17]. Recently, we demonstrated that even experimentally induced gingivitis - a low-level inflammation in response to bacterial biofilm formation after a few days of suspended oral

hygiene - triggers a systemic increase of surrogate markers of atherosclerotic plaque development [18]. However, the changes from health to disease as well as the mechanisms responsible for increased occurrence of pathogens are poorly understood. Likewise, studies of the human microbiome revealed that even healthy individuals could differ extremely in their bacterial community structure in same habitats, with much of this inter-individual difference remaining unexplained [2]. Thus, more insight into “healthy” consortia in oral biofilms is crucial for understanding development, prevention and treatment of oral diseases.

To evaluate potential shifts in bacterial biofilm composition during maturation and due to different bacterial colonizer pools in various hosts, we investigated oral bacterial colonization profiles close to the teeth in 32 young healthy adults (both male and female) after 5 different maturation intervals between 1 and 14 days, by 16S rDNA amplicon sequencing using a high-throughput sequencing approach.

Materials and Methods

Human Samples

All procedures related to subjects were approved by ethics committee of Hannover Medical School, registered at the International Clinical Trials Register Platform of the WHO (ID: DKRS00003366) and follow the guidelines of the Declaration of Helsinki. All participants gave written informed consent and were recently involved in our earlier study that investigated the systemic effects of experimental gingivitis.

Criteria for Participation

Human subjects agreeing following criteria were selected: (a) 20–30 years of age, (b) non-smokers, (c) no clinical signs of gingival inflammation, (d) no probing pocket depth >3 mm at any site and (e) no alveolar bone loss. Criteria for exclusion were: (a) systemic diseases (e.g. diabetes mellitus), (b) pregnancy or breastfeeding, (c) history of drug abuse, (d) allergic diathesis, (e) medications (e.g. antibiotics) within 3 months before entering the study, (f) untreated carious lesions and/or insufficient restorations, implants, crowns and (g) mouth breathing.

Biofilm Formation and Sampling

In order to obtain sufficient plaque material for sequencing maxillary and mandibular impressions were obtained and used to fabricate individual acrylic splints. Membrane filters (MilliporeExpress® PLUS) were attached to the splints next to the teeth and gingiva by surgical sutures. In each case four membranes on the upper and four on the lower jaw on buccal and oral sides of the teeth rows. Participants were advised to carry these splints for the time intervals 1, 3, 5, 9 and 14 days and to store them in provided humidified chambers during the meals and daily oral hygiene procedures. On every sampling day all 8 filters were removed from splints, washed with sterile PBS and immediately frozen in liquid nitrogen until further processing.

Nucleic Acid Isolation

Genomic DNA of dental plaque was isolated from a pool of 2 different membrane filters using the RTP® Bacteria DNA Mini Kit (STRATEC Molecular, Birkenfeld/Germany) following the manufacturer's instruction (protocol 4).

PCR Amplification and Deep Sequencing Analysis

The hypervariable regions 1 to 2 (V1–2) of the 16S rRNA gene was amplified from isolated genomic DNA using universal primer Pyro_27F (5'CTATGCGCCTTGCCAGCCCGCTCAGTCA-GAGTTTGATCCTGGCTCAG3') and barcoded reverse primer 338R (5'CGTATCGCCTCCCTCGGCCAT-CAGXXXXXXXXXXCATGCTGCCTCCCGTAGGAGT3'). The primer contained the 454 Life Sciences Adaptor B (forward) and A (reverse) denoted by italics and the underlined sequences represent the broadly conserved bacterial primers 27F and 338R. A two-base linker sequence (TC/CA) and four-base key (TCAG) were added as recommended by Roche (454). A unique 10mer multiplex identifier (designated X) was added to every reverse primer to tag each PCR product. Template DNA (100 ng) was added to a 25 µL PCR reaction mix including Phusion Hot Start DNA Polymerase (Finnzymes, Vantaa/Finland). Cycling conditions started with an initial denaturation step for 30 s at 98°C, followed by 35 cycles of 9 s at 98°C, 30 s at 55°C and 30 s at 72°C and ended with a final extension for 10 min at 72°C. All reactions were performed in duplicates and combined after PCR. Amplicons were size-checked, purified with the MiniElute Gel Extraction Kit (Qiagen, Hilden/Germany) and quantified with the Quant-iT dsDNA Broad-Range Assay Kit (Invitrogen, Darmstadt/Germany) using a NanoDrop 3300 fluorometer. Equimolar amounts of purified PCR product were pooled and further purified using Agencourt AMPure XP (Beckman Coulter, Krefeld/Germany). A sample of each library was run on an Agilent Bioanalyzer prior to emulsion PCR and sequencing as recommended by Roche. Amplicon libraries were sequenced on a 454 GS-FLX using Titanium sequencing chemistry (Roche, Mannheim/Germany).

Sequence Processing

All steps of sequence processing were conducted with the program mothur v1.27.0 [19]. Raw sequencing data in standard flowgram format were first assigned to samples according to their multiplex identifiers (MIDs) with the forward primer sequence removed. Only reads between 360 and 720 flows, with a maximum homopolymer count of 8 and no differences to MIDs/forward primer were retained (command *trim.flows*). Data were denoised (*shhh.flows*) and kept if their mean phred score [20,21] was ≥ 25 after denoising (*trim.seqs*). Sequences were aligned (*align.seqs*) to the SILVA reference alignment based on the SSURef database (v102) of bacterial sequences [22] provided on http://www.mothur.org/wiki/Silva_reference_files. The alignment was optimized by deleting the shortest and longest 2.5% of the sequences, respectively (*screen.seqs*), followed by removal of gap-only columns as well as columns containing missing data at both ends of the alignment (*filter.seqs*). The resulting alignment had 696 positions with sequence lengths ranging from 239 to 315 nucleotides. In order to further reduce PCR/pyrosequencing errors, reads with one mismatch to a more abundant sequence were merged with the latter (*pre.cluster*). Chimeras were eradicated employing mothur's implementation of Perseus [23] (*chimera.perseus, remove.seqs*). Initial sequence classification was conducted by a Bayesian approach [24] using a k-mer size of 8 and a bootstrap threshold of 60% (*classify.seqs*). The RDP [25] reference taxonomy (http://www.mothur.org/w/images/4/4a/Trainset7_112011.pds.zip) was used with reference sequences trimmed to the V1–V2 primer region to improve accuracy of the classification [cf. [26]. The dataset was screened for chloroplast sequences to be removed (*remove.lineage*) as these sequences would obviously have originated from vegetable food rather than from bacteria. A random subset of 1,042 sequences per sample (corresponding to the smallest number of reads above 1,000 across samples) was generated (*sub.sample*) to eliminate bias due to unequal sampling effort. A coverage of about 1,000 sequences per sample is suggested as a good balance between number of samples and depth of sampling [27]. Three samples (p35d01, p35d03, and p36d09) containing fewer sequences (855, 594, and 957, respectively) were kept in the dataset with reservation. The aligned and subsampled dataset was used to compute a distance matrix (*dist.seqs*) for binning sequences into operational taxonomic units (OTU) by average neighbor clustering (*cluster.split*). Based on a 97% similarity threshold (roughly corresponding to species level distinction) 1,779 OTUs were identified. A sample-by-OTU table was generated (*make.shared*) which for each sample states the number of sequences belonging to a certain OTU. This table was the basis for subsequent bioinformatic analyses. OTUs were classified according to the CORE reference database, a phylogenetically curated 16S rDNA database of the core oral microbiome [28], as described above for the RDP reference taxonomy (*classify.seqs*, followed by *classify.otu*).

Sequence Abundance Plots

All downstream computations were performed in R v2.15.2 [29] with custom scripts (available from the authors on request). Initial characterization of bacterial community composition was performed by summarizing OTU abundances at genus level. Genus-level abundances were normalized by dividing their number by the total number of sequences per sample and visualized as stacked bar plots.

Alpha Diversity Analysis

Effective OTU richness (also known as Shannon numbers equivalent, ¹D) [30,31] was calculated from the sample-by-OTU table using the R package *vegan* [32]. Initial data visualization was

performed with the R package *lattice* [33]. The study design corresponds to a mixed-effects model in which the fixed effect “Day” (i.e., maturation time of the biofilm) is nested within the random effect “Subject”. To estimate the effect of maturation time on effective OTU richness, 1D was fitted to “Day” in a generalized linear mixed model using function *gamm* of R package *mgcv* [34] with either random intercept or random intercept and slope. Models were compared by means of the Akaike Information Criterion (AIC) with restricted maximum likelihood estimation as described by Zuur *et al.* [35].

Beta Diversity Analysis

Redundancy Analysis (RDA) was used to explore the extent of change in OTU composition (also known as turnover) in oral microbial communities within subjects over time. OTU abundances were subjected to Hellinger transformation in order to make them compatible with RDA. This transformation down-weights highly abundant OTUs, providing a good compromise between linearity and resolution [36]. RDA was thus performed on Hellinger distances between samples (i.e. Euclidean distance of Hellinger-transformed data). The model was evaluated with *vegan*'s function (*rda*) and consisted of the fixed effect “Day” and the “Subject” effect as conditioning term: OTU count data \sim “Day” + Condition (“Subject”). The model was tested for significance with function *anova.cca* using 1,000 random permutations stratified within subjects. Homoscedasticity of the fixed effect was assessed using *vegan*'s *betadisper* function, followed by *permutest* with permutations stratified within subjects.

Consensus Clustering of Species

OTU abundances were normalized by total counts per sample and summarized at species level (henceforward called ‘species abundances’ for simplicity). Taxa that could not be distinguished at the species level or with terms “uncultured” or “unclassified” added to their names were removed from the dataset. Species relative abundances were used for the resulting sample-by-species table which was subjected to Principal Component Analysis (PCA) after Hellinger transformation. Based on this ordination configuration, weighted-average scores of species were calculated by *vegan*'s (*wascor*) function for projection of species points into the PCA space of samples (scaling 1). Consistent clusters of species were determined by average-linkage clustering based on Spearman rank correlations of relative species abundances. Cluster number and robustness were assessed by consensus clustering [37] with package *ConsensusClusterPlus* [38] based on 1,000 random species subsamples (drawing without replacement) with a sampling proportion of 0.8. The optimal number of clusters was determined by assessing the relative change in the area under the consensus cumulative distribution function [cf. [37]]. Robust cluster members were defined as species whose item consensus was ≥ 0.6 for the cluster they were assigned to and ≤ 0.4 for any other cluster. The multivariate coefficient of variation (RV) was calculated for each pair of robust species clusters based on rank-transformed species abundances. Clusters of robust species were projected as “spider” graphs into the PCA space of the samples.

Consensus Clustering of Subjects

Partitioning of human subjects in relation to the clusters of robust species was assessed as follows. For each subject, mean relative species abundances were calculated across all time points. The resulting subject-by-species table was subjected to PCA after Hellinger transformation. The matrix of subjects' PCA scores (scaling 1) was subjected to consensus clustering as described above with 10,000 random subject subsamples, using Partitioning

Around Medoids [39] as a robust grouping algorithm. The resulting subjects' partitioning was evaluated by Analysis of Similarities (ANOSIM) on Euclidean distances of the Hellinger-transformed subject-by-species table with 100,000 random permutations.

3D Visualization

A 3D plot of combined clustering and ordination data was produced in kinemage format [40] using the in-house developed R package *R2Kinemage* and displayed in *KiNG* v2.21 [41].

Nucleotide Sequence Accession Numbers

Sequence data were submitted to the NCBI (National Center for Biotechnology Information) Sequence Read Archive under accession no. SRP027013. Table S1 of file S1 lists multiplex identifiers and corresponding information for samples in the respective sequence libraries.

Results

Characterization of Bacterial Biofilm Composition during Maturation

Oral biofilm formation on membranes close to the gingiva was monitored in 32 healthy adults over 14 days. Aiming to examine the bacterial biofilm composition during the establishment, five different time points in biofilm formation were collected (day 1, 3, 5, 9 and 14). A total of 160 biofilm samples were analyzed after DNA extraction and amplifying the hypervariable region V1–V2 of the 16S rDNA by 454 pyrosequencing. 1,042 sequences per sample were retained after random subsampling, binned into a total of 1,779 operational taxonomic units (OTUs) of which about only 15 were highly abundant across all samples (see Tables S2 and S3 for a list of raw OTU counts and corresponding taxonomy in file S1, respectively). Variability of sample diversity was high, ranging from 7 to 130 OTUs, distributed over 8 major phyla/candidate divisions (Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, Spirochaetae, SR1 and TM7). The phyla could further be subdivided into 15 identified classes (Bacilli, Clostridia, Erysipelotrichi, Negativicutes, α -, β -, γ -, ϵ -Proteobacteria, Actinobacteridae, Coriobacteridae, Bacteroidia, Flavobacteria, Sphingobacteria, Fusobacteria, Spirochaetes) (see Table S4 in file S1). Comparison of the bacterial composition of all individuals over all time points by relative abundance plots revealed highly variable colonization profiles (Fig. 1).

One subject (p35) exhibited a conspicuously low level of *Streptococcus* in combination with elevated levels of *Prevotella*, *Fusobacterium* and other minor taxa over time in comparison with the other subjects (Fig. 1). Since measures strongly deviating from the mean can have adverse effects on regression-type analyses as performed here, we chose to exclude subject p35 from subsequent analyses.

As a measure of alpha diversity, we employed effective OTU richness 1D . Initial data visualization did not reveal conspicuous general trends in 1D with time (Fig. S1 in file S1). To check for a possible less obvious temporal pattern, we fitted a generalized linear mixed model with “Day” as fixed effect. Because residuals showed clear deviation from normality (validated by visual inspection of a quantile-quantile plot), a gamma distribution with log link was used to model 1D . Comparison by AIC suggested the random intercept model (the intercept of the regression line may vary between subjects) as the best choice. While the model was valid (no conspicuous residual patterns were observed), the effect of variable “Day” was not significant ($p=0.13$), thus no time-related change in alpha diversity of oral microbiota was observed.

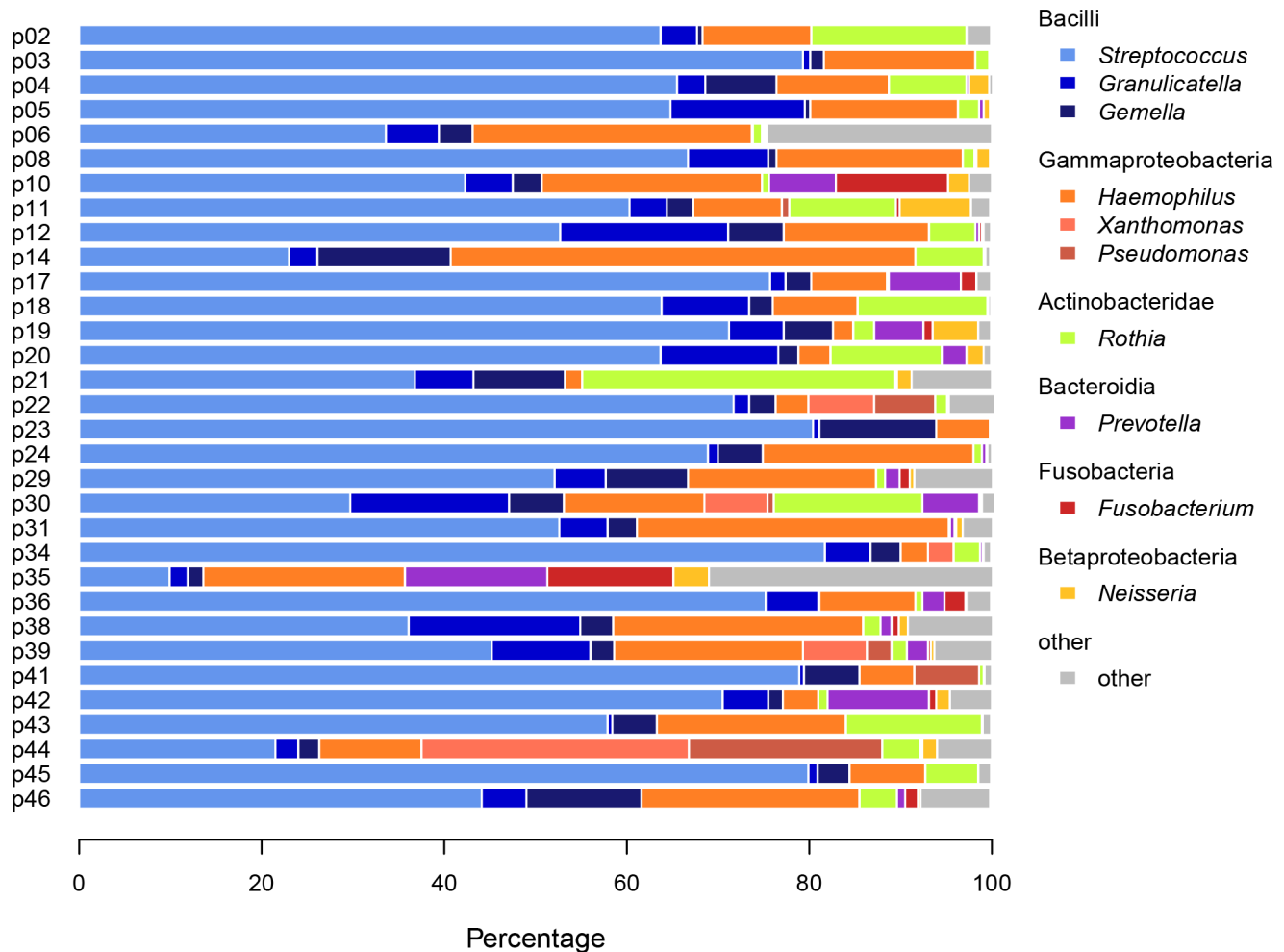


Figure 1. Mean bacterial composition of oral biofilms over time in individual subjects. Bacterial communities of oral biofilms in 32 different human subjects were analyzed by 16S rDNA amplicon deep sequencing (V1–V2 region). Taxa (mean relative abundance across subjects $\geq 1\%$) are shown at class and genus levels. doi:10.1371/journal.pone.0087449.g001

In beta diversity analysis, Hellinger-transformed OTU counts were subject to RDA with “subjects” as conditioning term to account for inter-individual variation. The effect of “Day” on the change of individual OTU abundances between samples of the same subject was not significant ($p \approx 0.1$). Accordingly, adjusted R^2 (a measure for the variance explained by the RDA model) was only 0.5%. Hence there was no obvious and consistent time-related change in relative OTU abundances within subjects. In contrast, a comparatively large portion of variance (35%) was attributed to the conditioning term “Subject”, arguing for a large inter-individual difference in microbial composition.

Evidence of Mutual Exclusions between Different Bacterial Complexes

Since alpha and beta diversity analysis did not show any significant time-related response in our data, we concentrated on evaluating correlations between individual species across all data points. For this purpose, we employed Spearman rank correlation for species abundances combined with consensus clustering - a simple and straightforward method of inferring robust clusters by resampling - to obtain robust results. A combined consensus clustering and unconstrained ordination analysis (Principal Com-

ponent Analysis) revealed three clusters of species mutually excluding each other across subjects and time points (Fig. 2A, B; see also Fig. S2 in file S1 for an interactive version of this image with additional display options such as taxon names, subject codes and individual subject time points; see Table S5 for a list representation of species clusters in file S1). These three clusters of species differed in their relative abundances (averaged over time) in three corresponding subject clusters (Fig. 2C). Separation of the latter was corroborated by a fairly large ANOSIM statistic ($R = 0.54$) with high statistical significance ($p < 10^{-5}$). The magenta-colored species cluster was dominated by different *Prevotella* species (“*Prevotella* cluster”), the green one contained β - and γ -Proteobacteria (“Proteobacteria cluster”) and the orange-colored cluster mainly contained *Streptococcus* species (“*Streptococcus* cluster”). The “*Prevotella* cluster” and the “*Streptococcus* cluster” showed a strong multivariate correlation ($RV = 0.3$; $p < 0.01$) and mutually exclude each other. A weak but still significant multivariate correlation ($RV = 0.07$; $p < 0.01$) was observed between the “Proteobacteria cluster” and the contrasting “*Prevotella* cluster”. No significant correlation could be observed between the “Proteobacteria cluster” and the “*Streptococcus* cluster”. Fig. 2C shows the relative species abundances (averaged over time and subjects) in the 3 detected subject clusters. Subject cluster 1 was

dominated by the “*Prevotella* cluster” (60%) and “*Streptococcus* cluster” (40%). In the second subject cluster the “*Streptococcus* cluster” dominated (66%), followed by members of the “*Prevotella* cluster” (32%) and a minority of the “*Proteobacteria* cluster” (2%). The third subject cluster exhibited the largest occurrence of the “*Proteobacteria* cluster” (27%), while the “*Prevotella* cluster” represented about half of the counts (54%) and the “*Streptococcus* cluster” was least abundant (20%).

Discussion

Oral microbial profiles in health and disease have been investigated in numerous *in vivo* studies [11,12,42]. In this respect, several bacterial species have been shown to be directly linked to specific diseases [12,43,44]. However, the transitional stages from health to disease as well as the mechanisms responsible for appearance of those pathogens are poorly understood. Thus, elucidating “healthy” complex consortia of oral biofilms in the course of time is crucial. In the present investigation we addressed this essential aspect by analyzing microbial composition of oral biofilms of 32 healthy human subjects during maturation over 14 days at 5 different time points.

Analysis of Oral Biofilms Over Time Revealed High Inter-individual Variation

Within the 16S rDNA amplicon sequencing approach of oral biofilms we analyzed a highly diverse dataset with more than 1,700 different OTUs at the 97% similarity level, although only 15 of them were highly abundant across all samples. The number of OTUs within each sample ranged from only 7 up to 130 consequently already suggesting a large variation between individuals. Further evidence for large inter-individual differences in microbial composition was obtained by RDA. This is in accordance with previous findings that the oral community is especially diverse in contrast to other body habitats, e.g. the vagina [2,45]. The consistency of our 16S rDNA profiles of oral biofilms is comparable to the keratinized gingiva baseline at the phylum level in healthy subjects as reported by the Human Microbiome Project [46]. Comparison to a lower level is impaired by differences due to methods and approaches used, e.g. primer set, database, sequences per sample and number of subjects.

We did not detect any systematic time-related patterns, neither in alpha nor in beta diversity. This suggests that both, the cumulative number of OTUs as well as their individual turnover in a subject, is influenced more by random (or unaccounted/unknown) effects than by time in the course of biofilm formation between 24 h and 14 days. These findings are in agreement with previous reports that propose a high variability in the oral microbiome between individuals [11,47] as well as a minimal temporal variability in individuals [45]. The Human Microbiome Project Consortium further reported on within-subject stability of the human microbiome by sampling an additional time point after approximately 220 days. They hypothesized that the stable individual microbial community may be another feature of the human microbiome specifically associated with health [2]. This assumption is supported by previous studies of microbial diversities of different body habitats, which has been linked to different diseases [48–50]. For instance the complex stool communities were markedly reduced in specific gut diseases and bacterial vaginosis is associated with a high microbial diversity, whereas healthy vaginal sides harbored simpler communities [2]. Our study of oral biofilms in healthy subjects over time correlates favorably with these findings and further supports the idea of stable complex oral microbiota in health that obviously differ among subjects.

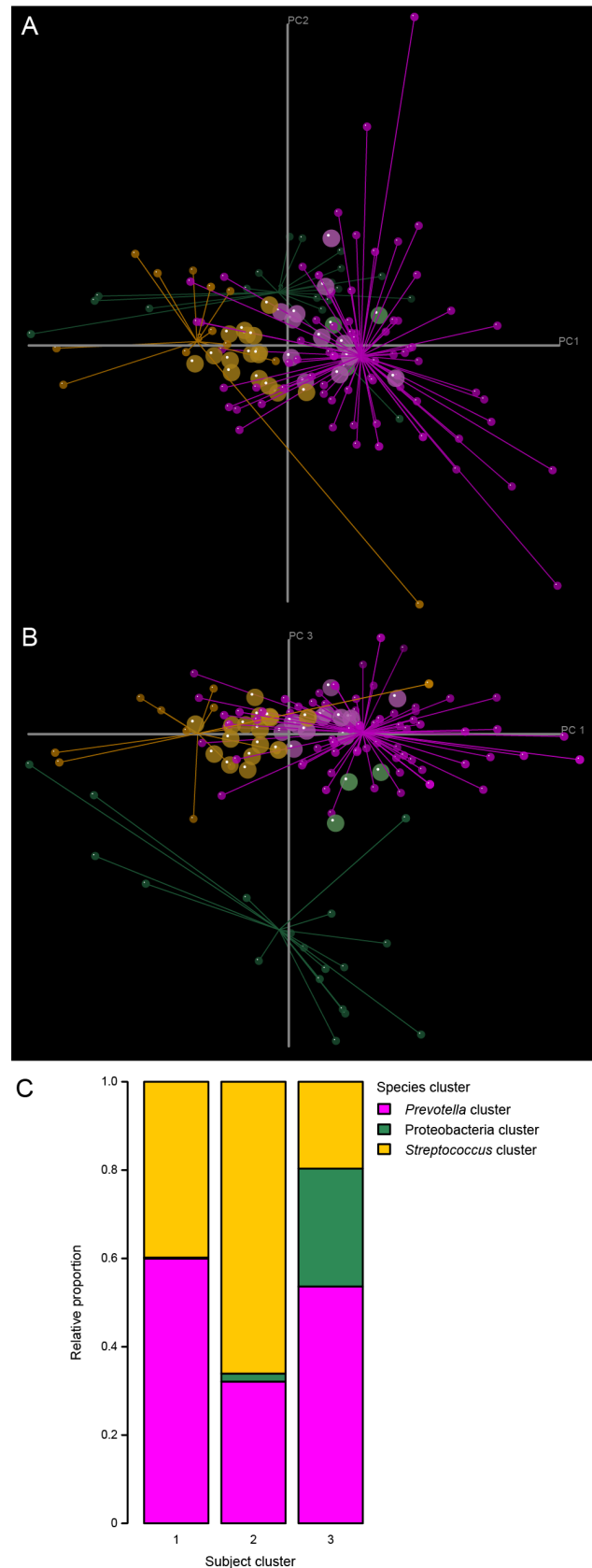


Figure 2. Species and subject clusters. Combined consensus clustering and ordination (PCA) of robust species and human subjects. (A) First (PC1) and second (PC2) axis and (B) first (PC1) and third axis

(PC3) of the ordination space of individual subject samples are shown. Species data points (small spheres) are projected into the ordination space as weighted averages and grouped into three clusters according to species consensus clustering: “*Prevotella* cluster” (magenta), “*Streptococcus* cluster” (orange), “*Proteobacteria* cluster” (green). Large spheres represent the centroids of individual sample points for each human subject, color-coded according to the result of subject consensus clustering. (C) Relative species abundances in subject clusters. Color coding of species clusters is analogous to (A) and (B). doi:10.1371/journal.pone.0087449.g002

Mutual Exclusion of Bacterial Consortia and their Assignment to Corresponding Subject Clusters

The most remarkable result obtained from our data set is the clustering of species into three groups that mutually exclude each other and differ in their relative abundances in three corresponding subject clusters (Fig. 2, Fig. S2 in file S1). To our best knowledge, this is the first study providing culture-independent evidence for species clusters in the human oral microbiome.

In particular, the strongest exclusionary relationship was observed between the “*Streptococcus* cluster” and “*Prevotella* cluster”. Mutualistic interactions as well as interspecies competition among microbial partnerships are often triggered simultaneously by several members of oral biofilms and are in the focus of current research [51–56]. A possible mechanism for such an “interspecies defense” might be the release of bacteriocins produced by several streptococci as well as by numerous Gram-negative bacteria of the oral cavity. These effectively act against a variety of further bacterial members within this habitat [57–60]. In addition, hydrogen peroxide produced by oral streptococci has been shown to inhibit the growth of other members of the oral cavity [54,61]. Certain oral streptococci have been shown to negatively impact biofilm formation of *Porphyromonas gingivalis* [62,63]. While *P. gingivalis* has not been detected in our dataset, this species is known to coaggregate with *Prevotella intermedia* [64], which is indeed a member of the “*Prevotella* cluster”. Moreover, the “*Prevotella* cluster” included other *Porphyromonas* spp. Hence, our study provides additional evidence for the reciprocal exclusion of streptococci and *Porphyromonas* spp.

Our results of cluster analyses are further in agreement with previous findings obtained by a co-culture experiment with cluster-representative species. Herein, Stingu *et al.* [65] observed significant bactericidal effects of oral streptococci, in particular *S. sanguinis* and *S. anginosus*, on *P. intermedia*. Further studies of Stingu and collaborators showed that the development of periodontitis is associated with an increased colonization of two *Prevotella* species (*P. intermedia* and *P. nigrescens*) as well as a reduced establishment of *S. sanguinis* [66,67].

Despite of the high inter-individual microbial variation in oral biofilms, our results revealed the existence of various colonizer pools in different hosts. We assume that even in health, the oral bacterial community features a broad potential compositional spectrum. The obtained composition of the oral biofilm of a certain individual may be indicative for this person’s ability to resist pathogens or disease susceptibility. Although all species in the identified clusters are members of the oral commensally community, there is evidence that some of these microorganisms are associated with gingivitis [68–70]. These include Gram-positive species (e.g., *Streptococcus* ssp. and *Parvimonas micra*), as well as numerous Gram-negative species (e.g., *Campylobacter gracilis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Veillonella*). Remarkably, all those bacteria are present in the identified “*Prevotella* cluster”. Moreover, another study [71] detected *Capnocytophaga* spp., which is also a member of the “*Prevotella* cluster”, on the onset of gingivitis and identified *Prevotella* spp. in areas with established

gingivitis. On the other hand, species that are associated with periodontal health - such as primary or early colonizers (e.g., *Streptococcus* and *Gemella*) [9,72] - have been predominantly found in the identified “*Streptococcus* cluster”. Since we know from our earlier study with the same subject cohort [18] that the human subjects responded differently to an experimentally induced gingivitis in terms of inflammation severity (Eberhard, unpublished data), we hypothesize a potential association of species cluster prevalence with disease susceptibility and inflammatory condition of human subjects. However, future investigations are mandatory to address this aspect.

Another conspicuity is the third subject cluster that consists of only three test persons. This cluster was shown to host a substantial proportion of the “*Proteobacteria* cluster”, which exhibits another - albeit weaker - contrasting relationship with the “*Prevotella* cluster”. The role of this cluster consisting primarily of aerobic β - and γ -*Proteobacteria* remains to be elucidated. However, in sufficient quantity it appears to replace the “*Streptococcus* cluster”.

Optimized Study Design and Application of Meta’omics Approaches may Reveal Trends Related to Time and Susceptibility to Inflammation

Due to the high inter-individual variability in microbial composition, an optimized study design is crucial to observe a potential time effect in oral biofilm formation. Even though our subject group was quite homogenous in terms of age and health status, several systemic and external factors such as diet, gender, hormone status and stress known to have an impact on the oral microbiome were not controlled for in the present study. This contributed to the observed highly diverse colonization profiles. A subsequent investigation with a stricter control of the subject set-up, optimally over periods longer than 14 days, may reveal clearer temporal trends in alpha diversity. Since a potential correlation between the oral microbiome and inflammatory diseases such as gingivitis might exist at the strain or even at the genomic/metabolic level, this correlation may not be reflected by 16S rDNA based phylogenetic analysis even after optimization of the experimental set up. Consequently, more advanced tools such as shotgun metagenomics or metatranscriptomics [73] should be preferentially employed to uncover such relationships.

In summary, our results provide evidence that, despite high microbial diversity in individuals, different consortia composition of oral microbiota exist in healthy hosts. External factors as well as physical predisposition of the host might be responsible and determine the structure of these consortia. Moreover first indications were obtained that community structures might be indicative for host’s disease susceptibility.

Supporting Information

File S1 Table S1: Multiplex identifiers and corresponding information on samples in the respective sequence libraries. Table S2: OTU counts in subjects over time. Table S3: OTU taxonomy. Table S4: Bacterial abundances at class-level in subjects over time. Table S5: List of species names in species clusters. Fig. S1: Alpha diversity analysis. Effective OTU richness 1D is shown within individual subjects over time. Fig. S2: Kinematic image representation of the combined consensus clustering and ordination (PCA) of robust species and human subjects shown in Fig. 2. Additional display options comprise species names, subject codes, individual subject time points, and means of time points over all subjects. Note: The free KiNG display software (Kinemage, Next Generation; <http://kinemage.biochem.duke.edu/software/king.php>) is needed to view the image.

(ZIP)

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Author Contributions

Conceived and designed the experiments: RAS DL JE. Performed the experiments: DL JE JFB SK. Analyzed the data: DL SCN RAS. Contributed reagents/materials/analysis tools: JE IS WH JFB SK. Wrote the paper: DL SCN RAS.

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