Oral health benefits of chewing gum
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Adhesion forces and composition of planktonic and adhering microbiomes

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Abstract

The oral microbiome consists of a planktonic microbiome as residing in saliva and an adhering microbiome; the biofilm adhering to oral hard and soft tissues. Here we hypothesize, that possible differences in microbial composition of the planktonic and adhering oral microbiome on teeth can be related to the forces by which different bacterial species are attracted to the tooth surface. The relative presence of seven oral bacterial species in saliva and biofilm collected from ten healthy human volunteers was determined twice in each volunteer using denaturing gradient gel electrophoresis. Analysis of both microbiomes showed complete separation of the planktonic from the adhering oral microbiome. Next, adhesion forces of corresponding bacterial strains with saliva coated enamel surfaces were measured using atomic force microscopy. Species that were found predominantly in the adhering microbiome had significantly higher adhesion forces to saliva coated enamel (-0.60 to -1.05 nN) than species mostly present in the planktonic microbiome (-0.40 to -0.55 nN). It is concluded that differences in composition of the planktonic and the adhering oral microbiome are due to small differences in the forces by which strains adhere to saliva coated enamel, providing an important step in understanding site- and material-specific differences in composition of biofilms in the oral cavity.
Introduction

The oral microbiome is a highly diverse and intricate system, consisting of more than 700 bacterial strains and species (1), although remarkably higher estimates of the microbial diversity in the oral cavity have been reported as well (2). The oral microbiome is different for every individual and specific to different sites and materials in the oral cavity. Minor disturbances in the delicate balance of the oral microbiome can lead to the onset of disease, such as caries and periodontitis (3).

Oral bacteria reside either in a planktonic state, as in saliva or in an adhering state, as in a biofilm on oral hard and soft surfaces. Although the oral microbiome is site-, material- and subject-specific, there is the concept of a general core microbiome, that plays an important role in the formation of oral biofilm (1,4). The formation of an oral biofilm is initiated by the adhesion of initial colonizers (5–7) to adsorbed salivary conditioning films. As such, oral biofilm can be regarded as a transition of bacteria from the planktonic microbiome to the adhering microbiome on oral hard and soft surfaces. This transition is mediated by an interplay between ligand-receptor interactions and non-specific Lifshitz-Van der Waals, acid-base and electrostatic forces.

Bacterial probe atomic force microscopy (AFM) has enabled the measurement of the forces by which bacteria are attracted to surfaces and relatively small differences in adhesion forces have been demonstrated to have major implications with respect to the ability of bacteria to adhere to a surface (8). Such observations lead us to hypothesize that differences in the composition of the planktonic and adhering oral microbiome on oral hard surfaces can be related to the force by which different bacterial strains and species are attracted to the tooth surface.

The aim of this study is to verify the hypothesis that differences in the composition of the planktonic microbiome and adhering oral microbiome on oral hard surfaces can be related to the force by which different bacterial strains and species are attracted to the tooth surface. To this end, we first determined the strains and species predominantly present in the planktonic and adhering microbiomes of ten healthy volunteers using Denaturing Gradient Gel Electrophoresis (DGGE) and related their prevalence to the average adhesion forces of strain representatives for different species to saliva coated enamel measured with bacterial probe AFM.
Materials and methods

Subjects and inclusion criteria
Ten healthy volunteers (6 females and 4 males, aged between 24 to 57 years) participated in this study. The study design was approved by the Medical Ethical Testing Committee of the University Medical Center Groningen (METc 2011/330) and all subjects signed a declaration of informed consent. All volunteers considered themselves in good health and had a dentition with at least 16 natural elements. Volunteers having used antibiotics up to three months prior to the study or having used a mouthrinse in the month prior to the study were excluded. Two weeks before entering the study, volunteers were requested to brush their teeth according to their habitual oral hygiene, but the use of a standard, fluoridated toothpaste without antimicrobial claims (Prodent Softmint, Sara Lee Household & Bodycare, The Hague, The Netherlands) was imposed, as during the entire study.

Sample collection

Biofilm
Volunteers were asked to come to the laboratory for biofilm collection immediately after breakfast, without having brushed their teeth. Biofilm was collected using a sterile hook and a cotton swab from the left upper quadrant of the dentition (buccal, palatal, occlusal and interproximal sides of the dentition). Biofilm was suspended in 1 ml sterile Reduced Transport Fluid (RTF) (9) and all biofilm samples were immediately put on ice after collection and sonicated 10 sec at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) to suspend bacterial clumps. The samples were stored in RTF at -20 °C for subsequent DGGE analysis.

Saliva
Volunteers were requested to collect 2 ml of unstimulated whole saliva. Saliva samples were put directly on ice after collection and sonicated two times 10 sec at 30 W. Subsequently, samples were centrifuged at 18000 g for 5 min (Eppendorf Centrifuge 5417R, Hamburg, Germany), supernatant was removed and the pellet was resuspended in 200 µL TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA) and stored at -20 °C for later DGGE analysis.

Biofilm and saliva samples were taken twice from each volunteer with a six weeks interval in between and treated as separate samples.
DGGE analyses of biofilm and saliva samples

DNA extraction, PCR and DGGE analysis are described in Appendix I. In order to compare the gels, reference markers were added, containing various species representing the oral microbiome in health and disease namely: *Streptococcus mutans* ATCC10449, *Streptococcus sanguinis* ATCC10556, *Streptococcus sobrinus* ATCC33478, *Streptococcus salivarius* HB, *Streptococcus mitis* ATCC9811, *Streptococcus oralis* ATCC35307 and an isolate of *Lactobacillus sp.*

Gelcompar II (v6.5 Applied Maths) was used for gel analysis. Presence of reference species in all samples was analyzed using band-based matching with 0.5% optimization and 0.5% band tolerance as accuracy settings. Presence of a band was taken indicative of the presence of the reference species in the sample, regardless of the staining intensity. Dice’s similarity coefficient was used to construct a similarity matrix. A dendrogram was calculated based on the non-weighted pair group method with arithmetic averages as clustering algorithm (10).

Atomic force microscopy measurements

AFM was used to measure adhesion forces of selected bacterial strains to saliva coated enamel surfaces. Nine oral bacterial strains, comprising a combination of different laboratory strains and clinical isolates, representative for the oral microbiome were included in AFM force measurements: *S. mutans* ATCC700610, *S. mutans* NS, *S. sanguinis* ATCC10556, *S. sobrinus* HG1025, *S. salivarius* HB, *S. mitis* BMS, *S. mitis* ATCC9811, *S. oralis* J22, *Lactobacillus acidophilus* JP and a *Lactobacillus* isolate. Bacteria were grown on blood agar plates for 24 h at 37 °C from frozen DMSO stock and inoculated in 10 ml Todd-Hewitt broth (Oxoid, Basingstoke, UK) for 24 h at 37 °C in ambient air. The pre-culture was used to inoculate a main culture which was grown for 16 h. Bacterial harvesting was done by centrifugation (5000 g, 5 min). Subsequently, the pellet was washed twice and resuspended in demineralized water. Bacterial clumps were suspended by sonicating 3 x 10 sec at 30 W.

Human whole saliva, stimulated by chewing parafilm, was collected of both genders in ice cooled beakers. All volunteers gave their informed consent agreeing with the rules as stated by the Medical Ethical Testing Committee of the University Medical Center Groningen (letter 06-02-2009). The saliva was pooled, centrifuged to remove particulate debris, dialyzed against demineralized water and subsequently lyophilized for storage. Lyophilized saliva was reconstituted at a concentration of 1.5 g/l in adhesion buffer (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8).
Enamel slabs were cut (0.6 x 0.6 x 0.2 cm) from the buccal surfaces of bovine incisors, grinded and polished, as described in Appendix II. Salivary conditioning films were created by immersing the enamel slabs into reconstituted saliva for 16 h at 4 °C. Before use in AFM experiments, the slabs were dipped three times in demineralized water to remove excess saliva.

Tipless AFM cantilevers (MP-010, Bruker, Billerica, USA) with immobilized bacteria were prepared and AFM was performed (BioScope Catalyst AFM Bruker, Billerica, USA), as described in Appendix III. For each combination of bacterial strains, at least 40 force-distance curves were recorded at randomly chosen spots with two to four bacterial probes and bacteria from at least two different cultures of each strain (for an example of a force-distance curve, see Appendix Fig. 1).

Statistics

DGGE profiles were assessed on predominant presence of bacterial species in the planktonic or adhering microbiome. Therefore a three way mixed effects ANOVA model was fitted to the bacterial presence among volunteers to investigate the effect of the microbiome for each species separately at a significance level of 0.05. The relative presence of species (adhering microbiome minus planktonic microbiome) in the model was taken random to be able to overcome the sparse data for the relative large number of species and address possible correlations, the microbiome and period were taken as fixed effects. The relative presence of species was estimated and accompanied with a 95% confidence interval. The residuals were investigated to evaluate the model fit.

Adhesion forces of individual bacterial strains displayed a skewed distribution (Shapiro-Wilk test, P < 0.01) and are presented as medians. Species averaged adhesion forces were calculated as weighted averages over the different strains used in AFM to represent a given species and presented as means with standard errors. SAS v9.3 (SAS institute inc., Cary, USA) and SPSS v20.0 (IBM Corp., Armonk, USA) were used to conduct statistical analysis.

Results

The planktonic and adhering oral microbiomes of nine out of all ten volunteers separated at the main branch of the clustering tree (Fig. 1) of their DGGE profiles (Appendix Fig. 2) at 36% similarity to each other. Samples taken on different occasions from each volunteer showed remarkably high similarities, on average 75%, indicating no periodic carry over effects.
This difference in microbial composition between both microbiomes was further analyzed by determining the species predominantly present in the adhering and planktonic microbiomes (Fig. 2). *S. mutans*, *S. sanguinis* and *Lactobacillus* strains were predominantly present in the adhering microbiome of the volunteers. *S. mutans* was not detected in saliva samples of any of the volunteers, but present in biofilm samples of 50% of all volunteers. *S. sanguinis* was present in 30% of all saliva samples, while found in 85% of all biofilm samples. No *Lactobacillus* strains were found in any of the saliva samples and only in 30% of all biofilm samples.

### Figure 1

Clustering tree from the DGGE profiles of the adhering and planktonic oral microbiomes, indicated as “biofilm” and “saliva” respectively as taken from ten healthy volunteers, on two different occasions from each volunteer (individual volunteers are indicated by numbers). Clustering tree was based on a band-based percentage similarity matrix.

Bacteria significantly more present in saliva samples were identified as *S. sobrinus* (60% in saliva samples versus 35% in biofilm samples) and *S. salivarius* (90% in saliva samples versus 35% in biofilm samples). In nine out of ten volunteers, *S. mitis* and *S. oralis* were found in both microbiomes.
Figure 2

The percentage of volunteers carrying specific bacterial species in their adhering and planktonic oral microbiomes, indicated as "biofilm" and "saliva" respectively. Error bars denote SD over ten volunteers, each measured on two occasions. Statistically significant differences in species occurrence in saliva and biofilm (P < 0.05) according to three way mixed effects ANOVA model, corrected for periodic effects, are indicated by an asterisk (*). The residuals of the model do not demonstrate outliers and the distribution is close to a normal distribution implying an appropriate description of the observed bacterial presence.

The relative presence (percentage of volunteers with the species present in the adhering microbiome minus the percentage occurrence in the planktonic microbiome) of different species toward the adhering microbiome increased with their adhesion forces to saliva coated enamel (Fig. 3). For species predominantly present in the adhering microbiome, species averaged adhesion forces varied from -0.60 nN to -1.05 nN. S. sanguinis and Lactobacillus sp. showed the highest adhesion forces followed by S. mutans. Bacteria predominantly present in the planktonic microbiome, S. salivarius and S. sobrinus, had lower species averaged adhesion forces between -0.40 and -0.55 nN. Bacterial species without a predominant presence in either microbiome, S. mitis and S. oralis, had adhesion forces in the range of -0.60 nN to -0.80 nN. Note that in Appendix Fig. 3, we present the adhesion forces of the individual strains used in AFM to represent a given species and obtain species averaged adhesion forces.
Figure 3
The relative presence of different species in human volunteers as a function of the species averaged adhesion force to saliva coated enamel surfaces, separated with respect to their predominant occurrence in the planktonic (saliva) or adhering (biofilm) microbiome. Adhesion forces are displayed as mean values over all representatives of a given species used in the AFM part of this study with standard errors. Linear regression was significant at P < 0.05 and 95% confidence intervals of the mean is indicated by two outer lines.

Discussion
This paper demonstrates the validity of our hypothesis that differences in the composition of the planktonic and adhering oral microbiome on oral hard surfaces are related to the forces by which different bacterial species adhere to the tooth surface, i.e. saliva coated enamel. Bacterial adhesion forces were higher for species predominantly residing in the
adhering microbiome than for species mostly present in the planktonic microbiome. To the
best of our knowledge, this is the first time that such an influence of bacterial adhesion
forces on the composition of oral biofilm versus bacterial composition in saliva is
demonstrated. As an important additional observation, residence in the planktonic or
adhering microbiome appears to be dictated by species averaged adhesion forces that
range between -0.40 and -1.05 nN. Species with adhesion forces stronger than -0.55 nN
end up in the adhering microbiome, while species with smaller adhesion forces become
member of the planktonic microbiome. A very similar adhesion force value of -0.5 nN has
been suggested to dictate whether *E. coli* would become a predominant member of the
adhering urogenital microbiome (8). It is amazing how such small differences in bacterial
adhesion forces can select strains from saliva to become member of the adhering
microbiome. Earlier, it has already been found that initial colonizers of dental hard surfaces
possess adhesion forces to saliva coated enamel that are only 0.1 nN stronger than of later
colonizing, more cariogenic strains (11). With respect to *Staphylococcus aureus* strains, a
difference in adhesion force of 0.28 nN appears to dictate whether a strain can invade
mammalian cells or not (12). Such observations endorse the concept that bacteria adhere
to a surface according to their own specific characteristics, such as their specific hydrogen
bonding capability and ability to form ligand-receptor bonds (13,14). In a first instance, it
appears as a limitation of our study that other interactions between bacteria, such as co-
adhesion between different strains and species, are not included in the analysis. However,
selection of appropriate co-adhesion partners also seems governed by the magnitude of
the adhesion forces between the co-adhesion partners (15) similar as to how the adhering
microbiome arises from adhesion force governed selection from the planktonic
microbiome, as we show in this paper.

The complete separation of the adhering and planktonic oral microbiomes as
found in our study (Fig. 1) is in line with literature results, showing that biofilm and saliva
samples display statistically significant clustering profiles with no significant differences
between PCR-DGGE profiles of males and females (16). *S. mitis* and *S. oralis* were found
in 90% of our volunteers (Fig. 2) and are indeed known to account for a major proportion of
all *Streptococcus* spp. (17) in the oral cavity. Note that DNA analysis in PCR-DGGE can
yield multiple and overlapping bands (18,19), as in the present case for *S. mitis* and *S.
oralis*, making it impossible to discriminate between these two species (Appendix Fig. 2).
Levels of *S. mutans* and *S. sobrinus* in the adhering microbiome relative to each other are
in line with literature, where the latter is rarely found in higher numbers than *S. mutans*
(20,21). Finally, the complete absence of sub-gingival species, like *Porphyromonas gingivalis*
in all samples (Appendix Fig. 2) is supported by literature as well (1). This
agreement with literature data demonstrates that the group of volunteers used can be
considered as a representative one and justifies its use to support evidence for our
hypothesis that the composition of the adhering microbiome is determined by the adhesion
force values of different strains and species.

Interestingly, some species (S. mutans, Lactobacillus sp.) were only found in the
adhering microbiome on oral hard surfaces, whereas the planktonic microbiome naturally
constitutes the species’ origin. This attests to our conclusion that the ability of a strain to
adhere determines its presence in either the adhering or planktonic microbiome. Evidently,
in case of strains present below the detection limit of DGGE (22), stronger adhesion forces
lead to selection followed by a detectable presence of these strains in the adhering
microbiome on oral hard surfaces. These observations support ongoing discussion in
literature that the microbial composition of saliva is of minimal value for the prediction of
dental caries and should only be used as an association instead of a causal relationship
(21,23–25).

The correlation between adhesion forces and the predominant presence of
bacterial strains and species in either the salivary or adhering microbiome demonstrated in
this research, is highly new and provides an important step in understanding the site- and
material-specific differences in composition of biofilms in the oral cavity.

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CHAPTER 5

References


Appendices

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Appendix I.
DNA Extraction, PCR and DGGE

DNA was isolated from thawed samples and centrifuged at 18000 g for 10 min. The pellet was washed by resuspension in 200 µL TE buffer and centrifuged again for 10 min. Isolation of chromosomal DNA for both biofilm and saliva samples was done as been described earlier (1). The DNA concentration was measured using a NanoDrop® Spectrophotometer (ND-100, NanoDrop Technologies Inc., Wilmington, DE, USA) at 230 nm. PCR was performed with 100 ng of DNA on a T-gradient thermocycler (Bio-rad I-Cycler, GENO-tronics BV, USA) to amplify the universal V3 region of the 16S rRNA gene in all samples with the F357-GC forward primer and R-518 as the reverse primer (2). The PCR products were applied on an 8% (w/v) polyacrylamide gel in 0.5 x TAE buffer (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 8.3). The denaturing gradient had a range of 30–80% and was made with a stock solution (100% denaturant equals 7 M urea and 37% formamide). A 10 ml stacking gel was made without denaturant and added on top. Electrophoresis conditions were set at 200 V for the first 10 min, and then set to 120 V at 60 °C overnight. Gels were stained for at least 10 min using a silver nitrate solution (0.2% AgNO₃ (w/v)) until maximal staining intensity was observed.

Appendix Figure 1
Example of a force distance curve between a bacterial probe (S. salivarius HB) and saliva coated enamel. The grey line shows the approach curve towards the surface, while the black line shows the retraction curve, with the maximum adhesion force occurring around 200 nm.

Appendix II.
Enamel preparation

Enamel slabs were cut from the buccal surface of bovine incisors into pieces of 0.6 x 0.6 x 0.2 cm under running tap water and grinded with 220 to 1200 grit sandpaper. The surface
was micropolished for 3 min using wet polishing pad with 0.05 µm alumina particles (Buehler Ltd., Lake Bluff, IL, USA) and subsequently cleaned with demineralized water and 2 min of sonication in a 35 kHz bath (Transsonic TP 690-A, Elma, Germany). Saliva conditioning films were created by immersing the enamel slabs into reconstituted saliva for 16 h at 4 °C. Before use in AFM experiments the slabs were dipped three times in demineralized water.

Appendix Figure 2
Dendrogram analysis of bacterial DGGE profiles of saliva (green) and biofilm samples (red). Numbers indicate different volunteers and first (A) or second (B) sampling moment. Positions of reference strains, used to identify bacterial species presence, are displayed above the DGGE profiles.
Appendix III.
Preparation of bacterial probes for AFM

For the immobilization of bacteria on tipless AFM cantilevers (MP-010, Bruker, Billerica, USA), cantilevers were first immersed for 1 min in a drop 0.01 % (w/v) poly-L-lysine (Sigma, Poole, UK). After air drying for 2 min, the tip of the cantilever was dipped in a bacterial suspension for 1 min to immobilize the bacteria to the tip. All cantilevers were used immediately after preparation for experiments. AFM measurements were initiated in contact mode on a BioScope Catalyst AFM (Bruker, Billerica, USA) in adhesion buffer at room temperature, with a scan rate of 0.5 Hz, a ramp size of 1.5 µm and a trigger threshold of 3 nN.

Bacterial adhesion forces were retrieved from force distance curves. To this end, the cantilevers spring constant, determined for each experiment, was used to convert the cantilever deflection into force values (nN). In order to verify that a bacterial probe enabled a single contact with the enamel surface, a scanned image in AFM contact mode with a loading force of 1-2 nN was made at the onset of each experiment and examined for double contour lines, which are indicative of multiple bacteria on the probe in contact with the enamel surface. Any probe exhibiting double contour lines was discarded. To ensure that a bacterial probe was not affected by previous measurements, force curves at 0 sec surface delay on clean glass were regularly compared to five initially measured control force curves on glass. If a continued measurement differed by more than 0.2 nN from the initial control force, data were discarded and a new probe prepared.
Appendix Figure 3
Median adhesion forces (nN) of individual bacterial strains to saliva coated enamel used to determine species average adhesion forces. Error bars denote SE, while colors indicate a predominant presence of a given species in a particular microbiome, as based on DGGE analysis.

References

