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The Effect of Inoculum Source and Fluid Shear Force on the Development of *In Vitro* Oral Multi-species Biofilms

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Abstract

Aim: Saliva has been previously used as an inoculum for *in-vitro* oral biofilm studies. However, the microbial community profile of saliva is markedly different from hard and soft tissue-associated oral biofilms. Here, we investigated the changes in the biofilm architecture and microbial diversity of *in-vitro* oral biofilms developed from saliva, tongue or plaque-derived inocula under different salivary shear forces. **Methods and Results:** Four inoculum-types (saliva, bacteria harvested from the tongue, toothbrush and curette-harvested plaque) were collected and pooled. Biofilms (n≥15) were grown for 20 h in cell-free human saliva flowing at three different shear forces. Stained biofilms were imaged using a confocal laser scanning microscope. Biomass, thickness and roughness were determined by image analysis and bacterial community composition analyzed using Ion Torrent. All developed biofilms showed a significant reduction in observed diversity compared to their respective original inoculum. Shear force altered biofilm architecture of saliva and curette-collected plaque and community composition of saliva, tongue and curette-harvested plaque. **Conclusions:** Different intraoral inocula served as precursors of *in-vitro* oral polymicrobial biofilms which can be influenced by shear. **Significance and Impact of the Study:** Inoculum selection and shear force are key factors to consider when developing multi-species biofilms within *in-vitro* models.

Keywords: Confocal laser scanning microscopy, saliva, dental plaque, tongue, biodiversity, microbiome, bacteria, 16S rRNA

Introduction

Oral biofilms are architecturally and taxonomically complex microbial communities that develop on teeth to form visually conspicuous dental plaque (Nyvad & Fejerskov, 1987, Mark Welch*, et al.*, 2016). Oral biofilms can contain hundreds of species of bacteria (Dewhirst*, et al.*, 2010). These biofilm communities develop through tightly orchestrated cell-cell interactions (Rickard*, et al.*, 2003, Hojo*, et al.*, 2009) and their formation is influenced by the colonizing species, the prevailing environmental conditions, and the topographical and physicochemical properties of the surface to which the colonizing bacteria adhere (Song*, et al.*, 2015). Through cell-cell and cell-environment interactions, which influence the species composition and architecture, oral biofilms can develop to cause caries and periodontal diseases (Jakubovics & Kolenbrander, 2010). The multi-species composition and the biofilm-specific lifestyle of the component bacteria are responsible for the recalcitrance of biofilms to physical and chemical control strategies (Gilbert*, et al.*, 2002, Marsh, 2003, ten Cate & Zaura, 2012).

In vitro model biofilm systems are commonly used to gain knowledge of changes in biofilm architecture and composition, especially when trying to understand the development of disease-causing biofilms and when evaluating the effectiveness of antimicrobial/anti-biofilm compounds (Kinniment*, et al.*, 1996, McBain, 2009, Zijnge*, et al.*, 2012, Salli & Ouwehand, 2015). However, many *in vitro* model systems are arguably not particularly representative of the conditions within the human oral cavity (McBain, 2009, Coenye & Nelis, 2010). Such a potential lack of representation is typically due to the use of artificial medium and/or the use of one or a few strains of bacteria (Saunders & Greenman, 2000, Guggenheim*, et al.*, 2001, Fernández*, et al.*, 2016). Given the bacterial

diversity of human oral biofilms, it would conceivably be preferable to use natural inocula to facilitate the development of *in vitro* biofilms to more broadly represent the *in vivo* community (Burmolle*, et al.*, 2014, Kistler*, et al.*, 2015). Recently, we developed an *in vitro* microfluidic oral biofilm system that uses filter-sterilized 25% pooled human saliva as the medium and pooled human saliva as the inoculum (Nance*, et al.*, 2013, Samarian*, et al.*, 2014, Kolderman*, et al.*, 2015). Using confocal laser scanning microscopy (CLSM), multi-species biofilms were shown to be architecturally complex and containing predominantly viable cells. The multi-species biofilms, also referred herein as polymicrobial biofilms, that formed within the system also contained species that are typically identified within *in vivo* supragingival dental plaque biofilms (Nance*, et al.*, 2013). This included species that are often regarded as being highly refractory to cultivation, including members of the candidate division TM7 (Soro*, et al.*, 2014, He*, et al.*, 2015). However, as opposed to our *in vitro* model system, the human oral cavity is composed of numerous surface-types, exposed to different environmental conditions and subject to colonization by different species in a site-dependent (and niche-dependent) manner (Aas*, et al.*, 2005). Cognizant of the anatomical and environmental diversity of the human oral cavity, we hypothesized that inocula derived from biofilms at different sites would give rise to taxonomically and architecturally distinct biofilms in our *in vitro* model system. Given that salivary flow also varies between sites *in vivo*, we hypothesized that shear might alter the architectural and taxonomic characteristics of the biofilms.

The aim of this study was to characterize differences in the biofilm architecture and microbial biofilm diversity of *in vitro* microfluidic-grown biofilms developed from saliva, tongue or plaque-derived inocula under different fluid shear. Findings from this study indicate that the architecture and biofilm community composition of developed *in vitro* oral biofilms is influenced by the source and harvesting approach to acquire inocula for this

in vitro model system. Furthermore, evidence suggested that salivary shear influenced the architecture and community composition/diversity of the biofilms in an inoculum-dependent manner.

Materials and Methods

Summary of Experimental Design

Saliva samples, bacteria harvested from the tongue, toothbrush-harvested plaque and curette-harvested plaque were collected from four healthy donors and pooled to make four inoculum-types, based upon the source from which they were harvested. Biofilms from these four inoculum-types were grown in cell free human saliva (CFS) in 24-channel Bioflux™ microfluidic plates for 20 h. CFS was flowed through the system at 0.1, 0.2 or 0.4 dyn cm^{-2} (fluid shear force) to yield a total of twelve experimental groups (four inoculum-types at three fluid shear forces). Developed biofilms were labeled with a vitality stain and imaged using a confocal laser scanning microscope. Biomass, thickness and roughness were calculated from the collected images. Experiments were performed in at least three independent assays (i.e. across three microfluidic plates). In each assay, between four to six channels supported biofilm growth from each experimental group. This facilitated the analysis of a total of 15 to 17 channels per group across three independent assays (i.e. n= 15-17). For each channel, three CLSM images were taken for analysis. For each experimental group, developed biofilms from three channels were harvested in order to assess community composition. Initial inocula (n= 3 per inoculum-type) and developed biofilms (n= 3 per inoculum-type) were sequenced with Ion Torrent PGM™ platform. Sequencing data were used to estimate alpha and beta diversity. The architectural measures and community composition of the biofilms were statistically compared considering fluid shear force and inoculum-type from which they were derived.

Patient Sampling

This study was approved by the University of Michigan Institutional Review Board for Human Subject Research (ID# HUM00101254). Samples were collected from four consenting healthy donors, who did not have any known underlying chronic disease and in good oral health. The donors had not received antibiotics for at least three months prior to collection. Collection of samples was performed in the morning for all volunteers. They were asked to refrain from ingesting food and brushing their teeth the morning of the collection.

To generate a saliva-inoculum, stimulated saliva was collected during mastication of parafilm until 5 mL was collected in a sterile plastic tub. To generate a tongue-inoculum, a sterile stainless steel tongue cleaner was drawn firmly over the dorsum of the tongue until all visible material had been removed. All the fluid collected was deposited in a sterile plastic tube. To generate a toothbrush-plaque-inoculum plaque was removed using a toothbrush. For this, donors were shown how to perform vertical movements in all the buccal and lingual surfaces for a total of two minutes. The plaque removed and the saliva accumulated in the mouth were collected in a sterile plastic tube. To generate the curette-harvested plaque-inoculum, visible accumulated plaque on dental surfaces was removed using a sterile curette. During sampling, plaque was collected in a sterile tube containing 500 µL of pre-reduced 10 mmol l^{-1} sodium phosphate buffer (pH 8.0) (Shu*, et al.*, 2007). Samples of each inoculum-type from the different donors were pooled, filtered using a 70 µm nylon filter to remove organic residual and glycerol was added to form a mixture containing a final concentration of 25% glycerol. Samples were stored in individual aliquots at -80ºC until required.

Cell-free pooled human saliva (CFS) was used as a natural nutrient source to mimic *in vivo* conditions inside the microfluidic biofilm model (Nance*, et al.*,

2013, Samarian*, et al.*, 2014). For this, around 30 ml stimulated saliva was collected from the same donors. The saliva was pooled and dithiothreitol was added at 2.5 mmol l^{-1} to prevent protein agglomeration. In order to remove visible particulate material, the pooled saliva was centrifuged for 30 min at 17,500 *g*. The resulting particulate-free saliva was diluted to 25% using deionized water and filter sterilized (0.22 µm polyethersulfone filter). Individual aliquots were stored at -80ºC until use.

Microfluidic Biofilm Model System

Biofilms were grown in a 24-channel Bioflux™ microfluidic system (Fluxion, South San Francisco, CA, USA) as described by Samarian et al., (2014). This model system contains 24 channels (6 mm long, 350 µm wide, 70 µm high) which are individually connected to an inlet- and outlet-well. Biofilms develop on the glass surfaces within the channels which are exposed continuously to growth medium (ie. CFS) flowing at a defined shear forces. Briefly, CFS was used to coat the channels of the microfluidic system for 20 min to simulate acquired pellicle formation. Each channel was subsequently inoculated with one of the four inoculum types (saliva samples, bacteria harvested from the tongue, toothbrush-harvested plaque and curette-harvested plaque) and incubated for 45 min at 37ºC. CFS was flowed in the system at 0.1, 0.2 or 0.4 dyn cm^{-2} (fluid shear force) during 20 h at 37ºC under aerobic conditions (i.e., saliva was not pre-reduced).

Biofilm Staining, Imaging, and Analysis.

After 20 h growth, developed biofilms were washed with PBS (pH 7.4) at 0.2 dyn cm^{-2} for 20 min. Live/Dead® reagent (Invitrogen, CA, USA) was diluted in PBS to contain 10 µmol l^{-1} SYTO 9 and 60 µmol l^{-1} propidium iodide,

introduced into the channels at 0.2 dyn cm^{-2}, and allowed to stain the biofilms for 45 min at room temperature. Subsequently, the biofilms were washed for 20 min with PBS to remove excess stain from the channels.

Three random representative image stacks were taken of the developed biofilm per channel using an inverted Leica SPE CLSM (Leica, Exton, PA, USA) equipped with a HCX PL APO 40X/0.85 CORR CS dry microscope objective (Leica, Exton, PA, USA). IMARIS Version 7.3.1 software (Bitplane, Zurich, Switzerland) was used to render images in 3D by using the *Surpass* visualization software component. Biomass, average thickness and roughness were calculated using COMSTAT2 software (Heydorn*, et al.*, 2000). Using the approach of Nance and colleagues (Nance et al., 2013), cell viability was calculated by determining the percentage of green pixels (from the total of green and red pixels) in each image stack using ImageJ software (Collins, 2007). All renderings and quantification analyses were performed on a PC equipped with Radeon 5850 1 Gb graphics card (AMD, Sunnyvale, CA, USA). Generated renderings were assembled in CorelDRAW v. X5 (Corel, Mountain View, CA, USA).

Harvesting of Samples and Genomic Analysis

Three channels from three different microfluidic plates (i.e. representing independent experiments) were randomly selected to harvest the developed biofilms. To perform this, the outlet-well was washed three times with sterile deionized water and all solution inside the inlet and outlet well completely removed. Following the washing step, 100 µL sterile deionized water was flowed through the channel that contained the biofilm at 20 dyn cm^{-2} in forward and reverse direction to remove the attached biofilm as described by Samarian *et al.* (2013).

DNA was extracted from harvested biofilms using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). An automated standard protocol was used in

concert with the QIACUBE (Qiagen, Hilden, Germany) to extract DNA reducing technical variation prior to sequencing. PCR primers for the V4 variable region (515-806) of the 16S rRNA gene were amplified in a single-step 30 cycle PCR using a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). This was performed using the following conditions: 94℃ for 3 min, followed by 28 cycles at 94℃ for 30 s, 53℃ f or 40 sand 72℃ for 1 min, with a final elongation step at 72℃ for 5 min. Sequencing was carried-out at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) using an Ion Torrent™ PGM and following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA USA).

Raw sequences were processed in-house with QIIME (ver. 1.9.0). Sequences with ambiguous base calls, an average Phred quality score below 25, homopolymer length of >6, primer mismatch exceeding 0, or read length that is below 200 bp were discarded. All sequences that remained after this filtering step, had primers, adaptors, and linker sequences truncated. Operational taxonomic units (OTUs) were clustered by 97% identity using an open-referenced OTU picking strategy with PyNAST sequence aligner against the CORE database (Caporaso*, et al.*, 2010a, Griffen*, et al.*, 2011). Taxonomy was assigned using the RDP Classifier (Wang*, et al.*, 2007) in QIIME. Singleton OTUs were filtered out as part of the default QIIME parameters. Additionally, OTUs constituting less than .05% of total reads were filtered out. The final OTU table was analyzed with QIIME (Caporaso*, et al.*, 2010b) and the Phyloseq package in R (McMurdie & Holmes, 2013). Downstream analytics include Shannon-Weaver within-sample diversity, community relative abundance, and unweighted UniFrac distances between samples. Outcomes were measured within the Phyloseq package and graphical output generated with R's ggplot package. For principal coordinates construction, the jackknifed_beta_diversity.py pipeline within QIIME was used.

Statistical Analysis.

Statistical analyses were performed using R (RStudio, Inc., version 0.99.489) for beta diversity and SPSS (IBM Corp., version 23.0, Armonk, NY, USA) for architecture outcomes and alpha diversity. For biomass and thickness differences among groups were analyzed using ANOVA/ Tukey's test. Biofilm roughness and Shannon-Weaver alpha diversity differences were tested using Kruskal-Wallis/Mann-Whitney. The effect of shear was also analyzed using a linear regression analysis. The significance threshold was set at .05 for all analyses.

Results

Architectural Properties of Biofilms Developed from Different Inoculum-Types

Each inoculum type developed architecturally complex biofilms under the three different shear forces. Representative images of these developed biofilms are presented in Figure 1. As inferred by the Live/Dead stain, by determining the ratio of red to green cells, the amount of viable (green fluorescently labeled) cells predominated over the damaged/dead (red fluorescently labeled) cells (Fig. 1). The average green fluorescence was always > 75% for the biofilms developed from each inoculum and under each shear force (90.35± 9.31% of viability; average± SD; n= 187 channels). While no unique architectural structures could be assigned to either the inoculum from which the biofilm was developed or the shear force applied, it was evident that the biofilms that were developed from the toothbrush- and curette-developed plaque biofilms were generally more homogenous in structure (i.e. lacking larger biofilm biomasses) and consisted of many small biofilm micro colonies, as compared to biofilms developed from saliva and tongue inoculums.

Of note, image analysis showed that different shear forces (0.1, 0.2 and 0.4 dyn cm^{-2}) exhibited quantifiable significant differences ($p < 0.05$) to affect the architectural properties (biomass, thickness, and roughness) of the saliva and curette plaque developed biofilms. In particular, regression analysis showed that increasing shear force, increased the biomass and thickness of biofilms derived from the saliva and curette-plaque-developed biofilms (Fig. 2). Compared to biofilms developed at the lowest shear (0.1 dyn cm^{-2}), the highest shear (0.4 dyn cm^{-2}) resulted in the development of two-fold thicker biofilms from curette-harvested plaque and the development of three-fold thicker biofilms from collected saliva. By analyzing the distribution of the biomass and thickness values, biofilms developed under 0.2-dyn cm^{-2} demonstrated the least variability in architectural outcomes (Fig. 2). Although, considering the large standard deviation of values of biomass and thickness, saliva-derived biofilms were also the most architecturally variable biofilms. Differences by shear force were only observed in roughness values for biofilms developed from saliva and tongue inocula, whereby increasing the shear significantly reduced the roughness values (Supporting Information: Table S1). Toothbrush-plaque and tongue were seemingly the least responsive to shear, showing very little change in architecture, except for roughness for biofilms developed from the pooled tongue inoculum.

It was observed that only biofilms developed at 0.2 dyn cm^{-2} exhibited significant architectural differences among inocula (Fig. 2 and Supporting Information: Table S1). At this shear, biomass and thickness of biofilms developed from saliva were greater and statistically different than curette-plaque-developed biofilm ($p < 0.05$), while toothbrush-plaque-developed biofilm was not statistically different to saliva and curette-plaque-developed biofilm. For biofilms developed from tongue inocula, only the biofilm biomass was significantly different to biofilms grown from saliva inocula (Supporting Information: Table S1). No significant differences in biofilm roughness were evident among inocula.

Community Composition Analysis

The most abundant genera in all our samples (pooled inocula and the respective biofilms that developed from the inocula) were those typically associated with the human oral microbiome and included *Streptococcus, Neisseria, Rothia, Fusobacterium and Veillonella.* For the pooled plaque inoculum and the biofilms developed from it, a large number of genera could not be assigned to a taxonomic group (Supporting Information: Figure S1). These unclassified genera belonged to a variety of families including *Neisseriaceae, Streptococcaceae, Actinomycetaceae, Aerococcaceae, Lachnospiraceae, Gemellaceae, Enterobacteriaceae*. Given the level of taxonomic resolution, the relative abundance at family level for each inoculum-type and their respective developed biofilms grown at 0.1, 0.2 and 0.4 dyn cm^{-2} were derived and are shown in Figure 3. At the family level (Fig. 3), the biofilm composition varied depending upon inoculum type and shear force applied (which was also reflected at the genus level, for those which could be classified; Supporting Information: Figure S1). Of particular note was that for all inocula, specific equally dominant bacterial families were abundant in all the inocula. These included members of the *Streptococcaceae*, *Neisseriaceae*, *Veillonellaceae*, *Micrococcaceae*, and *Actinomycetaceae*. In addition to members of these dominant bacterial families, curette-derived plaque inocula also contained an abundance of members of the *Fusobacteriaceae*. Developed from each of the four inocula types, biofilms communities with substantially altered ratios of family members were developed (Figure 3). In particular, members of the *Streptococcaceae* and *Neisseriaceae* dominated at the expense of the other bacterial families. In order to further quantify and compare the differences across each community, alpha and beta diversity were assessed.

i. Alpha Diversity

The alpha diversity, estimated by determining the Shannon-Weaver Index for each inoculum and biofilm sample, showed that each type of inoculum possessed the largest alpha diversity, compared to the biofilms that developed from them (Fig. 4). For the different inocula, the curette-plaque derived inoculum possessed the largest alpha diversity, followed by the toothbrush-derived plaque inoculum, and the inocula derived from the tongue and saliva. Also, the shear influenced the alpha diversity of some of the biofilms (Fig. 4). Increasing the imposed shear to 0.4-dyn cm^{-2} decreased alpha diversity of saliva-developed biofilms. Conversely, for biofilms derived from the pooled tongue inoculum, the lowest alpha diversity was observed at the intermediate shear of 0.2 dyn cm^{-2}. This was different when compared with biofilms developed at 0.1 dyn cm^{-2}, but not statistically different with biofilms developed at 0.4 dyn cm^{-2} (Fig. 4). Biofilms developed from toothbrush-plaque derived inocula were unaffected by shear ($p > 0.05$). Curette-plaque-developed biofilms seemingly showed greater alpha diversity when developed under 0.4-dyn cm^{-2} but not statistically different with slower run shears ($p > 0.05$).

ii. Beta Diversity

The beta diversity analysis is presented graphically in Figure 5 as a principal coordinate analysis (PCoA) plot. In the plot, each colored point represents a sample and the distance between samples represent the differences in community composition (membership and bacteria abundances) among individual samples. Three inoculum and three biofilm samples from each inoculum source (toothbrush-derived plaque, curette-derived plaque, saliva and tongue) are plotted using a color-coordinated approach (Fig. 5). As visibly noticeable in Figure 5 and shown quantitatively in Table 1, the inoculum and biofilm samples clustered into groups according to the sites from which they were harvested. Unifrac distance values presented in Table 1 show the magnitude of the differences in community composition among

the groups presented in Figure 5. Toothbrush-plaque and curette-plaque inocula were close (0.191 Unifrac distance) reflecting similar community composition, while saliva (0.301) and tongue (0.327) were more distant to plaque-derived inocula but closer between them (0.209) (Table 1 and Fig. 5, A). Developed biofilms were far removed from the original inoculum (Table 1), nevertheless, they remained clustered by inoculum type in the PC2 (vertical-axis) (Fig. 5, B). Of the four inoculum types, developed curette-plaque biofilms were the most dissimilar from its initial inoculum (Table 1, 0.664; 0.662 and 0.709 for 0.1 -0.2 -0.4 dyn cm^{-2}) and tongue the most similar (Table 1, 0.504, 0.540 and 0.489 for 0.1 -0.2 -0.4 dyn cm^{-2}). In addition, developed biofilms derived from the same inoculum and exposed to different shear forces were clearly clustering together (Fig. 5). Shear-induced clustering was statistically significant for biofilms developed from saliva and toothbrush-plaque (Table 1).

Discussion

Inocula harvested from different oral niches facilitated the development of *in vitro* polymicrobial oral biofilms within a microfluidic system. Even though the developed biofilms had a reduced diversity compared to the original inoculum, different inocula facilitated the development of relatively specific biofilms, as highlighted by the beta diversity analyses (Fig. 5 and Table 1). Because the model simulates a constant salivary flow, the effect of three different velocities to generate different shear forces was also tested. Biofilm architecture (Fig. 1 and 2) and microbial community composition (Fig. 3 to 5) were seemingly influenced by shear in an inoculum-dependent manner. Such a role for shear and inoculum-type in the development or oral multi-species biofilms has received only limited attention to date (Saunders & Greenman, 2000, Signori*, et al.*, 2016).

Our findings support previous reports indicating that the community composition of oral biofilms is site specific (Segata*, et al.*, 2012, Simon-Soro*, et al.*, 2013b) and that each type of inoculum develops taxonomically unique communities that exhibit some similarity to the communities from the original *in vivo* sites (Rudney*, et al.*, 2012). In agreement with our findings, other studies have illustrated the differences between saliva and plaque inocula (Rudney*, et al.*, 2012, Simon-Soro*, et al.*, 2013b), and showed that the microbial composition of saliva is more similar to the composition of tongue than it is to dental plaque (Mager*, et al.*, 2003) (Fig. 5). Such a similarity could relate to the shear effects on the tongue and subsequent seeding of biofilm associated cells into the saliva, as opposed to less pronounced shear effects on tooth-associated dental plaque biofilms.

When considering the stimulated saliva inoculum used in this study, a concern could be that the community composition could be different from unstimulated saliva. Evidence indicates that the stimulation of saliva production (e.g. chewing parafilm) likely increased the release of bacteria (Dawes*, et al.*, 2001). Work by Simon-Soro and colleagues indicated that differences in diversity between stimulated and unstimulated saliva can be present. Although, in their study, differences between stimulated and unstimulated were variable; whereby stimulated saliva showed a lower diversity than unstimulated saliva in one individual, and the reverse relationship was found in the other individual (Simon-Soro*, et al.*, 2013b). Conversely, another study reported that the microbial profiles of unstimulated and stimulated saliva samples collected from the same person have comparable composition (Belstrom*, et al.*, 2016). Thus, we decided to use stimulated saliva as surrogate of unstimulated saliva for our studies (Belstrom*, et al.*, 2016). Since the inocula we used in our study may overlap "niches" (e.g. toothbrush-harvested inoculum mixes saliva and supra-gingival plaque), the samples for each inoculum type were collected on different days over the period of a week. Human oral biofilms have been

454 shown to be stable over a week (Belstrom*, et al.*, 2016), so the different
455 composition observed among inocula are likely related to the oral site/niche
456 and not related to temporal differences.
457
458 Few studies focus on the potential to develop biofilm communities that
459 are representative of specific the sites in the human oral cavity. For caries
460 research the use of supra-gingival dental plaque has been indicated to be
461 ideal source of bacteria to develop *in vitro* microcosms, but its collection is
462 sometimes not practical since human volunteers, qualified clinicians, and
463 specific equipment are needed. Another complexity associated with growing
464 representative biofilms *in vitro* from dental plaque inoculum is the specific
465 collection site. Dental plaque composition is influenced by teeth localization
466 (upper/lower, anterior/posterior), site of accumulation (proximal, cervical,
467 occlusal) and also the mineral status of the teeth (sound/demineralized,
468 active/inactive) (Simon-Soro*, et al.*, 2013a, Simon-Soro*, et al.*, 2013b)
469 which generates a potentially large variation in community composition in
470 the *in vitro* developed biofilm, depending on the place of sampling. For this
471 reason, some studies use specific areas for collection (Reilly*, et al.*, 2014),
472 while others pooled samples from different locations (Shu*, et al.*, 2007) to
473 attempt to reduce variability. For this work, we compared four different
474 types of pooled inoculums; saliva, bacteria harvested from the tongue,
475 toothbrush-harvested plaque and curette-harvested plaque. Toothbrush-
476 plaque and curette-plaque developed biofilms had more consistent biomass
477 and thickness values (more homogenous data) than saliva and tongue-
478 developed biofilm (Figure 2). Saliva developed biofilms with the most
479 variable architecture outcomes. Regarding community composition, all
480 biofilms grown from the different inocula showed a reduction in diversity.
481 *Streptococcus* and *Neisseria* dominated within the *in vitro* developed
482 biofilms. The species identity of these two genera as well as members of
483 other genera in the oral biofilms was difficult to determine due to the low
484 resolution to identify taxonomic units at genus level. This was especially for

curette-plaque and toothbrush-plaque developed biofilms. Such a problem is explained by the short read lengths that traverse through V4 region in the 16S rRNA gene.

While it was observed that all the developed biofilms exhibited a reduction in diversity, the use of inocula harvested from oral sites, facilitated the development of multi-species biofilms that contains bacteria typically isolated in dental plaque (Dewhirst*, et al.*, 2010).Our findings indicate that toothbrush-plaque-developed biofilms retained the greatest diversity, while biofilms developed from curette-harvested plaque exhibited a substantial reduction of diversity. Despite this reduction in diversity, the use of human inocula to develop *in vitro* microcosms still can be considered more representative than the use of single species (Ccahuana-Vásquez & Cury, 2010, Fernández*, et al.*, 2016) or a defined consortia of bacteria (Saunders & Greenman, 2000, Guggenheim*, et al.*, 2001). Additionally, the community composition of the developed-biofilms collected from different channels and from different experiments remained similar as highlighted by the clustering in the PCoA (Fig. 5). This indicated reproducibility of our *in vitro* model. Although some variation in viability, as inferred by image analysis, was observed between developed biofilms, the average percent of green fluorescence was always >75% in all groups. Differences between some groups within the upper 25% range were observed but such differences are difficult to evaluate in regard to viability (especially in polymicrobial communities) (Netuschil*, et al.*, 2014) and may be a consequence of experimental variation. Using the same microfluidic model we have observed that the shifts in green fluorescence are considerably greater after treatment to antimicrobials (Nance et al., 2013).

Changes in fluid shear have been shown to cause alterations in biofilm morphology (Klapper*, et al.*, 2002, Stoodley*, et al.*, 2002), thickness (Rittman, 1982) and diversity (Rickard*, et al.*, 2004). In addition to the

changes in physical aspects of the biofilm, fluid shear can impact the production of exopolysaccharides, mass transfer, and influence metabolic/genetic behaviors (Liu & Tay, 2001, Liu & Tay, 2002). Some of these observations could also be explained by the effect of fluid flow conditions on cell-cell signaling (quorum-sensing) (Kim*, et al.*, 2016). In our system, shear force seemed to influence biofilm architecture and community composition but the effect was not evident for all inoculum types. Biomass and thickness increased when shear force was increased, but only in biofilms formed from saliva and curette-plaque inocula (Fig. 2). We hypothesize that shear force affects biofilm architecture and community composition by favoring the initial and subsequent attachment/retention of some species and by altering the substrate availability.

Developed biofilm communities contained species that were present in the inoculums from which they were derived (Fig. 3). However, these biofilms communities were altered with respect to which bacterial families dominated, as compared to the inoculums from which they were developed. A number of reasons could account for the expansion of certain bacterial families at the expense of others. For example, as compared to planktonic populations, biofilm communities are subject to differences in environmental factors, such as pH, dissolved oxygen, and substrate availability which exert a large effect on the composition of oral microcosms grown *in vitro* (Brown & Gilbert, 1993, Marsh, 2009, Zaura*, et al.*, 2009). These differences will also conceivably change in a spatiotemporal manner as the biofilms develop. In our model, specifically, members of the *Streptococcaceae* and *Neisseriaceae* were seemingly selected for within the biofilms and were consequently the most abundant. At the genera level, our data showed that *Neisseria* was more prevalent in saliva developed biofilms. This observation is in agreement with previous *in vitro* biofilm models where *Neisseria* was the predominant species in multi-species developed biofilms (Saunders & Greenman, 2000, Nance*, et al.*, 2013, Kistler*, et al.*, 2015) and as part of

the healthy 'core microbiome' of the human oral cavity (Zaura*, et al.*, 2009). Also, it has been observed previously within an *in situ* model, that *Streptococcus* and *Neisseria* dominated in the early phase of biofilm development (Wake*, et al.*, 2016). Given that our model system is aerobic, aerobic bacterial and facultative aerobic species were expected to be common biofilm members. Future studies could explore the effect of anaerobic conditions and longer periods to evaluate shifts in community composition.

In conclusion, within a saliva-based *in vitro* model, different intraoral inocula serve as precursors of oral biofilms. The biofilms developed in the model had reduced bacterial diversity compared to the original inocula. Our data indicates that inoculum selection and hydrodynamic shear force can influence biofilm architecture and community composition. Thus, inoculum type and shear are key factors to carefully consider when developing multi-species biofilms within *in vitro* models. These findings offer valuable insight into understanding the parameters that influence the development of multi-species biofilms within the laboratory.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. M.W. Dodds and M.B. Aspiras are current employers of the Wm. Wrigley Jr. Company, Chicago, IL, USA.

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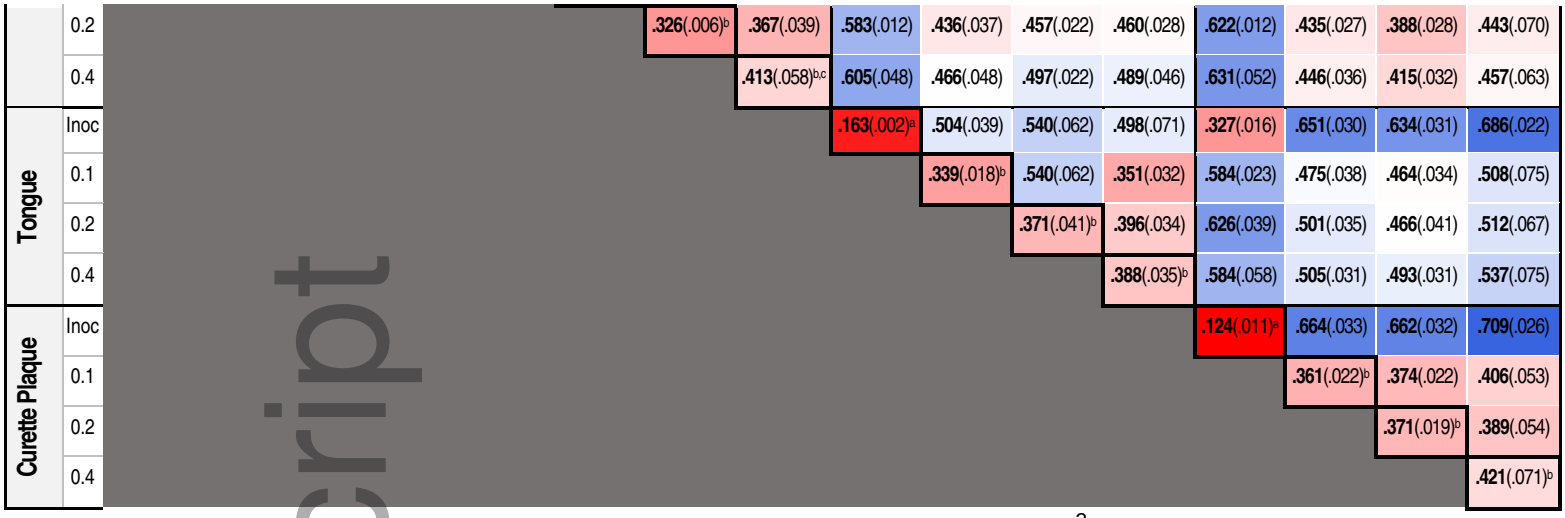
Table 1. Heat map of unweighted UniFrac distances [**average**(sd)]. Shades of red (lowest values) and blue (largest values) indicate the magnitude of the differences in community composition between compared groups. Bolded borders to cells highlight the distances between inoculum and the respective developed biofilms at 0.1, 0.2 and 0.4 dyn cm^{-2} for each inoculum-type. Statistically significant differences are represented by different letters. The number of pairwise comparisons was three for within group and nine for between groups.

		Experimental Groups															
		Saliva				**Toothbrush Plaque**				**Tongue**				**Curette Plaque**			
		Inoc	0.1	0.2	0.4	Inoc	0.1	0.2	0.4	Inoc	0.1	0.2	0.4	Inoc	0.1	0.2	0.4
Saliva	Inoc	**.177**(.006)[a]	**.569**(.011)	**.666**(.016)	**.591**(.009)	**.229**(.011)	**.527**(.027)	**.570**(.021)	**.587**(.060)	**.209**(.006)	**.518**(.029)	**.555**(.063)	**.510**(.069)	**.301**(.008)	**.647**(.029)	**.634**(.034)	**.684**(.026)
	0.1		**.329**(.026)[b]	**.419**(.032)	**.365**(.024)	**.605**(.006)	**.373**(.012)	**.367**(.012)	**.419**(.040)	**.575**(.017)	**.409**(.039)	**.426**(.026)	**.419**(.030)	**.650**(.005)	**.459**(.032)	**.424**(.018)	**.468**(.071)
	0.2			**.393**(.043)[c]	**.412**(.025)	**.691**(.014)	**.470**(.046)	**.423**(.024)	**.469**(.035)	**.666**(.015)	**.480**(.037)	**.447**(.045)	**.489**(.053)	**.712**(.013)	**.470**(.024)	**.433**(.029)	**.461**(.059)
	0.4				**.399**(.006)[c]	**.631**(.006)	**.420**(.030)	**.402**(.023)	**.439**(.045)	**.596**(.010)	**.419**(.032)	**.417**(.021)	**.426**(.032)	**.659**(.008)	**.470**(.026)	**.441**(.024)	**.485**(.060)
Toothbrush Plaque	Inoc					**.158**(.020)[a]	**.552**(.021)	**.594**(.018)	**.609**(.061)	**.277**(.011)	**.563**(.025)	**.600**(.051)	**.558**(.060)	**.191(.006)**	**.660**(.028)	**.652**(.034)	**.700**(.027)
	0.1						**.314**(.018)[b]	**.345**(.034)	**.375**(.065)	**.557**(.020)	**.433**(.027)	**.475(.016)**	**.449**(.028)	**.584**(.017)	**.448**(.028)	**.415**(.023)	**.477**(.067)



Inoc: Initial inoculum; 0.1-0.2-0.4: shear force (dyn cm^{-2})

Figure Legends

Figure 1. 3D reconstruction of the developed biofilm after 20 h of growth in cell-free saliva medium at fluid shears of 0.1, 0.2 and 0.4 dyn cm^{-2}.

Figure 2. Biofilm architecture (biomass and thickness) quantification of developed biofilms (average (SD): n=16 per group). Different letters indicate statistical differences among groups ($p<0.05$; ANOVA/Tukey).

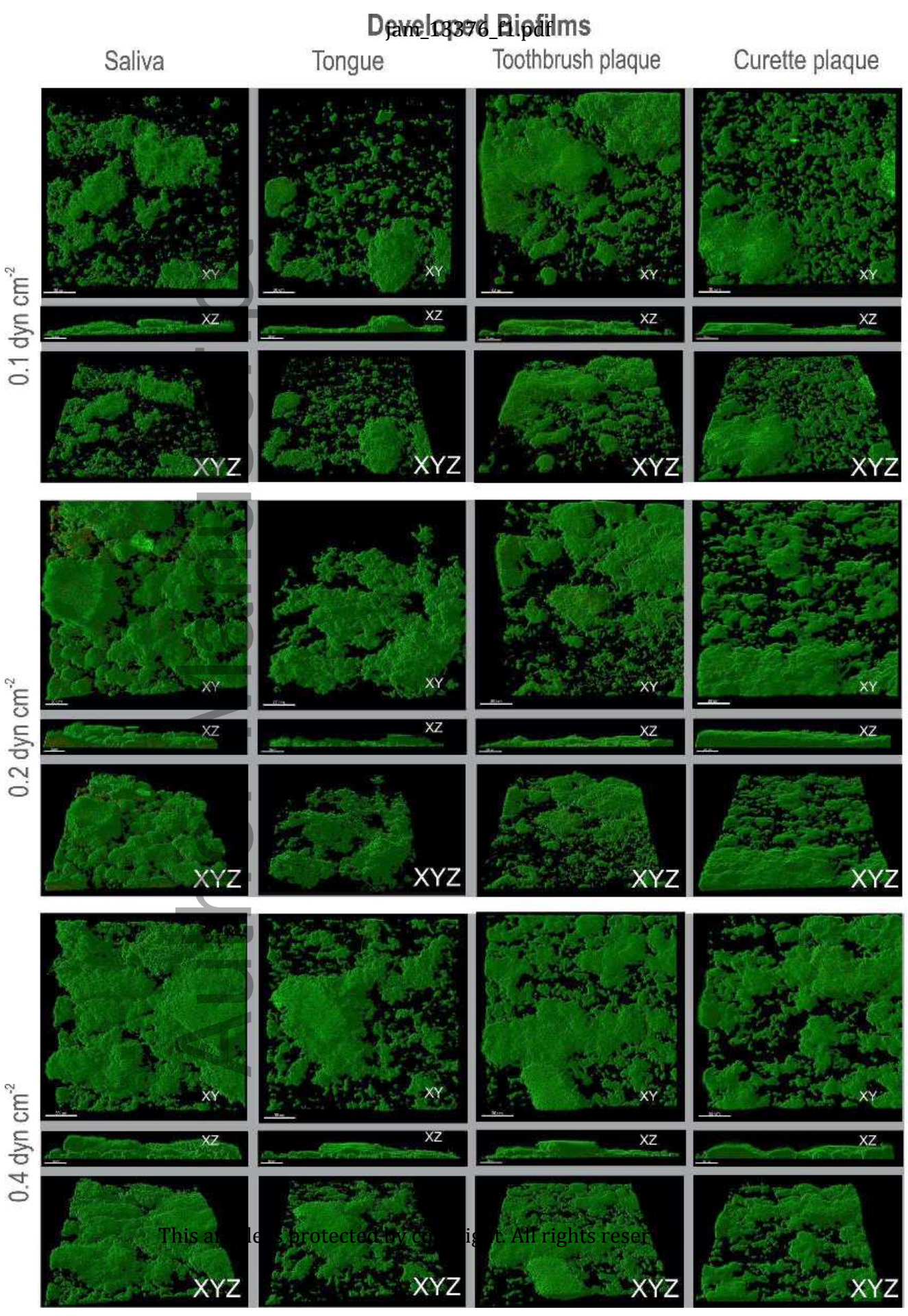
Figure 3. Relative abundance by family composition (n=3 samples per group). Each original inoculum and the respective developed-biofilms grown at fluid shears of 0.1, 0.2 or 0.4 dyn cm^{-2} are represented by inoculum type.

Figure 4. Box plot of Shannon-Weaver Index values (n=3 samples per group) to characterize the Alpha Diversity of the original inoculum and the respective developed-biofilms. Developed biofilm are separated by level of shear force used during its development (0.1, 0.2 or 0.4 dyn cm^{-2}). Different lower case letters indicate statistical differences among groups ($p<0.05$; n=3; Kruskal Wallis/Man-Whitney tests).

Figure 5. Principal coordinate analysis (PCoA) plot is based on community variation using Unifrac distance. The figure represents original inocula (A) [blue (curette-plaque), purple (toothbrush-plaque), red (saliva) and green (tongue)] and the respective developed biofilms (B) grown at three different fluid shear force levels. Color-scale of blue (curette-plaque), purple (toothbrush-plaque), red (saliva) and green (tongue) represent the developed biofilms grown at 0.1, 0.2 or 0.4 dyn cm^{-2}. Circle lines represent clustering by inoculum type.

Table S1. Quantification of biofilm architecture of developed biofilms (**average** (SD): n=16 per group).

Figure S1. Relative abundance by genus composition (n=3 samples per group). Each original inoculum and the respective developed-biofilms grown at fluid shears of 0.1, 0.2 or 0.4 dyn cm^{-2} are represented by inoculum type.



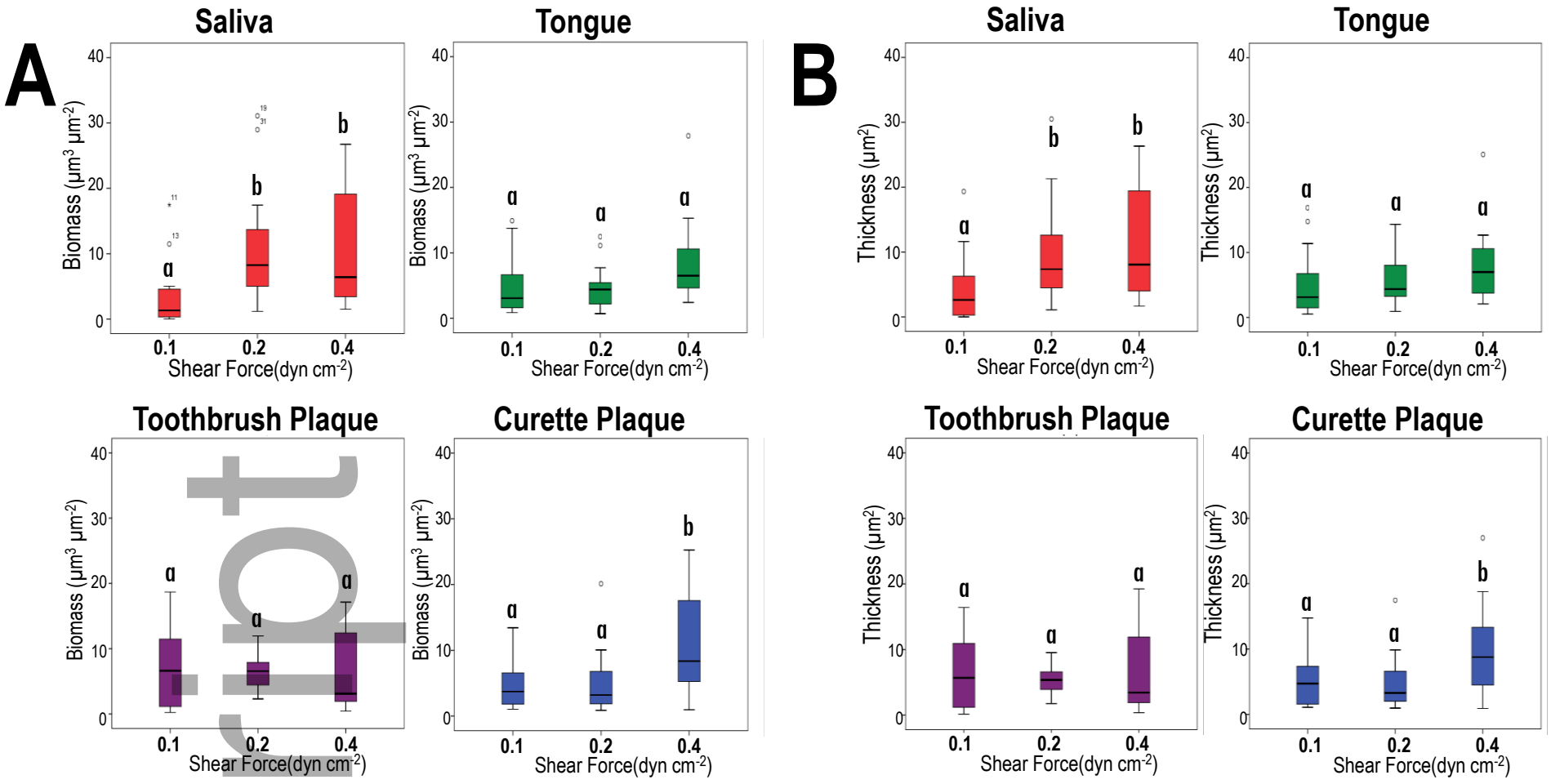


Fig 2

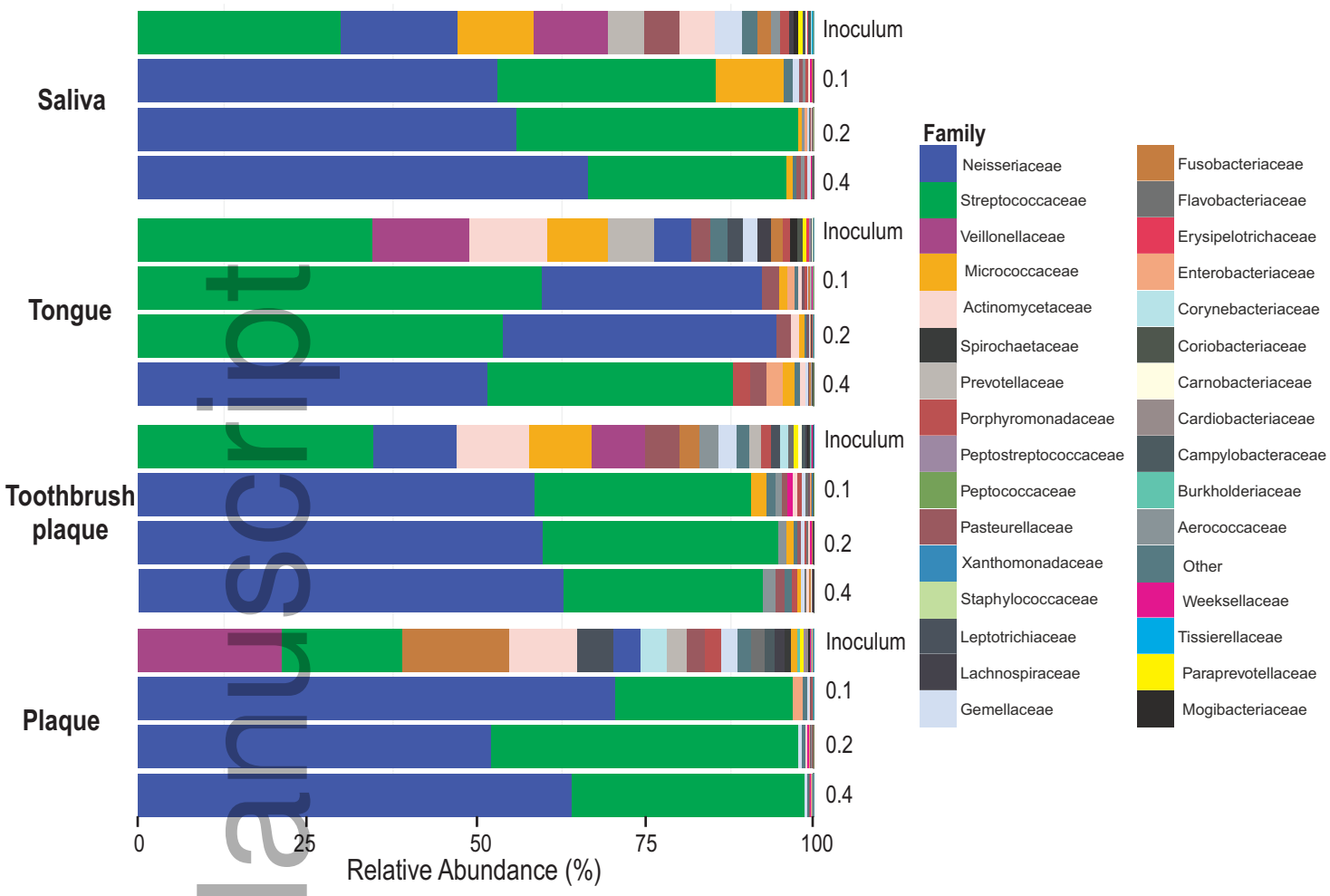


Fig 3

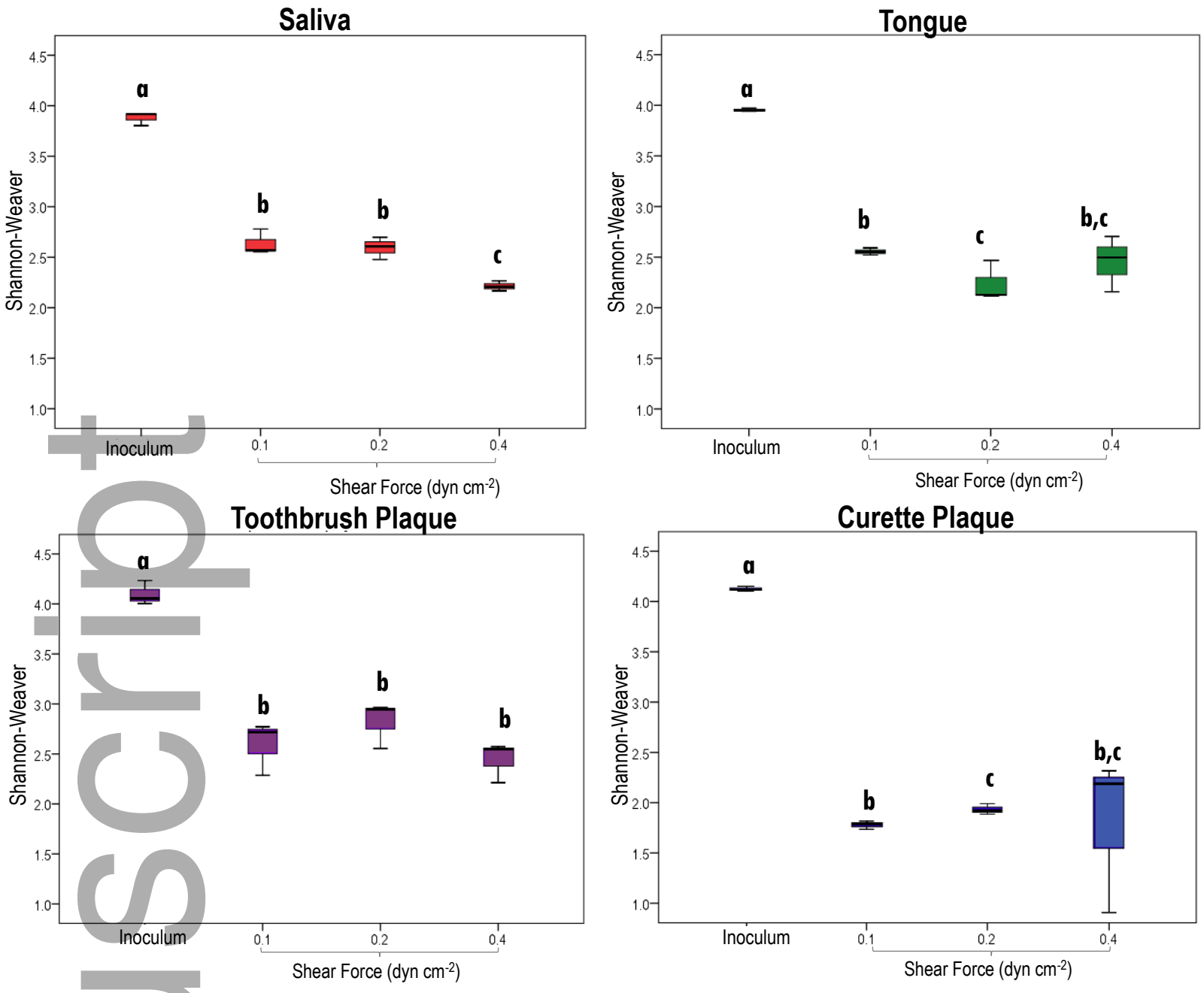


Fig 4

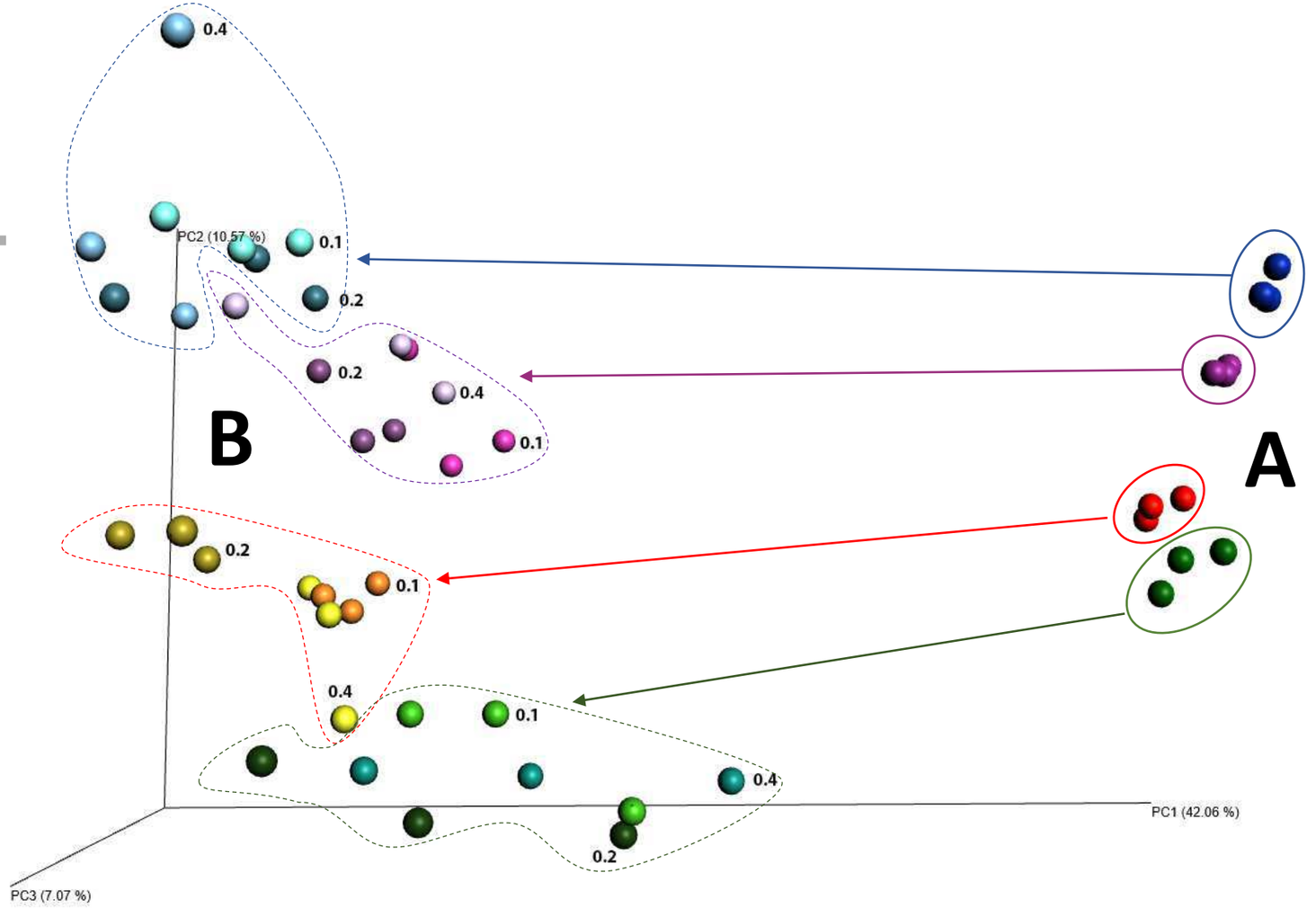


Fig 5