The human salivary microbiome exhibits temporal stability in bacterial diversity

Short Title: Temporal Variability of the Salivary Microbiome

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Abstract (197 Words)

The temporal variability of the human microbiome may be an important factor in determining its relationship with health and disease. In this study, the saliva of 40 participants was collected every two months over a one year period to determine the temporal variability of the human salivary microbiome. Salivary pH and 16S rRNA gene copy number was measured for all participants, with the microbiome of ten participants assessed through 16S rRNA amplicon sequencing. In February 2013, 16S rRNA gene copy number was significantly (P<0.001) higher, with individual changes between time points significant (P=0.003). Salivary pH levels were significantly (P<0.001) higher in December 2012 than in October 2012 and February 2013, with significant (P<0.001) individual variations seen throughout. Bacterial α-diversity showed significant differences between participants (P<0.001), but not sampling periods (P=0.801), and a significant positive correlation with salivary pH (R^2=7.8%; P=0.019). At the phylum level, significant differences were evident between participants in the Actinobacteria (P<0.001), Bacteroidetes (P<0.001), Firmicutes (P=0.008), Fusobacteria (P<0.001), Proteobacteria (P<0.001), Synergistetes (P<0.001), and Spirochaetes (P=0.003) phyla. This study charted the temporal variability of the salivary microbiome, suggesting that bacterial diversity is stable, but that 16S rRNA gene copy number may be subject to seasonal flux.

Introduction

The role that the human microbiome plays in health and disease has become a major area of interest, and has revealed a number of novel links to disease (Cho & Blaser, 2012). The human microbiome is closely linked to the physiological state of the host, and the state of the immune system in particular can have substantial effects on its structure and function. Understanding the temporal variability of the human microbiome may give novel insights into the pathways leading to microbiome-related conditions (Grice *et al.*, 2009).

The human oral cavity consists of a number of well-defined areas (tongue dorsum, lateral sides of tongue, buccal epithelium, hard palate, soft palate, supragingival plaque of tooth surfaces, subgingival plaque, maxillary anterior vestibule, and tonsils), which have been shown to have distinct microbiomes (Aas *et al.*, 2005). Culture-independent study of the human oral microbiome has identified over 600 bacterial species which are prevalent, with distinct bacterial populations present at different spatial regions (Dewhirst *et al.*, 2010). Other studies have shown the microbiome to be an important component of some oral diseases, such as periodontal disease (Dahan *et al.*, 2004; Liu *et al.*, 2012; Schwarzberg *et al.*, 2014) and dental caries (Yang *et al.*, 2012; Scannapieco, 2013). Interestingly, the oral microbiome has also been related to systemic diseases, including cardiovascular disease (Seymour *et al.*, 2007), ischemic stroke (Joshipura *et al.*, 2002), and diabetes (Genco *et al.*, 2005).

Due to the ease of sampling, saliva has been one of the most widely studied oral features in humans. However, the microbiome found within human saliva is distinct from the microbiomes of other oral structures, such as the tongue, tonsils, throat, and gingiva. Using culture-independent sequencing the microbiome of saliva is dominated at the phylum level by the Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria whilst resolving down to the genus indicated , that

Streptococcus, *Veillonella*, *Prevotella*, *Neisseria*, and *Fusobacterium* genera accounted for the majority of the microbiome (Segata *et al.*, 2012).

The variation in saliva microbiomes in ten saliva samples obtained from each of the twelve sampling locations around the world was assessed but it was not possible to link microbial diversity to geographical origins (Nasidze *et al.*, 2009). The primary observation of this study was that there was a high degree of differences between individuals within populations; estimated at approximately 13.5%. Interestingly, this is also similar to the total variance in neutral genetic markers within the human population; suggesting that the composition of the oral microbiome is largely determined by non-genetic factors, such as environmental features. In line with this, a longitudinal study of the salivary microbiome of monozygotic and dizygotic twins suggested that age and the environment has a higher impact on the composition of the oral microbiome than the host's genetic make-up (Stahringer *et al.*, 2012).

The regulation of the human body in response to, or in anticipation of, changing environmental conditions is an evolutionary advantage; allowing for physiological and behavioural changes to occur. Seasonal alterations in physiological and behavioural responses including weight and reproductive changes, are well established in mammals and linked to the effects of melatonin (Barrett & Bolborea, 2012). Melatonin has also been shown to be responsible for seasonal changes in the human immune system, namely cytokine production, neutrophil activity, and the differentiation and proliferation of lymphocytes (Klink *et al.*, 2012). There are also seasonal trends in upper respiratory illnesses, particularly those related to viral infections (Linder *et al.*, 2013), which have been associated with increased bacterial loads (Chappell *et al.*, 2013). Taking these data together it may be that the salivary microbiome will also show seasonal variability which may reflect host physiology, immunological status and biochemistry.

To investigate this possibility, we sampled 40 participants over a one year period, collecting saliva samples every two months. For all participants, we measured salivary pH and used quantitative PCR to determine salivary 16S rRNA gene copy number. The microbiome was assessed in sub-group of ten participants, whom were selected based on their lifestyle similarities, through amplicon sequencing of the V3 to V4 region of the 16S rRNA gene. These analyses suggest a seasonal change in 16S rRNA gene copy number in late winter, with no stage of the year exhibiting a change in salivary bacterial diversity.

Materials and Methods

Ethics Statement

This study received ethical approval from the Aberystwyth University Research Ethics Committee. Written informed consent was obtained from all participants at least 24 hours before the first sample was donated and additional consent forms were obtained before each subsequent sample was donated. All participant information obtained was link anonymised prior to subsequent data analysis.

Participant Recruitment and Sampling

Saliva samples were obtained from 40 participants consisting of staff and students at Aberystwyth University, over a one year period, from October 2012 to October 2013. During this period, a total of seven samples were collected every two months, each over a twelve day period, i.e. October 2012 (10/09/2012 to 21/09/2012), December 2012 (10/12/2012 to 21/12/2012), February 2013 (11/02/2013 to 22/02/2013), April 2013 (08/04/2013 to 19/04/2013), June 2013 (10/06/2014 to 21/06/2014), August 2013 (12/08/2013 to 23/08/2013), and October 2013 (14/10/2013 to 25/10/2013). Participants donated 5 mL of saliva into a sterile 50 mL centrifuge tube and stimulated additional saliva if necessary. All participant donations were completed in one time point. Participants were not restricted in eating or drinking prior to donating a saliva sample. At each sampling, information on oral hygiene practice, antibiotic use, smoking history and diet was collected.

Sample Processing and DNA Extraction

All saliva samples were checked to ensure a 5 mL volume of sample was present. Any excess saliva above 5 mL was removed. Samples then underwent centrifugation at 10,000 x g for 20 minutes at 4°C, after which 2 mL of the saliva supernatant was transferred to a PCR grade microcentrifuge tube. The remaining saliva supernatant was removed and destroyed, and the saliva pellet transferred to a

PCR grade microcentrifuge tube. The pellet was stored at -80°C until DNA extraction was completed within seven days of sample collection. All salivary supernatant samples were stored at -80°C until all sampling time points had been completed. Genomic DNA was extracted from 200 µL of the saliva pellet using a FastDNA SPIN kit for soil (MP Biomedical, Santa Ana, USA) following manufacturer's instructions. Bead beating was carried out in a FastPrep-24 machine (MP Biomedical) with three cycles at speed setting 6.0 for 30 sec, with cooling on ice for 60 sec between cycles. Genomic DNA was eluted with 50 µL of DES (DNase/Pyrogen-Free Water) and dsDNA concentration determined, in duplicate, using 2 µL on the Epoch spectrometer system (BioTek, UK).

16S rRNA Quantitative PCR

To calculate the 16S rRNA gene copy number within salivary DNA extracts, standards with known 16S rRNA gene copy numbers were created through amplification of the entire 16S rRNA gene of five randomly selected October 2012 samples. Creation of standards was completed as previously described by Jones *et al.*, (2014). In brief, PCR reactions were completed in a 20 µL reaction volume consisting of 10 µL of 2 x BioMix (BioLine), 0.25 µL each of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1389r (5'-ACG GGC GGT GTG TAC AAG-3') primers (Hongoh *et al.*, 2003) to give a final concentration of 500 nM, 1 µL of neat extracted DNA and 9.5 µL of PCR Grade Water (Roche). PCR consisted of 94 °C for 2 min, 30 cycles of 94 °C for 45 sec, 55 °C for 45 sec, and 72°C for 90 sec, followed by a final elongation step of 72 °C for 7 min. The resulting PCR products were combined and purified using an Isolate II PCR and Gel Extraction purification kit (BioLine, UK), following manufacturer's instructions and quantified with an Epoch spectrometer. After determination of gene copy number, serial dilutions of 10^{10}, 10^{8}, 10^{6}, 10^{4}, 10^{2}, and 10^{0} were made and used in subsequent quantitative PCR reactions.

Quantitative PCR was completed on neat extracted DNA with each reaction completed in 25 µL volumes, each consisting of 12.5 µL 2 x SYBR Green Mastermix (Life Technologies), 0.25 µL of each

EUBF1 (5'-GTG STG CAY GGY TGT CGT CA-3') and EUBR1 (5'-ACG TCR TCC MCA CCT TCC TC-3') primers (Maeda *et al.*, 2003), in a final concentration of 400 nM, 9 µL of PCR Grade Water (Roche) and 3 µL of neat DNA extract. Reactions were run using a C100 thermal cycler (BioRad, Hercules, USA) and CFX96 optical detector (BioRad), with data captured using CFX Manager software (BioRad), under conditions of 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec followed by a melt curve consisting of a temperature gradient of 60 °C to 95°C in 0.5°C increments, each for five seconds. The CFX Manager software created a standard curve of Cq values for each of the six standards with known 16S rRNA gene copy number and used this to calculate the estimated 16S rRNA gene copy number for each of the salivary DNA extracts with an unknown concentration based on the Cq value of each individual sample.

Selection of Participants for 16S rRNA Amplicon Sequencing

Of the 40 recruited participants in this study, a subgroup of ten was selected for 16S rRNA amplicon sequencing of all seven monthly samples collected. This subgroup was selected based on supporting information given at each bi-monthly sample, with a view to selecting a group of participants with minimal differences. Participants were selected based on oral hygiene practices (no history of mouthwash but a history of flossing at least weekly), smoking history (no current smokers and past smokers with a cessation period greater than ten years), allergen history (no asthma or hay fever), diet (only individuals with a meat and vegetable diet), antibiotic exposure (no antibiotic use within sampling period and six months prior to start) but with no restriction on age or gender.

16S rRNA Amplicon Preparation

Sequencing of the 16S rRNA gene was carried out via amplification of the V3 to V4 region and subsequent amplicon sequencing on the Illumina MiSeq platform. Firstly, the V3 to V4 region of the 16S rRNA gene was amplified through duplicate PCR with locus specific primers, alongside negative water controls. In a 25 µL reaction volume, 12.5 ng of extracted DNA or 2.5 µL of PCR grade water for

negative controls, was added to 12.5 µL of 2 x Accuzyme Mix (BioLine) and 5 µL each of a 1 µM concentration of 319f primer (5'– CCT ACG GGN GGC WGC AG–3') with Illumina forward overhang adapter sequence (5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3') and 806r primer (5'– GAC TAC HVG GGT ATC TAA TCC–3') with Illumina reverse overhang adapter sequence (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3') as detailed by Klindworth *et al.*, (2013). PCR consisted of 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72°C for 30 sec with a final elongation step (72 °C, 5 min). Each duplicate PCR volume was confirmed through visualisation on a 2% agarose gel. Subsequently, reaction volumes were combined and purified using an Isolate II PCR and Gel Extraction kit (BioLine) eluting into 20 µL of kit buffer. Following purification, a second PCR was completed to attach Illumina adaptors to amplified products to allow multiplexed amplicon sequencing on the Illumina MiSeq platform. To a final reaction volume of 25 µL, 2.5 µL of purified PCR product was added to 12.5 µL of 2 x Accuzyme Mix (BioLine), 5 µL of PCR Grade Water (Roche) and 2.5 µL each of the relevant Nextera XT Index Primer 1 (N7##) and Nextera XT Index Primer 2 (S5##) (Illumina, USA) as detailed in Supplementary Information Table S1. The reaction mix underwent a limited cycle PCR consisting of 95 °C for 3 min, eight cycles of 95 °C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec with a final elongation step (72 °C, 5 min). To remove non-combined adaptors, the entire reaction volume was fractionated on a 2% agarose gel. The gel was visualised using a DR195M Transilluminator (Clare Chemical Research, Colorado, USA) and each PCR product excised using a sterile scalpel blade. PCR products were purified using an Isolate II PCR and Gel Extraction kit (BioLine) with elution into 20 µL of kit buffer and quantified using a Quant-iT dsDNA High Sensitivity assay kit and a Qubit fluorometer (Life Technologies, UK).

16S rRNA Amplicon Sequencing and Analysis

Individual sample libraries were pooled together in equimolar concentration and sequenced on the Illumina MiSeq platform using MiSeq v3 reagents for a 2 x 300 bp run at the IBERS Translational Genomics Facility, Aberystwyth University, UK. As a control for low diversity sequences, 20% PhiX

DNA was also sequenced. Sample reads were demultiplexed and trimmed for quality, with overlapping reads merged using FLASH (Magoč & Salzberg, 2011). Merged reads were analysed using the MG-RAST metagenomics analysis pipeline (Meyer *et al.*, 2008). Taxonomic alignments of sequences was completed using 'Best Hit Classification' facility within MG-RAST against the Ribosomal Database Project (Cole *et al.*, 2009) facility, with only those sequences with a minimum alignment identity of 97%, maximum e-value of 1×10^{-5}, and a minimum alignment cut-off of 15 being used. Sequences were exported from MG-RAST into Microsoft Excel 2010 where sequence numbers for each sample were normalised as a percentage composition of the total volume of sequences for each taxonomic level of classification for that sample. All sequence files are available under the MG-RAST project ID 11549: 'Charting Temporal Variability in the Salivary Microbiome'. Raw sequence reads are available at the European Nucleotide Archive under primary accession number PRJEB9010 and secondary accession number ERP010064.

pH Measurements of Saliva

Measurements of the pH of saliva supernatant was carried out using a B-212 Twin pH Meter (Horiba, Kyoto, Japan) after two point calibration using pH 7 and pH 4 buffers. For pH measurements, 200 µL of saliva supernatant was used. After each reading, the sensor was washed with ultrapure water and blotted dry.

Data and Statistical Analysis

Arithmetic means and standard deviations were calculated, and data figures created in Microsoft Excel 2010. Additional analyses, including one-way analysis of variances and regression analyses were completed in the MINITAB 14 package. Multivariate analysis, including principal component analysis, was completed using the MetaboAnalyst platform (Xia *et al.*, 2012). Where shown, *P* values indicate the significance of one-way ANOVA tests unless otherwise stated. In some figures, significance thresholds are indicated using the standard format of *** = $P < 0.001$, ** = $P < 0.01$, and * = $P < 0.05$.

Results

Participant Recruitment and Collection

Saliva was collected from 40 participants over one year, with sampling occurring over a two week period every two months, from October 2012 to October 2013. Participant information for the complete sample group is detailed in Table 1, alongside the characteristics of the sub-group of ten participants selected for 16S rRNA amplicon sequencing based on their lifestyle similarities. Full participant information is detailed in Supplementary Information Table S2.

16S rRNA Bacterial Gene Concentrations

Mean 16S rRNA gene copy numbers for all 40 participants measured through qPCR are given in Figure 1a, with average individual changes from one time point to the next, and from October 2012 to October 2013, Figure 1b. One-way ANOVA showed that the February 2013 time point had a significantly ($P < 0.001$) higher 16S rRNA gene copy number than all other time points. Considering differences in 16S rRNA gene copy number between consecutive time periods, significant ($P < 0.001$) changes can be seen with February 2013 to April 2013 and June 2013 to August 2013 changes showing a net decrease.

16S rRNA Bacterial Diversity

To investigate if 16S rRNA gene copy number changes reflected changes in microbiome diversity, amplicon sequencing of the 16S rRNA gene was completed. Amplicon sequencing statistics are detailed in Supplementary Information Table S3 and showed no significant differences in total sequence base pairs by participant ($P = 0.268$), or month ($P = 0.537$), or total sequence number by participant ($P = 0.247$) or month ($P = 0.542$). However, sequence lengths by participant were significantly different ($P < 0.001$) with a range of approximately 15 bp. However, no such differences

were seen in sequence length by month (P = 0.101). The GC content of sequences was also significantly different by participants (P < 0.001), but not by month (P = 0.896).

Modelling using PCA shows that significant separation is not completely possible between participants, although a number of participants are clearly significantly different from a large number of samples from other participants (Figure 2a). No significant separation was evident between sampling month (Figure 2b).

Analysis of species diversity within a sample at each time point was calculated using the MG-RAST online platform. Averages of α-diversity are given in Figure 3 by (a) participant and (b) month. Significant differences were seen between participants (P < 0.001) but not between sampling months (P = 0.801).

From PCA modelling and α-diversity values, it is evident that the variation between participants is substantially, and significantly, greater than that seen between sampling time points. This suggested relative temporal stability in taxonomic diversity within the salivary microbiome. Although large-scale differences are not seen within the taxonomic diversity of the salivary microbiome, micro-level changes, at the genus level could be present.

To investigate this possibility, one-way ANOVAs were completed to identify genera that may be significantly altered in their abundance over the sampling time course. The genera *Rhodococcus* (P = 0.006) and *Variovorax* (P < 0.050) were shown to have significantly different abundances over the time course of sampling. However, both of these genera were very low in abundance and were present in less than 50% of all samples and indeed, *Variovorax* was only present in two samples. Therefore, it is likely that these significance values were statistical artefacts of the genera's low abundances.

Focusing on significant individual differences in the taxonomic composition of the salivary microbiome, difference at the phylum level were initially established. The Actinobacteria ($P < 0.001$), Bacteroidetes ($P < 0.001$), Firmicutes ($P = 0.008$), Fusobacteria ($P < 0.001$), Proteobacteria ($P < 0.001$), Synergistetes ($P < 0.001$), and Spirochaetes ($P = 0.003$) were shown to be significantly different between participants (Figure 4). Although the number of unclassified sequences, with a suspected bacterial origin, contributed a substantial proportion of the total bacterial reads (up to 50% of reads in some samples), Firmicutes was the largest of the phyla.

Assessment of Salivary pH

The pH of any environment can be an important factor in the ability of microorganisms to inhabit and grow and could influence microbiome community composition. As with 16S rRNA gene copy number, the pH of saliva samples was measured at each time point, and the time point averages (Figure 5a) and average individual time point differences (Figure 5b) were calculated. Salivary pH was shown to be significantly ($P = 0.003$) higher in December 2012 compared to October 2012 and February 2013. Although over the one year period there was no net overall change, there were significant ($P < 0.001$) changes from one point to the next (Figure 5b).

When attempting to correlate pH changes with other variables measured in this study, it was shown to have no significant ($P = 0.219$) relationship with 16S rRNA gene copy number. However, salivary pH levels were shown to have a small but significant positive correlation with α-diversity values ($R^2 = 7.8\%$, $P = 0.019$).

Discussion

The human microbiome may have an important role in health; with dysbiosis of the human microbiome linked to a number of diseases (The Human Microbiome Consortium, 2012). In further understanding its role, its temporal variability needs to be definitively established. We have previously suggested that the 16S rRNA gene copy number of human saliva may be an *in vivo* marker of immunity because previous work has shown an increase in this measurement over the winter months (Jones *et al.*, 2014). However, this study focused only on the salivary microbiome of physically-active males. Here, we found that the highest level of salivary 16S rRNA gene copy number were observed when sampling around February. Others have suggested that there may be a link between salivary bacterial load and *de novo* plaque formation (Dahan *et al.*, 2004) although this has been disputed by others (Rowshani *et al.*, 2004). Salivary bacterial load has also been suggested not to be associated with common dental conditions such as gingivitis and periodontal disease (Mantilla Gomez *et al.*, 2001). However, these studies relied on the use of culture-dependent techniques such as counting of colony forming units. Thus, it may be that there is no link between the bacterial load of cultureable bacteria and common dental diseases but a link with difficult-to-culture bacteria cannot be dismissed. It may be possible that the use of culture-independent techniques, such as quantitative PCR, may detect associations between salivary bacterial load and dental disease.

In this current study, no relationships were seen between salivary 16S rRNA gene copy number and salivary pH level or α-diversity of the salivary microbiome. This suggests that the key variable(s) associated with the change were not measured in this study. Such variables could be markers for human immunological status such as immunoglobulin factors. However, when markers for the human immune system were measured by Jones *et al*., (2014) no association with 16S rRNA gene copy number was observed (Jones *et al.*, 2014).

An early work which examined temporal and spatial differences in the human microbiome when sampled from several body sites found that spatial differences were more significantly than temporal differences. However, samples were only collected over a small time period with the first and last collection separated by four months (Costello *et al.*, 2009). In another study, temporal variation across four body sites samples (right and left palms, gut, and tongue) was examined. This demonstrated a high degree of temporal variability so that no core temporal microbiome could be determined. This flux in bacterial populations notwithstanding, the microbiomes at each body site remained distinctive (Caporaso *et al.*, 2011).

Taken together, our analyses of the salivary microbiome indicated that participant differences were the major source of variation. Our work was also noteworthy for its length of study which, to our knowledge, appears to be unique within the published literature. The results imply that in terms of salivary microbiome composition, sampling from any time point within the year could be valid. The microbiomes of some individuals appeared to cluster more closely than others suggesting greater consistency in some study participants compared to others. In line with this, estimations of α-diversity, were also shown to be determined more by participant than by sampling time point.

The source of this individual variation appears to not have been measured as a variable of this study, but its expansion to cover a larger population could reveal a contribution of diet, climate, innate genetic variation in the human population or suggest that it reflects random buccal bacterial colonisation events in; for example, childhood. For example, Stahringer et al., (2012) found that the human salivary microbiome appears remarkably stable once in adulthood, which may be as a result of a stabilisation in diet, oral hygiene, and other lifestyle factors (Stahringer *et al.*, 2012). Over a shorter time period, namely three months, the oral cavity and other body sites displayed a high degree of temporal stability (Costello *et al.*, 2009).

When considered against the background of considerable individual-to-individual variation in salivary microbiomes it was significant that there was an increase in salivary 16S rRNA gene copy number in February 2013. This was towards the end of a winter period when individuals could be immunocompromised (Mourtzoukou & Falagas, 2007). Interestingly, no relationship between α-diversity and salivary 16S rRNA gene copy number was observed, suggesting that the increase in time point is an equal increase in all bacteria, rather than specific taxa.

At the phylum level of classification, seven phyla were seen to have significantly different abundances between participants. The large number of unclassified bacterial sequences evident in samples, with an average range of between 30% and 50%, is noteworthy. It may be possible that significant differences are indeed present within the taxonomic composition of the salivary microbiome, but that these differences exist within poorly defined taxa.

Considering possible sources for participant associated changes in α-diversity, it could be relevant that a significant correlation was observed with salivary pH, though only 7.8% of variation was explained. The positive correlation between salivary pH and bacterial diversity suggests that as saliva becomes increasingly acidic, the range of bacteria able to tolerate these conditions decreases. This could reflect differential pH sensitivities for key enzymes in a particular range of species. Salivary pH is an important determinant in bacterial colonisation and growth. Indeed, lower salivary pH levels have been linked to oral diseases, such as dental caries (Humphrey & Williamson, 2001). However, in this study we observed changes in salivary pH between 6.8 and 7.4, which is arguably a small-scale change. The extent that these changes are able to impact intracellular or periplasmic enzyme function is hitherto unknown, and it is possible that the microbiome is able to tolerate this level of change without significant impact. This was not possible to measure in this study because of its observational design, though it may be an interesting principle to establish for future work.

Through sequencing of the 16S rRNA gene in this portion of work, only the taxonomic make-up of the salivary microbiome could be established. To establish the functional capacity of the salivary microbiome, metagenomic sequencing of the entire DNA found within a sample would be required. This method of sequencing however requires substantial resources which were not available to this project. Additionally, metagenomic sequencing allows for the assignment of species or even strain-level taxonomy, and it may be that temporal variation exists within these classifications (Weinstock, 2012).

To summarise, our work on the taxonomic composition and diversity of the salivary microbiome in this portion of work appeared to be determined by individual differences, rather than temporal changes over the one year sampling period. Crucially, 16S rRNA gene copy number, which may be indicative of bacterial load, did differ at the end of the winter months and, if linked to an immunocompromised state, could lead to it being exploited clinically to indicate a patient's immunological status.

References

441 Aas JA, Paster BJ, Stokes LN, Olsen I & Dewhirst FE (2005) Defining the Normal Bacterial Flora of the
442 Oral Cavity. *J Clin Microbiol* **43**: 5721–5732.
443 Barrett P & Bolborea M (2012) Molecular Pathways Involved in Seasonal Body Weight and
444 Reproductive Responses Governed by Melatonin. *J Pineal Res* **52**: 376–388.
445 Caporaso JG, Lauber CL, Costello EK, *et al.* (2011) Moving Pictures of the Human Microbiome.
446 *Genome Biol* **12**: R50.
447 Chappell K, Brealey J, Mackay I, Bletchly C, Hugenholtz P, Sloots T, Sly P & Young P (2013) Respiratory
448 Syncytial Virus Infection is Associated with Increased Bacterial Load in the Upper Respiratory
449 Tract in Young Children. *J Med Microbiol Diagnosis* **S1**.
450 Cho I & Blaser MJ (2012) The Human Microbiome: At the Interface of Health and Disease. *Nat Rev*
451 *Genet* **13**: 260–270.
452 Cole JR, Wang Q, Cardenas E, *et al.* (2009) The Ribosomal Database Project: Improved Alignments
453 and New Tools for rRNA Analysis. *Nucleic Acids Res* **37**: D141–D145.
454 Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI & Knight R (2009) Bacterial Community
455 Variation in Human Body Habitats Across Space and Time. *Science (80-)* **326**: 1694–1697.
456 Dahan M, Timmerman MF, Van Winkelhoff AJ & Van der Velden U (2004) The Effect of Periodontal
457 Treatment on the Salivary Bacterial Load and Early Plaque Formation. *J Clin Periodontol* **31**:
458 972–977.
459 Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, Lakshmanan A & Wade WG (2010) The
460 Human Oral Microbiome. *J Bacteriol* **192**: 5002–5017.
461 Genco RJ, Grossi SG, Ho A, Nishimura F & Murayama Y (2005) A Proposed Model Linking
462 Inflammation to Obesity, Diabetes, and Periodontal Infections. *J Periodontol* **76**: 2075–2084.
463 Grice EA, Kong HH, Conlan S, *et al.* (2009) Topographical and Temporal Diversity of the Human Skin
464 Microbiome. *Science (80-)* **324**: 1190–1192.
465 Hongoh Y, Ohkuma M & Kudo T (2003) Molecular Analysis of Bacterial Microbiota in the Gut of the
466 Termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). *FEMS Microbiol Ecol* **44**: 231–242.

467 Humphrey SP & Williamson RT (2001) A Review of Saliva: Normal Composition, Flow, and Function. *J*
468 *Prosthet Dent* **85**: 162–169.
469 Jones AW, Cameron SJS, Thatcher R, Beecroft MS, Mur LAJ & Davison G (2014) Effects of Bovine
470 Colostrum Supplementation on Upper Respiratory Illness in Active Males. *Brain Behav Immun*
471 **39**: 194–203.
472 Joshipura KJ, Hung H-C, Rimm EB, Willett WC & Ascherio A (2002) Periodontal Disease, Tooth Loss,
473 and Incidence of Ischemic Stroke. *Stroke* **34**: 47–52.
474 Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M & Glöckner FO (2013) Evaluation of
475 General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-
476 Based Diversity Studies. *Nucleic Acids Res* **41**: e1.
477 Klink M, Bednarska K, Blus E, Kielbik M & Sulowska Z (2012) Seasonal Changes in Activities of Human
478 Neutrophils *in vitro*. *Inflamm Res* **61**: 11–16.
479 Linder JE, Kraft DC, Mohamed Y, Lu Z, Heil L, Tollefson S, Saville BR, Wright PF, Williams J V & Miller
480 EK (2013) Human Rhinovirus C: Age, Season, and Lower Respiratory Illness Over the Past Three
481 Decades. *J Allergy Clin Immunol* **131**: 69–77.
482 Liu B, Faller LL, Klitgord N, *et al.* (2012) Deep Sequencing of the Oral Microbiome Reveals Signatures
483 of Periodontal Disease. Highlander SK, ed. *PLoS One* **7**: e37919.
484 Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F &
485 Takashiba S (2003) Quantitative Real-Time PCR Using TaqMan and SYBR Green for
486 *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ*
487 Gene and Total Bacteria. *FEMS Immunol Med Microbiol* **39**: 81–86.
488 Magoč T & Salzberg SL (2011) FLASH: Fast Length Adjustment of Short Reads to Improve Genome
489 Assemblies. *Bioinformatics* **27**: 2957–2963.
490 Mantilla Gomez S, Danser MM, Sipos PM, Rowshani B, van der Velden U & van der Weijden GA
491 (2001) Tongue Coating and Salivary Bacterial Counts in Healthy/Gingivitis Subjects and
492 Periodontitis Patients. *J Clin Periodontol* **28**: 970–978.

493 Meyer F, Paarmann D, D'Souza M, *et al.* (2008) The Metagenomics RAST Server: A Public Resource
494 for the Automatic Phylogenetic and Functional Analysis of Metagenomes. *BMC Bioinformatics*
495 **9**: 386.
496 Mourtzoukou EG & Falagas ME (2007) Exposure to Cold and Respiratory Tract Infections. *Int J Tuberc*
497 *Lung Dis* **11**: 938–946(6).
498 Nasidze I, Li J, Quinque D, Tang K & Stoneking M (2009) Global Diversity in the Human Salivary
499 Microbiome. *Genome Res* **19**: 636–643.
500 Rowshani B, Timmerman MF & Van der Velden U (2004) Plaque Development in Relation to the
501 Periodontal Condition and Bacterial Load of the Saliva. *J Clin Periodontol* **31**: 214–218.
502 Scannapieco FA (2013) The Oral Microbiome: Its Role in Health and in Oral and Systemic Infections.
503 *Clin Microbiol Newsl* **35**: 163–169.
504 Schwarzberg K, Le R, Bharti B, *et al.* (2014) The Personal Human Oral Microbiome Obscures the
505 Effects of Treatment on Periodontal Disease. *PLoS One* **9**: e86708.
506 Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, Huttenhower C & Izard J (2012)
507 Composition of the Adult Digestive Tract Bacterial Microbiome Based on Seven Mouth Surfaces,
508 Tonsils, Throat and Stool Samples. *Genome Biol* **13**: R42.
509 Seymour GJ, Ford PJ, Cullinan MP, Leishman S & Yamazaki K (2007) Relationship Between Periodontal
510 Infections and Systemic Disease. *Clin Microbiol Infect* **13 Suppl 4**: 3–10.
511 Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, Knight R & Krauter KS (2012)
512 Nurture Trumps Nature in a Longitudinal Survey of Salivary Bacterial Communities in Twins
513 from Early Adolescence to Early Adulthood. *Genome Res* **22**: 2146–2152.
514 The Human Microbiome Consortium (2012) Structure, Function and Diversity of the Healthy Human
515 Microbiome. *Nature* **486**: 207–214.
516 Weinstock GM (2012) Genomic Approaches to Studying the Human Microbiota. *Nature* **489**: 250–
517 256.

Xia J, Mandal R, Sinelnikov I V, Broadhurst D & Wishart DS (2012) MetaboAnalyst 2.0: A Comprehensive Server for Metabolomic Data Analysis. *Nucleic Acids Res* **40**: W127–W133.

Yang F, Zeng X, Ning K, *et al.* (2012) Saliva Microbiomes Distinguish Caries-Active from Healthy Human Populations. *ISME J* **6**: 1–10.

Table 1. Lifestyle History of Whole Participant Group and Sequencing Sub-Group

Group means of whole sample group (n=40) and sequencing sub-group (n=10). Group means are shown alongside standard deviations in brackets where appropriate.

Table 1. Lifestyle History of Whole Participant Group and Sequencing Sub-Group		
Lifestyle Factor	Whole Group	Sequencing Sub-Group
Age	41.75 (13.14)	44.90 (14.86)
Gender Ratio (Male : Female)	24 : 16	7: 3
Current Smoker	4/40	0/10
Smoking Pack Years	2.19 (2.10)	0.00 (0.00)
Past Smoker	9/40	2/10
Smoking Pack Years	9.47 (8.24)	10.50 (6.36)
Cessation Period (Years)	14.80 (10.42)	27.50 (3.54)
Never Smoker	27/40	8/10
Asthma History	3/40	0/10
Hay Fever History	5/40	0/10
Mouthwash Use	19/40	0/10
Antibacterial Mouthwash Use	17/19	0/10
Manual Toothbrush Use	23/40	3/10
Electric Toothbrush Use	17/40	7/10
Flossing	26/40	10/10
Flossing Frequency (Days Per Week)	3.46 (2.39)	2.90 (2.64)
Diet including meat (1 to 3 days per Week)	9/40	4/10
Diet including meat (4 to 7 days per Week)	26/40	6/10
Vegetarian	5/40	0/10
Group means of whole sample group (n=40) and sequencing sub-group (n=10). Group means are shown alongside standard deviations in brackets where appropriate.		

Figure 1. 16S rRNA Gene Copy Number

Copy numbers of the 16S rRNA gene were measured through qPCR. Mean 16S rRNA gene copy number (a) time point show a significantly ($P < 0.001$) higher level in February 2013 than at all other time points. Additionally, average (b) individual changes from one time point to the next show a significant ($P < 0.001$) level of flux, with net decreases shown only in the February 2013 to April and June 2013 to August 2013 time point. Error bars in figures show one standard deviation around the mean. Letters indicate statistical groupings based on significance of one-way ANOVA tests.

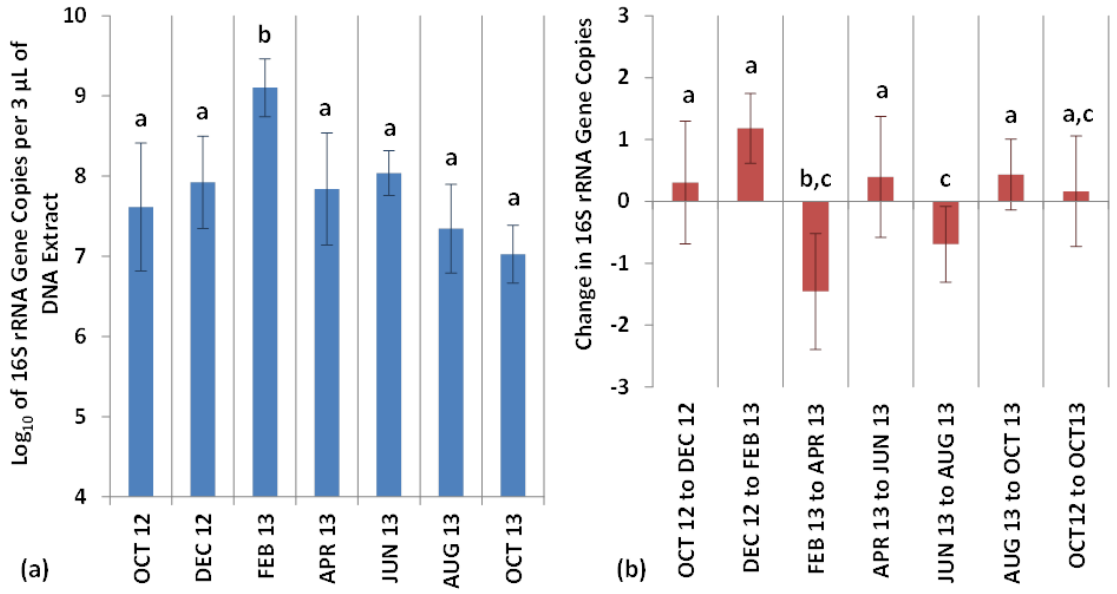

Figure 2. Principal Component Analysis of 16S rRNA Taxonomy

PCA modelling was completed using genus-level taxonomic assignments, after normalisation for sequence number, and the MetaboAnalyst analysis pipeline. Resulting plots show partial separation by (a) participant, but not by (b) sampling month. Shaded areas indicate 95% confidence intervals of significant groupings by colour.

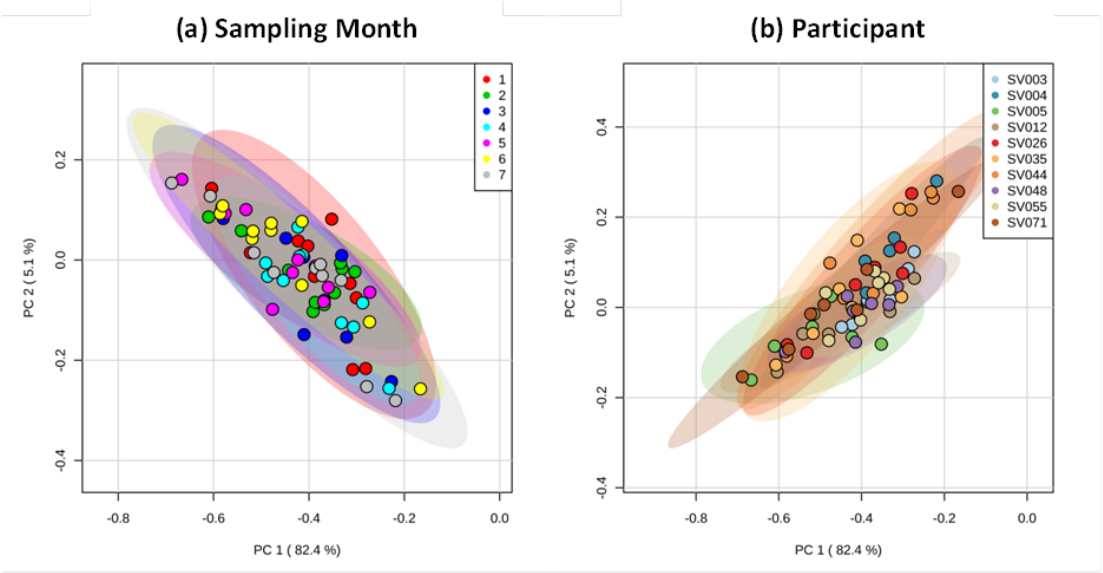

Figure 3. α-Diversity Values by Participant and Month

Species diversity within a sample at each time point was calculated using the MG-RAST online platform, with averages of α-diversity given by (a) participants, and by (b) month. Significant ($P < 0.001$) differences were observed between participants, but not between sampling months ($P = 0.801$). Errors bars display one standard deviation around the mean. Letters indicate statistical groupings based on significance of one-way ANOVA tests.

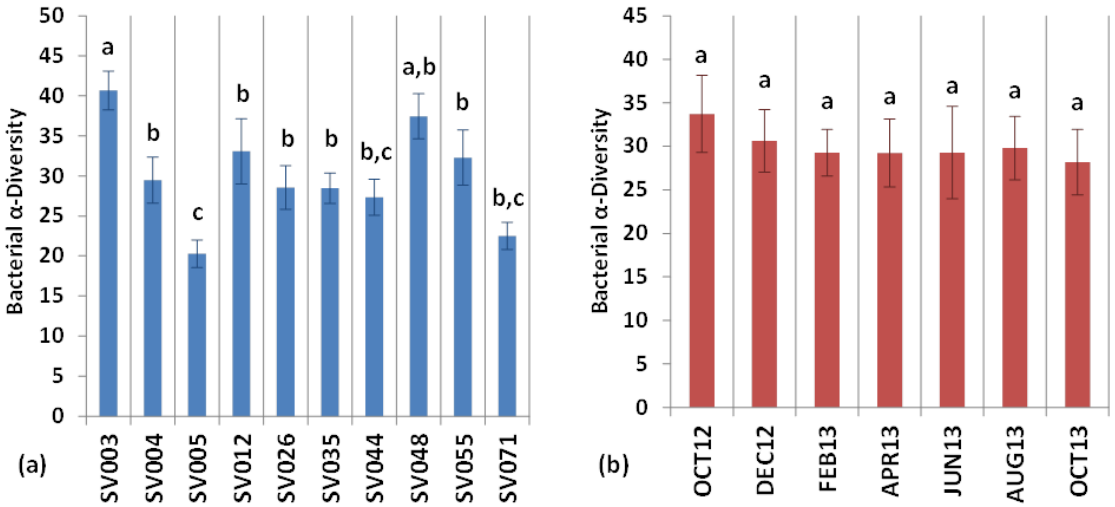

Figure 4. Average Phylum Level Taxonomy for 16S rRNA Sequencing Sub-Group

Individual differences have been shown to be more substantial in determining the taxonomic composition of the salivary microbiome than any temporal or seasonal factors. At the phylum level of classification, these individual differences are pronounced, with a number of phyla displaying significantly different abundances between participants. Significance thresholds, as determined through one-way ANOVAs, are indicated in figure legend (*** = $P < 0.001$; ** = $P < 0.01$).

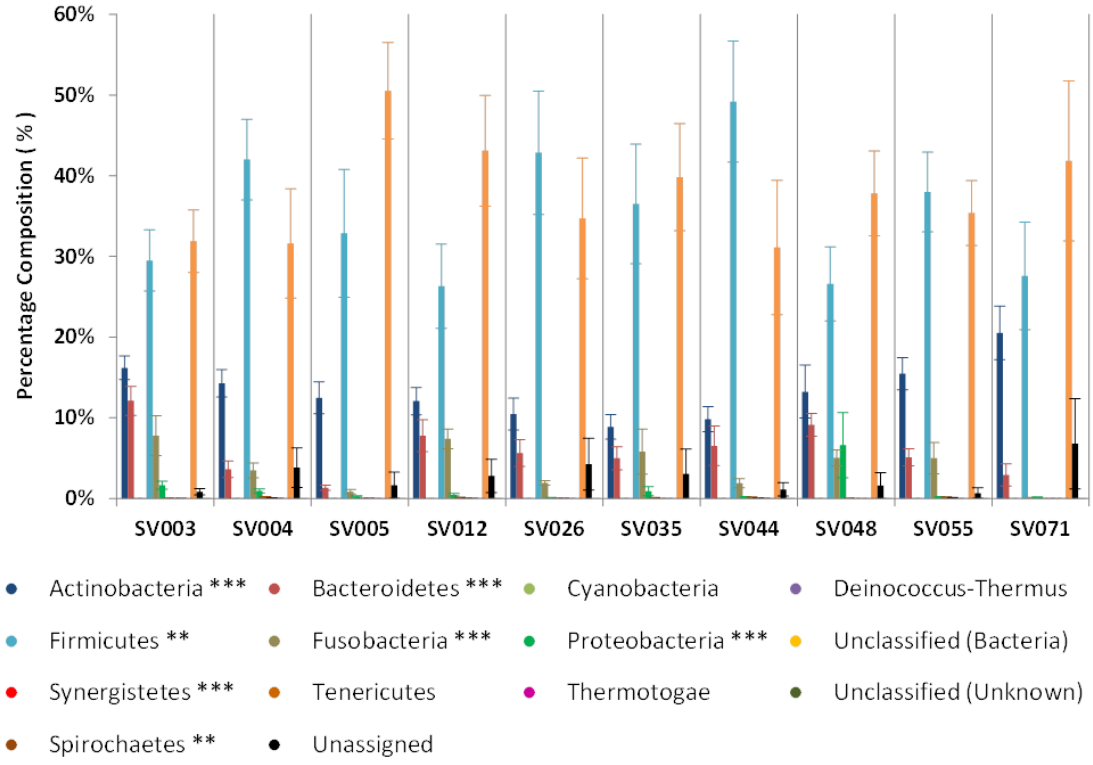

Figure 5. Salivary pH Levels

Salivary pH average for each (a) time point, and (b) individual changes between each time point, were measured. The December 2012 time point was shown to have a significantly ($P = 0.003$) higher pH than the October 2012 and February 2013 time points only. Individual differences between time points were significant ($P < 0.001$), though there was no overall net change over the entire sampling period. Error bars shown are one standard deviation around the mean. Letters indicate statistical groupings based on significance of one-way ANOVA tests.