

## Aberystwyth University

### *The human salivary microbiome exhibits temporal stability in bacterial diversity*

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1 **The human salivary microbiome exhibits temporal stability in bacterial diversity**

2

3 **Short Title:** Temporal Variability of the Salivary Microbiome

4

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13 **Key Words:** Saliva ☒ Microbiome ☒ 16S rRNA ☒ Seasonal Variability ☒ Temporal Variability

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27 **Abstract (197 Words)**

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29 The temporal variability of the human microbiome may be an important factor in determining its  
30 relationship with health and disease. In this study, the saliva of 40 participants was collected every  
31 two months over a one year period to determine the temporal variability of the human salivary  
32 microbiome. Salivary pH and 16S rRNA gene copy number was measured for all participants, with the  
33 microbiome of ten participants assessed through 16S rRNA amplicon sequencing. In February 2013,  
34 16S rRNA gene copy number was significantly ( $P<0.001$ ) higher, with individual changes between  
35 time points significant ( $P=0.003$ ). Salivary pH levels were significantly ( $P<0.001$ ) higher in December  
36 2012 than in October 2012 and February 2013, with significant ( $P<0.001$ ) individual variations seen  
37 throughout. Bacterial  $\alpha$ -diversity showed significant differences between participants ( $P<0.001$ ), but  
38 not sampling periods ( $P=0.801$ ), and a significant positive correlation with salivary pH ( $R^2=7.8\%$ ;  
39  $P=0.019$ ). At the phylum level, significant differences were evident between participants in the  
40 Actinobacteria ( $P<0.001$ ), Bacteroidetes ( $P<0.001$ ), Firmicutes ( $P=0.008$ ), Fusobacteria ( $P<0.001$ ),  
41 Proteobacteria ( $P<0.001$ ), Synergistetes ( $P<0.001$ ), and Spirochaetes ( $P=0.003$ ) phyla. This study  
42 charted the temporal variability of the salivary microbiome, suggesting that bacterial diversity is  
43 stable, but that 16S rRNA gene copy number may be subject to seasonal flux.

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53 **Introduction**

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

61

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

73

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

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

81

82 The variation in saliva microbiomes in ten saliva samples obtained from each of the twelve sampling  
83 locations around the world was assessed but it was not possible to link microbial diversity to  
84 geographical origins (Nasidze *et al.*, 2009). The primary observation of this study was that there was  
85 a high degree of differences between individuals within populations; estimated at approximately  
86 13.5%. Interestingly, this is also similar to the total variance in neutral genetic markers within the  
87 human population; suggesting that the composition of the oral microbiome is largely determined by  
88 non-genetic factors, such as environmental features. In line with this, a longitudinal study of the  
89 salivary microbiome of monozygotic and dizygotic twins suggested that age and the environment has  
90 a higher impact on the composition of the oral microbiome than the host's genetic make-up  
91 (Stahringer *et al.*, 2012).

92

93 The regulation of the human body in response to, or in anticipation of, changing environmental  
94 conditions is an evolutionary advantage; allowing for physiological and behavioural changes to occur.  
95 Seasonal alterations in physiological and behavioural responses including weight and reproductive  
96 changes, are well established in mammals and linked to the effects of melatonin (Barrett & Bolborea,  
97 2012). Melatonin has also been shown to be responsible for seasonal changes in the human immune  
98 system, namely cytokine production, neutrophil activity, and the differentiation and proliferation of  
99 lymphocytes (Klink *et al.*, 2012). There are also seasonal trends in upper respiratory illnesses,  
100 particularly those related to viral infections (Linder *et al.*, 2013), which have been associated with  
101 increased bacterial loads (Chappell *et al.*, 2013). Taking these data together it may be that the  
102 salivary microbiome will also show seasonal variability which may reflect host physiology,  
103 immunological status and biochemistry.

104

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

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131 **Materials and Methods**

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133 **Ethics Statement**

134 This study received ethical approval from the Aberystwyth University Research Ethics Committee.

135 Written informed consent was obtained from all participants at least 24 hours before the first sample

136 was donated and additional consent forms were obtained before each subsequent sample was

137 donated. All participant information obtained was link anonymised prior to subsequent data analysis.

138

139 **Participant Recruitment and Sampling**

140 Saliva samples were obtained from 40 participants consisting of staff and students at Aberystwyth

141 University, over a one year period, from October 2012 to October 2013. During this period, a total of

142 seven samples were collected every two months, each over a twelve day period, i.e. October 2012

143 (10/09/2012 to 21/09/2012), December 2012 (10/12/2012 to 21/12/2012), February 2013

144 (11/02/2013 to 22/02/2013), April 2013 (08/04/2013 to 19/04/2013), June 2013 (10/06/2014 to

145 21/06/2014), August 2013 (12/08/2013 to 23/08/2013), and October 2013 (14/10/2013 to

146 25/10/2013). Participants donated 5 mL of saliva into a sterile 50 mL centrifuge tube and stimulated

147 additional saliva if necessary. All participant donations were completed in one time point.

148 Participants were not restricted in eating or drinking prior to donating a saliva sample. At each

149 sampling, information on oral hygiene practice, antibiotic use, smoking history and diet was

150 collected.

151

152 **Sample Processing and DNA Extraction**

153 All saliva samples were checked to ensure a 5 mL volume of sample was present. Any excess saliva

154 above 5 mL was removed. Samples then underwent centrifugation at 10,000 x g for 20 minutes at

155 4°C, after which 2 mL of the saliva supernatant was transferred to a PCR grade microcentrifuge tube.

156 The remaining saliva supernatant was removed and destroyed, and the saliva pellet transferred to a

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

165

#### 166 **16S rRNA Quantitative PCR**

167 To calculate the 16S rRNA gene copy number within salivary DNA extracts, standards with known 16S  
168 rRNA gene copy numbers were created through amplification of the entire 16S rRNA gene of five  
169 randomly selected October 2012 samples. Creation of standards was completed as previously  
170 described by Jones *et al.*, (2014). In brief, PCR reactions were completed in a 20 µL reaction volume  
171 consisting of 10 µL of 2 x BioMix (BioLine), 0.25 µL each of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3')  
172 and 1389r (5'-ACG GGC GGT GTG TAC AAG-3') primers (Hongoh *et al.*, 2003) to give a final  
173 concentration of 500 nM, 1 µL of neat extracted DNA and 9.5 µL of PCR Grade Water (Roche). PCR  
174 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,  
175 followed by a final elongation step of 72 °C for 7 min. The resulting PCR products were combined and  
176 purified using an Isolate II PCR and Gel Extraction purification kit (BioLine, UK), following  
177 manufacturer's instructions and quantified with an Epoch spectrometer. After determination of gene  
178 copy number, serial dilutions of 10<sup>10</sup>, 10<sup>8</sup>, 10<sup>6</sup>, 10<sup>4</sup>, 10<sup>2</sup>, and 10<sup>0</sup> were made and used in subsequent  
179 quantitative PCR reactions.

180

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

183 EUBF1 (5'-GTG STG CAY GGY TGT CGT CA-3') and EUBR1 (5'-ACG TCR TCC MCA CCT TCC TC-3')  
184 primers (Maeda *et al.*, 2003), in a final concentration of 400 nM, 9 µL of PCR Grade Water (Roche)  
185 and 3 µL of neat DNA extract. Reactions were run using a C100 thermal cycler (BioRad, Hercules,  
186 USA) and CFX96 optical detector (BioRad), with data captured using CFX Manager software (BioRad),  
187 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  
188 melt curve consisting of a temperature gradient of 60 °C to 95°C in 0.5°C increments, each for five  
189 seconds. The CFX Manager software created a standard curve of Cq values for each of the six  
190 standards with known 16S rRNA gene copy number and used this to calculate the estimated 16S  
191 rRNA gene copy number for each of the salivary DNA extracts with an unknown concentration based  
192 on the Cq value of each individual sample.

193

#### 194 **Selection of Participants for 16S rRNA Amplicon Sequencing**

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

203

#### 204 **16S rRNA Amplicon Preparation**

205 Sequencing of the 16S rRNA gene was carried out via amplification of the V3 to V4 region and  
206 subsequent amplicon sequencing on the Illumina MiSeq platform. Firstly, the V3 to V4 region of the  
207 16S rRNA gene was amplified through duplicate PCR with locus specific primers, alongside negative  
208 water controls. In a 25 µL reaction volume, 12.5 ng of extracted DNA or 2.5 µL of PCR grade water for

209 negative controls, was added to 12.5  $\mu$ L of 2 x Accuzyme Mix (BioLine) and 5  $\mu$ L each of a 1  $\mu$ M  
210 concentration of 319f primer (5'– CCT ACG GGN GGC WGC AG–3') with Illumina forward overhang  
211 adapter sequence (5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3') and 806r primer (5'–  
212 GAC TAC HVG GGT ATC TAA TCC–3') with Illumina reverse overhang adapter sequence (5'- GTC TCG  
213 TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3') as detailed by Klindworth *et al.*, (2013). PCR  
214 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  
215 30 sec with a final elongation step (72 °C, 5 min). Each duplicate PCR volume was confirmed through  
216 visualisation on a 2% agarose gel. Subsequently, reaction volumes were combined and purified using  
217 an Isolate II PCR and Gel Extraction kit (BioLine) eluting into 20  $\mu$ L of kit buffer. Following purification,  
218 a second PCR was completed to attach Illumina adaptors to amplified products to allow multiplexed  
219 amplicon sequencing on the Illumina MiSeq platform. To a final reaction volume of 25  $\mu$ L, 2.5  $\mu$ L of  
220 purified PCR product was added to 12.5  $\mu$ L of 2 x Accuzyme Mix (BioLine), 5  $\mu$ L of PCR Grade Water  
221 (Roche) and 2.5  $\mu$ L each of the relevant Nextera XT Index Primer 1 (N7##) and Nextera XT Index  
222 Primer 2 (S5##) (Illumina, USA) as detailed in Supplementary Information Table S1. The reaction mix  
223 underwent a limited cycle PCR consisting of 95 °C for 3 min, eight cycles of 95 °C for 30 sec, 55°C for  
224 30 sec, and 72°C for 30 sec with a final elongation step (72 °C, 5 min). To remove non-combined  
225 adaptors, the entire reaction volume was fractionated on a 2% agarose gel. The gel was visualised  
226 using a DR195M Transilluminator (Clare Chemical Research, Colorado, USA) and each PCR product  
227 excised using a sterile scalpel blade. PCR products were purified using an Isolate II PCR and Gel  
228 Extraction kit (BioLine) with elution into 20  $\mu$ L of kit buffer and quantified using a Quant-iT dsDNA  
229 High Sensitivity assay kit and a Qubit fluorometer (Life Technologies, UK).

230

### 231 **16S rRNA Amplicon Sequencing and Analysis**

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

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

247

#### 248 **pH Measurements of Saliva**

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

253

#### 254 **Data and Statistical Analysis**

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

261 **Results**

262

263 **Participant Recruitment and Collection**

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

269

270 **16S rRNA Bacterial Gene Concentrations**

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

278

279 **16S rRNA Bacterial Diversity**

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

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

288

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

293

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

298

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

304

305 To investigate this possibility, one-way ANOVAs were completed to identify genera that may be  
306 significantly altered in their abundance over the sampling time course. The genera *Rhodococcus* ( $P =$   
307  $0.006$ ) and *Variovorax* ( $P < 0.050$ ) were shown to have significantly different abundances over the  
308 time course of sampling. However, both of these genera were very low in abundance and were  
309 present in less than 50% of all samples and indeed, *Variovorax* was only present in two samples.  
310 Therefore, it is likely that these significance values were statistical artefacts of the genera's low  
311 abundances.

312

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

320

### 321 **Assessment of Salivary pH**

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

329

330 When attempting to correlate pH changes with other variables measured in this study, it was shown  
331 to have no significant ( $P = 0.219$ ) relationship with 16S rRNA gene copy number. However, salivary  
332 pH levels were shown to have a small but significant positive correlation with  $\alpha$ -diversity values ( $R^2 =$   
333 7.8%,  $P = 0.019$ ).

334

335

336

337

338 **Discussion**

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

355

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

362

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

371

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

379

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

388

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

395

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

401

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

414

415 Through sequencing of the 16S rRNA gene in this portion of work, only the taxonomic make-up of the  
416 salivary microbiome could be established. To establish the functional capacity of the salivary  
417 microbiome, metagenomic sequencing of the entire DNA found within a sample would be required.  
418 This method of sequencing however requires substantial resources which were not available to this  
419 project. Additionally, metagenomic sequencing allows for the assignment of species or even strain-  
420 level taxonomy, and it may be that temporal variation exists within these classifications (Weinstock,  
421 2012).

422

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

429

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511

512

512

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

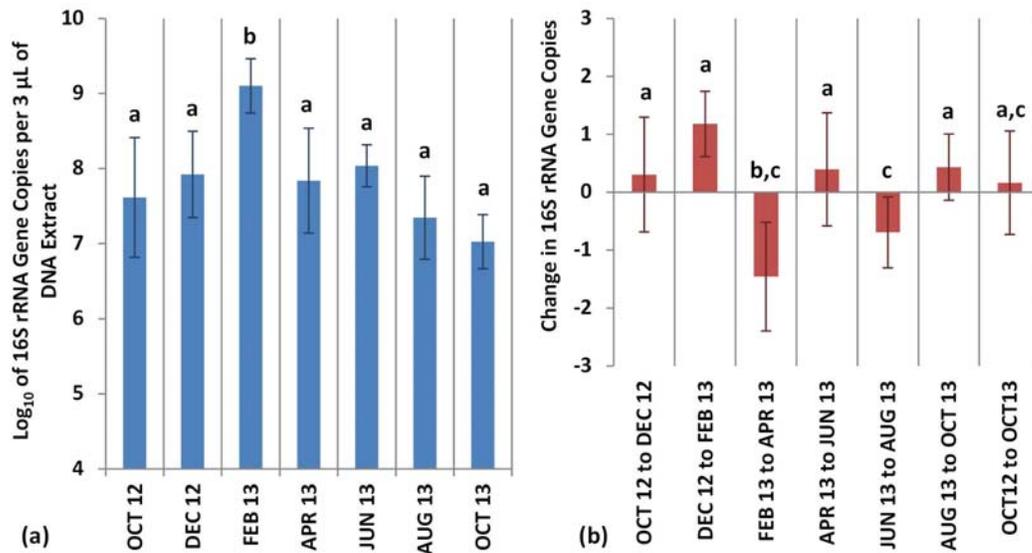
Lifestyle Factor	Whole Group	Sequencing Sub-Group
Age	41.75 (13.14)	44.90 (14.86)
Gender Ratio (Male : Female)	24 : 16	7: 3
Current Smoker	4/40	0/10
Smoking Pack Years	2.19 (2.10)	0.00 (0.00)
Past Smoker	9/40	2/10
Smoking Pack Years	9.47 (8.24)	10.50 (6.36)
Cessation Period (Years)	14.80 (10.42)	27.50 (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

514

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

517

518

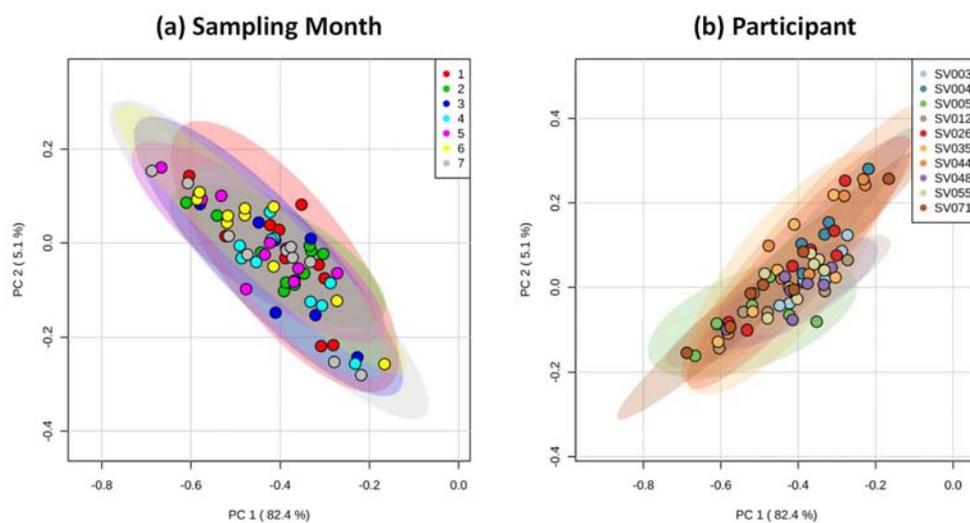


**FIGURE 1**

518

519 **Figure 1.** 16S rRNA Gene Copy Number. Copy numbers of the 16S rRNA gene were  
 520 measured through qPCR. Mean 16S rRNA gene copy number (a) time point show a  
 521 significantly ( $P < 0.001$ ) higher level in February 2013 than at all other time points.  
 522 Additionally, average (b) individual changes from one time point to the next show a  
 523 significant ( $P < 0.001$ ) level of flux, with net decreases shown only in the February 2013 to  
 524 April and June 2013 to August 2013 time point. Error bars in figures show one standard  
 525 deviation around the mean. Letters indicate statistical groupings based on significance of one-  
 526 way ANOVA tests.

527

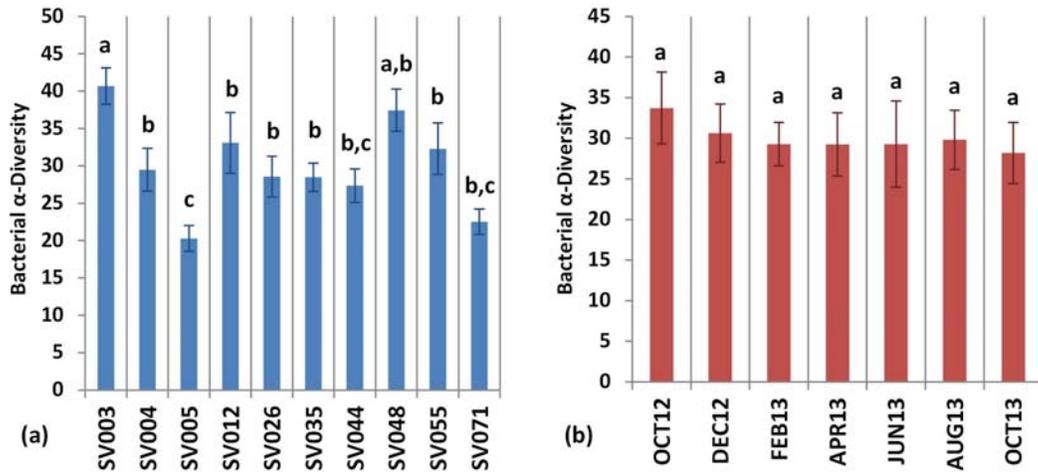


**FIGURE 2**

527

528 **Figure 2.** Principal Component Analysis of 16S rRNA Taxonomy. PCA modelling was  
 529 completed using genus-level taxonomic assignments, after normalisation for sequence  
 530 number, and the MetaboAnalyst analysis pipeline. Resulting plots show partial separation by  
 531 (a) participant, but not by (b) sampling month. Shaded areas indicate 95% confidence  
 532 intervals of significant groupings by colour.

533

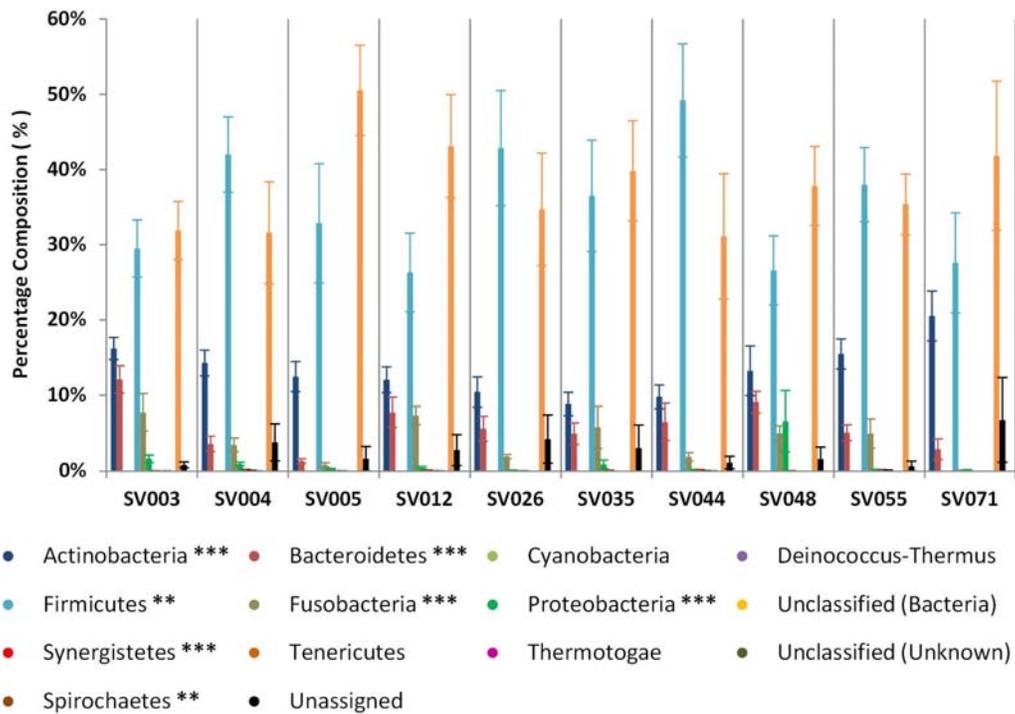


**FIGURE 3**

533

534 **Figure 3.**  $\alpha$ -Diversity Values by Participant and Month. Species diversity within a sample at  
 535 each time point was calculated using the MG-RAST online platform, with averages of  $\alpha$ -  
 536 diversity given by (a) participants, and by (b) month. Significant ( $P < 0.001$ ) differences were  
 537 observed between participants, but not between sampling months ( $P = 0.801$ ). Errors bars  
 538 display one standard deviation around the mean. Letters indicate statistical groupings based  
 539 on significance of one-way ANOVA tests.

540

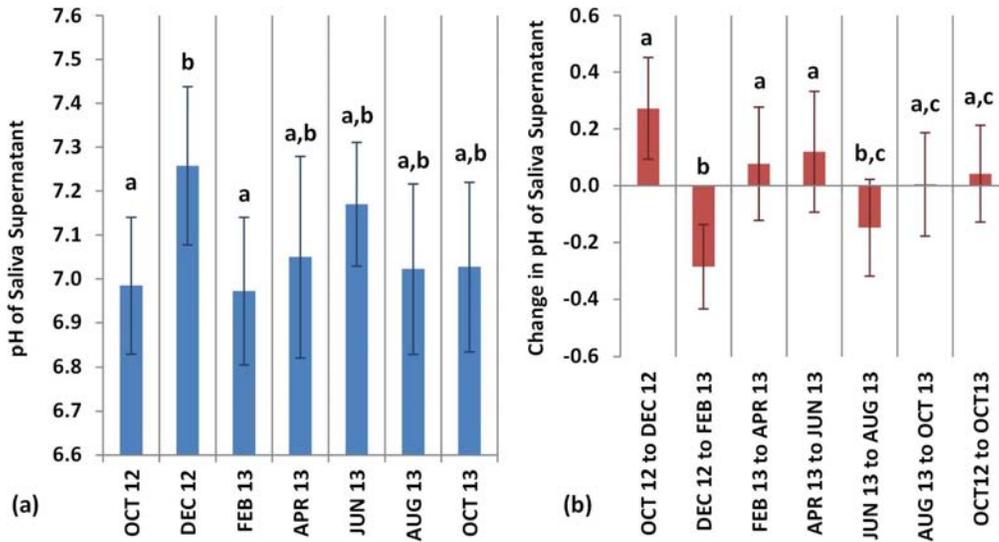


**FIGURE 4**

540

541 **Figure 4.** Average Phylum Level Taxonomy for 16S rRNA Sequencing Sub-Group.  
 542 Individual differences have been shown to be more substantial in determining the taxonomic  
 543 composition of the salivary microbiome than any temporal or seasonal factors. At the phylum  
 544 level of classification, these individual differences are pronounced, with a number of phyla  
 545 displaying significantly different abundances between participants. Significance thresholds, as  
 546 determined through one-way ANOVAs, are indicated in figure legend (\*\*\*) =  $P < 0.001$ ; \*\* =  
 547  $P < 0.01$ ).

548



**FIGURE 5**

548

549 **Figure 5.** Salivary pH Levels. Salivary pH average for each (a) time point, and (b) individual  
 550 changes between each time point, were measured. The December 2012 time point was shown  
 551 to have a significantly ( $P = 0.003$ ) higher pH than the October 2012 and February 2013 time  
 552 points only. Individual differences between time points were significant ( $P < 0.001$ ), though  
 553 there was no overall net change over the entire sampling period. Error bars shown are one  
 554 standard deviation around the mean. Letters indicate statistical groupings based on  
 555 significance of one-way ANOVA tests.

556