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4 A new technique for use in the study of the microbiome: An evaluation
5 of a three-dimensional cell culture technique in maintaining the
6 gastrointestinal microbiome of four Balb/c female mice and implications
7 for future studies

8 Maintaining the integrity of an explant microbiome and implications for
9 future studies

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11 Everest Uriel Castaneda^{1,2}, Jeff Brady¹, Janice Speshock²

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17 ¹Texas A&M AgriLife Research and Extension Center, Stephenville, Texas, United States of
18 America

19 ²Department of Biological Sciences, Tarleton State University, Stephenville, Texas, United
20 States of America

21

22 *Corresponding author

23 Email: Speshock@Tarleton.edu

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Abstract (Level 1)

25 Fluctuations in oxygen, pH, nutrients, or other factors such as food or pharmaceuticals, may
26 perturb the microbiota of the gastrointestinal (GI) tract. This environmental variation is a cause
27 for concern given dysbiosis of the microbiome is correlated with disease states; thereby, model
28 organisms are utilized to study microbial communities during, after, or before shifts in microbes
29 since intact *ex vivo* microbiomes have historically been challenging to utilize. The objective of
30 this study is to culture an explant microbiome of 4 Balb/c, laboratory bred mice to develop an *ex*
31 *vivo* tool for future microbiome studies. We cultured homogenates of the distal colon of 4 mice
32 in three dimensional, 24 well plate culture dishes. These dishes were incubated for 24 hours in
33 two different oxygen concentration levels, 0% and 20%. The pH of the plate was tested before
34 and after incubation. To analyze the integrity of the microbiome, we utilized 16S sequencing.
35 Further, we utilized 16S metagenomics to characterize fecal samples and colon samples to
36 speculate whether future studies may utilize feces in constructing an explant microbiome to spare
37 animal lives. We found that pH and familial relationship had a profound impact on community
38 structure while oxygen did not have a significant influence. The feces and the colon were similar
39 in community profiles, which lends credence to utilizing feces in future studies. In addition, our
40 efforts successfully cultured archaea, which included difficult to culture strains such as
41 Miscellaneous Crenarchaeota group (MCG) and Methanobacteria. Ultimately, further attempts to
42 culture and preserve an animal's microbiome needs to control for and maintain stable pH.

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Introduction (Level 1)

47 The microbiome forms a symbiotic relationship with its host [1]. Essentially, microbes have a
48 cooperative role in the GI tract and contribute to a host's immune system and metabolism [2–5].

49 Although the natural relationship between the microbiome and the host is essential,
50 overpopulation by an undesirable species, or dysbiosis, has been linked to particular diseases and
51 phenomena such as autism spectrum disorder [6], cancer [3], and obesity [7]. Historically,
52 researchers have utilized animal models such as gnotobiotic mice to study microbiome-animal
53 interactions but experimentation with such mice is expensive [8–10]. Thus, an *ex-vivo* model of
54 the microbiome that provides cost-effective, reproducible, and reliable results is highly desirable
55 in studying this dynamic [11].

56 Mouse microbiome studies have expanded our views on the impact of prokaryotes on digestion,
57 disease, and even behavior, but many microbial species cannot persist in culture [12,13];
58 therefore, most research currently relies on germ-free mice for microbiome studies, which can be
59 cost-prohibitive for many laboratories [7,8,14]. To further assess this transient mixture of
60 microbiota [15], scientists have been utilizing culture independent, next-generation sequencing to
61 inquire about shifts within the microbiome and what stimuli affect these changes in composition
62 [16]. With the decreasing cost of next-generation sequencing, an influx of research has been
63 possible in this area [17]. It is worth noting the financial and ethical burden of raising,
64 sacrificing, and housing model organisms [9,10]; therefore, it would be beneficial to develop
65 techniques to save organisms and further decrease costs.

66 In this study, we cultured and maintained the GI microbiomes of 4 laboratory-bred female Balb/c
67 mice in three-dimensional (3D) well plates, partitioned into 2 oxygen levels. Due to the variable

68 nature of the oxygen levels of the GI tract [18], we cultured 3D plates in both a conventional
69 incubator and an anaerobic chamber, both at 37 degrees Celsius. Additionally, we determined the
70 microbial composition of the mouse stool and the distal colon to observe if future studies may
71 utilize feces and avoid sacrificing organisms altogether. We utilized sequencing methods to
72 verify final proportional community composition of each sample.

73 **Materials and Methods (Level 1)**

74 **Subjects (Level 2)**

75 The study was performed under a protocol approved by the Tarleton State University
76 Institutional Animal Care and Usage Committee (Animal Use Protocol 12-009-2016-A1). 4
77 Balb/c females 8 weeks in age were utilized in this experiment. Females were housed together
78 and raised on identical chow diets and similarly weaned. Mice 1 and 2 were siblings while mice
79 3 and 4 were siblings. The siblings were born from different dams and sires. All mice were held
80 in sterile containers and euthanized with 150 µL of sodium pentobarbital delivered
81 intraperitoneally. Post injection, mice shed two to three samples of stool which were recovered
82 utilizing sterile forceps and immediately frozen. Once deceased, 2.5 cm of the large distal colon
83 were removed. After, two to three small additional 0.5 cm samples of the large distal colon were
84 excised from the specimen and immediately frozen. Colon tissue extractions were added to a
85 sterile tissue grinder along with 5 mL of Dulbecco's Modified Eagle Medium (DMEM). The
86 sample was manually homogenized into a liquid solution. Homogenate was checked for pH by
87 applying a small droplet with a mechanical pipette onto litmus paper.

88 **Culture method (Level 2)**

89 Cultures were established in sterile 24 well plates with multiwell tissue culture inserts, 8 μ m pore
90 size (Corning Incorporated, New York). Corning PuraMatrix Peptide Hydrogel was prepared
91 using 8 mL of molecular grade water and 20 ml of hydrogel to create a 0.25% solution. 150 mL
92 of the prepared solution were added to each of the 12 well inserts. In addition, 500 mL of
93 supplementary DMEM was added under each well insert. This technique is a modified version of
94 3D tissue culture repurposed for culturing our *ex-vivo* microbiome. Once the 3D culture plates
95 were prepared, 250 mL of the homogenized colon were added to each of the prepared 12 well
96 inserts. Plates were checked for baseline pH by transferring a small drop of medium with a
97 mechanical pipette onto litmus paper. The plates were then added to a single incubator, but to
98 create an anoxic environment, half of the plates were incubated in an anaerobic chamber. Plates
99 were incubated for 24 hours. Each insert's medium was then tested for pH again and transferred
100 into sterile 2.5 mL storage tubes and frozen for future DNA extraction.

101 **DNA extraction and library production (Level 2)**

102 DNA was extracted from each sample using a solid phase extraction protocol from Brady et al.
103 [19]. After extraction, DNA was amplified utilizing prokaryote-specific primers, 519F 5'-
104 CAGCMGCCGCGGTAA-3') and 785R (5'- TACNVGGTATCTAATCC-3'), that target the
105 V4 region of the 16Ss rRNA [20,21]. PCR amplification was accomplished through denaturation
106 at 95°C for 3 minutes, followed by 35 cycles of 95°C for 10 seconds, 55°C for 30 seconds, and
107 72°C for 30 seconds. Dual 6 bp DNA barcodes were added to sequencing libraries using the
108 same PCR protocol and Illumina P5 and P7 flowcell binding adapters [22]. Sequencing libraries
109 were size-selected with a Pippin Prep instrument (Sage Science, Beverly, MA) to a length of
110 300-600 base pairs. Sequencing was conducted on a MiSeq instrument using 600 cycle paired
111 end v3 sequencing kits at the Texas A&M University Genomics Core Facility. Raw sequences

112 were processed with QIIME [23] and USEARCH [24]. Taxonomy was assigned using the
113 Greengenes 13.8 database [25] as a reference with UCLUST [24], and reference-based
114 Operational Taxonomic Unit (OTU) picking was conducted at 97% sequence similarity using the
115 RDP [26] method in QIIME.

116 **Statistical analysis (Level 2)**

117 Cumulative Sum Scaling [27] was utilized to normalize the data. Biom files were constructed
118 with QIIME [28] and transferred into R [29] and Microsoft Excel for further statistical analysis.
119 Phyloseq [30], ggplot2 [31], and vegan [32] packages were utilized to evaluate alpha and beta
120 diversity with seed set at 1400. Alpha diversity was assessed using the Shannon diversity index.
121 Variation in alpha diversity for oxygen, pH, mouse, feces, and colon comparisons were first
122 checked for normality using the Shapiro-Wilk test for normality [33]. The data was non-normal
123 in distribution (Shapiro-Wilk test, $w=0.9506$, $p<0.01$); therefore, comparisons were made with
124 non-parametric tests. All multivariate tests were corrected using false discovery rate (FDR) [29].
125 Comparisons of alpha diversity were conducted using Kruskal Wallis one-way analysis of
126 variance (KW ANOVA) and Wilcoxon rank sums test (Wilcoxon test) while comparisons of beta
127 diversity were assessed with unweighted unifrac distance metrics at 1000 permutations using
128 permutational multivariate analysis of variance (PERMANOVA). Dunn's test, post-hoc analysis
129 was conducted using the dunn.test package in R [34]. In addition, non-parametric t-tests were
130 used for comparisons of mean abundance in individual bacterial strains between samples.
131 Principle coordinate analysis (PCoA) and canonical correspondence analysis (CCA) were
132 performed at the level of OTU using unweighted unifrac distance metrics. PRIMER 7 [35] was
133 used for the hypothesis testing utilizing square root transformed Bray-Curtis ordination data at
134 9999 permutations. A microbial network was constructed using the Co-occurrence Network

135 Interferences (CoNet) [36] application for Cytoscape [37]. Feces and colon data were removed
136 before CoNet analysis. CoNet has been utilized in previous studies to investigate defined
137 interactions between microbes [38–40]. Spearman correlation coefficient with a cutoff ratio of
138 0.6 was utilized, and to focus the network, only microbes with sequence counts greater or equal
139 to 20 were included. 1,000 permutations were accomplished through a bootstrapping method
140 with an FDR correction [39].

141 **Results (Level 1)**

142 **pH and oxygen (Level 2)**

143 pH readings of each plate were taken before and after incubation. As shown in Table 1, pH
144 fluctuated from the original homogenate and baseline (before 24-hour incubation). In addition,
145 mouse samples maintained varying levels of pH which correlated with differences in oxygen
146 concentration (Table 1).

147 **Table 1. Sample size, pH, and oxygen level.**

Mouse	Homogenate pH	Sample Size	Oxygen Level	Plate Baseline pH	Plate Final pH
M1	7.5	12	20%	8	10
		11	0%	8	9
M2	7.5	11	20%	8	10
		12	0%	8	9
M3	7.5	12	20%	8	7
		11	0%	8	6
M4	7.5	12	20%	8	7
		12	0%	8	6

148

149 **16s rRNA sequencing results (Level 2)**

150 We analyzed microbiomes from the 4 mice at varying oxygen levels after culturing them *in vitro*.
151 In the 24 well plate system, we utilized 12 membrane inserts to culture the microbiomes. After
152 mice succumbed to euthanasia, colon samples were harvested to complement fecal shedding.
153 Mouse 1, 2, and 4 shed two fecal samples each while mouse 3 shed three fecal samples;
154 therefore, we had a total of 9 colon and 9 fecal samples across 4 mice. After sequence quality
155 filtering we had a total sample size of 111 samples and 3,133,666 sequences total (Table 2).

156 **Table 2. Total sample size and sequence count per mouse.**

Mouse	Sample Size	Total Sequence Count	Average Sequence Count
M1	27	234051	8669
M2	27	384215	14230
M3	29	1243981	40128
M4	28	1194017	45924

157

158 **Fecal and colon comparison (Level 2)**

159 The feces and the colon samples were characterized for microbial composition at the phylum and
160 family level (Figs 1A and 1B, respectively). Community composition was dominated by the
161 phyla Firmicutes and Bacteroidetes (Fig 1A), with means of 47% and 49%, respectively, and
162 standard deviations (SD) of 23%. In addition, the *Bacteroidales* family S24-7 was highly
163 abundant with a mean of 42% and a SD of 20% (Fig 1B). These results are consistent with recent
164 microbiome studies of mice [41–43]. Beta diversity analysis for each of the feces and colon
165 samples showed no difference in composition (Table 3), and alpha diversity analysis (Fig 1C)
166 also revealed no difference (KW ANOVA, chi-squared = 6.64, df = 7, p = 0.47). Therefore, all
167 colon samples were pooled together, and all feces samples were pooled together for a statistical
168 comparison of feces and colon. The Shannon diversity index was utilized for comparison of the

169 bulk samples (Fig 1D). Results showed no difference between the feces and colon (Wilcoxon
170 test, $p = 0.44$). In addition, beta diversity comparison showed no difference (PERMANOVA,
171 Pseudo $F = 1.06$, $p = 0.37$). Since we found that feces and colon samples are similar, we pooled
172 sequences from all feces and colon samples together into one bulk sample, named “microbiome,”
173 for diversity comparisons with cultured microbiomes.

174 **Table 3. Results of the pairwise PERMANOVA tests between feces and colon samples.**

Comparison	Pseudo F	p-score
M1 Feces vs M2 Feces	3.16	0.32
M1 Feces vs M3 Feces	2.75	0.34
M1 Feces vs M4 Feces	2.60	0.31
M2 Feces vs M3 Feces	3.15	0.33
M2 Feces vs M4 Feces	2.8	0.34
M3 Feces vs M4 Feces	3.01	0.30
M1 Colon vs M2 Colon	1.47	0.34
M1 Colon vs M3 Colon	1.46	0.34
M1 Colon vs M4 Colon	1.40	0.33
M2 Colon vs M3 Colon	1.39	0.31
M2 Colon vs M4 Colon	1.29	0.33
M3 Colon vs M4 Colon	1.41	0.33

175

176 **Fig 1. Community composition of fecal and colon samples.** (A) Relative abundance of
177 bacterial phyla. Phyla with observations less than 1% are pooled into “Other” category. The first
178 two characters represent the mouse from which the organ and stool were dissected. “S” denotes
179 the different samples acquired. (B) Relative abundance of bacterial families. Families with
180 observations less than 1% are pooled into “Other” category (C) Comparison of Shannon
181 diversity between feces and colon samples. “ns” means non-significant, $p > 0.05$. (D) Shannon
182 diversity comparison of pooled feces and colon samples.

183 **Microbiome comparison (Level 2)**

184 In the explanted microbiomes, Firmicutes and Bacteroidetes were the dominant phyla (Fig 2A).
185 Firmicutes had the highest average relative abundance, 70% (SD 28%), with Bacteroidetes
186 averaging 18% (SD 16%). Across all cultures, *Enterococcus* was highly abundant (Fig 2B)
187 having a mean of 47% (SD 32%). The Shannon diversity index was different between the

188 explanted cultures and the microbiome of the mice (KW ANOVA, chi-squared = 73.58, df = 8, p
189 < 0.01). Post-hoc analysis shows that, compared to the microbiome, mouse cultures 1 and 2 were
190 the same while mouse cultures 3 and 4 differed (Fig 2C). The microbial profile of each sample
191 revealed a difference in beta diversity between the plates and the microbiome (Table 4), which is
192 also reflected in the PCoA plot (Fig 2D).

193 **Table 4. Results of the pair-wise PERMANOVA tests between cultured plates and**
194 **harvested samples.**

Comparison	Pseudo F	p-score
M1 0% vs Microbiome	11.09	p < 0.01
M1 20% vs Microbiome	18	p < 0.01
M2 0% vs Microbiome	11.93	p < 0.01
M2 20% vs Microbiome	15.21	p < 0.01
M3 0% vs Microbiome	14.94	p < 0.01
M3 20% vs Microbiome	7.95	p < 0.01
M4 0% vs Microbiome	18.83	p < 0.01
M4 20% vs Microbiome	21.321	p < 0.01

195

196 **Fig 2. Community composition of cultures and comparison of microbiome.** (A) Relative
197 abundance between bacteria Phyla. Phyla with observations less than 1% are pooled into “Other”
198 category. The first two characters represent the mouse, and the percent oxygen used in culture
199 conditions is noted. (B) Relative abundance of bacterial genera. Genera with observations less
200 than 5% are pooled into “Other” category. (C) Results of the post-hoc, Shannon Diversity,
201 pairwise comparisons between cultures and the microbiome. p<0.05 is noted by “*”, p<0.0001 is
202 noted by “****”, and non-significance is noted by “ns.” (D) Unweighted unifrac PCoA plot for
203 plates and microbiome comparison. 12 well plates are denoted by the mouse in which they were
204 derived from, “M,” and the percent oxygen.

205 **Environmental variables (Level 2)**

206 A community profile of cultural composition due to varying levels of oxygen and pH was
207 constructed at the phylum and genus levels (Fig 3A-D). No difference in alpha diversity existed
208 between the two oxygen levels (Wilcoxon test, p = 0.34; Fig 3E). Results showed similarity in
209 beta diversity (PERMANOVA, Pseudo F = 1.25, p = 0.21). Multivariate analysis shows a
210 significant difference in alpha diversity associated with differences in pH (KW ANOVA, chi-

211 squared = 58.13, df = 3, p < 0.01). Post-hoc analysis revealed that plates reaching a pH of 6 and
212 7 were similar while all other comparisons differed (Fig 3F). Comparisons of microbial
213 communities also showed a marked difference between plates of varying pH levels (Table 5).
214 Additionally, CCA and PERMANOVA revealed that oxygen did not contribute to community
215 clustering (Table 6; Fig 4).

216 **Table 5. Results of the pairwise PERMANOVA test between pH groups.**

Comparison	Pseudo F	p-score
pH 6 vs pH 7	1.86	0.04
pH 6 vs pH 9	17.15	p < 0.01
pH 6 vs pH 10	25.46	p < 0.01
pH 7 vs pH 9	15.27	p < 0.01
pH 7 vs pH 10	22.31	p < 0.01
pH 9 vs pH 10	1.62	0.03

217

218 **Table 6. Hypothesis testing for sources of variation based on PERMANOVA analysis.**

Source	df	SS	MS	Pseudo F	P(perm)	Unique perms
Oxygen	1	3432.6	3432.6	1.6677	0.0756	9903
Sibling Relationship	1	52930	52930	25.716	0.0001	9921
Oxygen x Sibling Relationship	1	2407.4	2407.4	1.1696	0.2437	9915
Residuals	89	1.8319E+05	2058.3			
Total	92	2.4194E+05				

df, degrees of freedom; SS, sum of squares; MS, mean square.

The only significant effect fitted in the PERMANOVA is sibling relationship (fixed factor) while oxygen (fixed factor) is not significant. Sibling relationship explains the changes in community composition as time elapsed in the incubators.

219

220 **Fig 3. Microbial structuring due to environmental factors.** (A) Relative abundance between
221 bacteria phyla. Phyla with observations less than 1% are pooled into “Other” category. (B)
222 Relative abundance of bacterial genera. Genera with observations less than 1% are pooled into
223 “Other” category. (C) Shannon diversity comparison between oxygen concentrations. Non-
224 significance is shown by “ns.”

225 **Fig 4. CCA for the effects of pH, oxygen, and sibling relationship on community**
226 **structuring.**

227 **Siblings (Level 2)**

228 A marked difference in cultural composition was noted by familial relationship (Table 6, Fig 4,
229 Fig 5A, B). Not only was clustering associated with siblings, shown in Fig 4, but Shannon
230 diversity significantly varied (KW ANOVA, chi-squared = 56.24, df = 3, p < 0.01). Post-hoc
231 analysis showed mouse 1 and mouse 2 were similar and varied from mouse 3 and mouse 4,
232 which were also similar (Fig 5C). Additionally, beta diversity varied according to familial
233 relationship (PERMANOVA, Pseudo F = 12.82, p < 0.01).

234 **Fig 5. Microbial composition by mouse.** (A) Relative abundance between bacteria phyla. Phyla
235 with observations less than 1% are pooled into “Other” category. (B) Relative abundance at the
236 level of genus. Observations less than 0.03% are pooled into “Other” category. (C) Shannon
237 diversity index comparison using mouse 2 as a reference group. “****” means significance
238 p<0.0001 while “ns” means non-significant.

239 **Individual Strain Comparisons (Level 2)**

240 *Enterococcus* significantly increased between mice cultures and the microbiome (Fig 6A).
241 Additionally, Proteobacteria strains were more abundant in cultures reaching a high pH;
242 although, cultures reaching pH 6 were equivalent in Proteobacteria compared to cultures
243 reaching pH 9 (Fig 6B). *Enterococcus* strains also followed a similar dynamic in which they
244 increased in cultures reaching a lower pH, 6 and 7 (Fig 6C). Subsequently, cultures with the
245 lowest pH, 6, had a significantly high abundance of *Lactobacillus* (Fig 6D). Archaea were more
246 abundant in plates with pH 9 and 10 (Fig 6E), and further, *Clostridium* strains were more likely
247 to be present in mouse 1 and 2 cultures compared to mouse 3 and 4 (Fig 6F).

248 **Fig 6. Comparison barcharts of individual microbial taxa.** (A) Comparison of the mean
249 abundance of *Enterococcus* in cultured plates compared to the microbiome. (B) Comparison of
250 the mean abundance of Proteobacteria in samples with varying pH. (C) Comparison of mean

251 abundance of *Enterococcus* in samples with varying pH. (D) Comparison of mean abundance of
252 *Lactobacillus* in samples with varying pH. (E) Comparison in mean abundance of archaea in
253 samples with varying pH. (F) Comparison in mean abundance of *Clostridium* in cultures
254 partitioned by which mouse, M, it originated. “ns”, non significant; “***”, $p < 0.001$; “**”, $p <$
255 0.01 ; “*”, $p \leq 0.05$.

256 **Microbial network (Level 2)**

257 The OTUs in the microbial network represent 88% of the relative sequence count for the cultured
258 well plates (Fig 7). Much of the interactions were positive in nature meaning copresence in a
259 shared-niche is the most abundant interaction type. Negative, mutually exclusive interactions are
260 only between OTUs from the genus *Enterococcus* and several OTUs from the order
261 Bacteroidales (Fig 7). The interaction between the 4 mutually exclusive OTUs account for 50%
262 of all sequences.

263 **Fig 7. Microbial network generated using Spearman’s rank correlation at the taxonomic**
264 **level of genus.** Most of the edges, 54 of 58, are of a positive correlation. The rest are negative.
265 Nodes sizes are configured based on abundance. Nodes greater than or equal 10% are the largest,
266 intermediate sized nodes are greater than or equal to 1%, and the smallest nodes are less than
267 1%.

268 **Discussion (Level 1)**

269 Many studies of the microbiome utilize germ-free mice, which are expensive to house and breed
270 and require sacrificing animals to study the GI microbiome [14,16]. In this study, we attempted
271 to culture an *ex vivo* microbiome in 3D well plates to decrease the cost associated with studying
272 animal microbiomes. We found that cultures for mouse 1 and 2 were comparable to the gut
273 microbiome in Shannon diversity (Fig 2C), which is very promising. Ultimately, our explant
274 microbiome was significantly different than the *in vivo* microbiome, but we were able to culture
275 a diverse number of prokaryotic strains utilizing our method. Optimizing efforts in culture
276 media, detection, and atmospheric gradients is extremely important in culturing desired microbes

277 [44]. With very little optimization, we were able to culture many gut microbial species including
278 difficult to culture strains such as Methanobacteria [45], mean of 0.57 (SD 4.31), and MCG [46],
279 mean of 218.59 (SD 633.71), which included the fecal B10 strain [47], (Fig 6E).

280 The upsurge of *Enterococcus* in cultured plates (Fig 6A) is explained by its competitiveness
281 outlined in Fig 7. A likely scenario is that *Enterococcus* outcompeted strains within the order
282 Bacteroidales, which makes up a high proportion of the gut microbiome [48,49]. *Enterococcus* is
283 a facultative anaerobe [50] that may respire aerobically in the presence of hemin using
284 cytochrome *bd* terminal oxidase, which reduces oxygen into water [51,52]. Since it is a known
285 pioneer colonizer of the GI tract, its presence possibly established and maintained the anoxic
286 environment by metabolically depleting the atmospheric oxygen, which allowed obligate
287 anaerobes to thrive [53,54].

288 Post-incubation, a shift in pH was seen amongst the various 12 wells of the plates (Table 1). This
289 shift in pH is accounted for by the increase in gram negative Proteobacteria in plates with a more
290 basic pH (Fig 6B). Creation of the amine groups from this phylum perhaps led to the increase in
291 pH [55–57]. Further, the decrease in pH may be related to the increase in the lactic acid
292 fermenter *Enterococcus* in plates with a pH of 7 (Fig 6C) [52] while plates with a pH of 6 may
293 be partially explained by the increase in both *Enterococcus* and *Lactobacillus* (Fig 6C, D)
294 [52,58,59]. Additionally, pH was a strong influencer in the growth of archaea. Archaea grew
295 more readily in plates with a higher pH (Fig 6E). Not only are archaea difficult to culture, but
296 also their diversity is not well studied in regard to the gut microbiome [60]. Results are
297 comparable to those of Ilhan et al. wherein pH had a strong influence on microbial composition
298 in fecal anaerobic cultures [59]. There was a possible interaction between differences in the
299 microbial communities seeding the culture plates and physio-chemical culture conditions causing

300 the pH in plates from different mice to swing in opposite directions. Additionally, culturing
301 plates in an anaerobic chamber instead of a CO₂ incubator may have exacerbated pH instability
302 [61]. Future efforts to stabilize pH may allow for additional growth of archaea and provide a
303 means to temporarily culture and study members that have in the past been recalcitrant to culture
304 methods.

305 The microbiome is passed on from mother to litter [8,62]. Our *ex vivo* microbiome was highly
306 impacted by familial relationship (Table 6, Fig 4). Further, mice differed in the amount of
307 *Clostridium* cultured. Mouse 1 and 2 had higher numbers of *Clostridium* than mouse 3 and 4 (Fig
308 7F). Not only were these mice siblings but also weaned by different mothers. The effects of
309 weaning are similar to Bian et al. wherein the abundance of an unclassified strain of
310 *Clostridiaceae* was affected by the nursing mother [63]. Our results reiterate the impact of the
311 mother on the microbiome, but also show this dynamic transfers even when explanted from the
312 source.

313 Essentially, our results indicate that the feces and large distal colon are highly similar; therefore,
314 it is reasonable to consider avoiding mouse sacrifice by culturing feces. Future experiments will
315 need to control pH shifts to avoid media-related population dynamics. Since none of the plates
316 maintained the original baseline pH or even homogenate pH, we assume that additional buffering
317 capacity or equilibrating media and culturing in a CO₂ incubator may create a more stable
318 explanted microbiome, possibly maintaining diversity more similar to *in vivo* microbiota. Even
319 with the pH swings seen here, we were able to culture bacteria that are difficult to routinely
320 culture. Ultimately, this study found that pH was a stronger influencer of community
321 composition than oxygen. pH has a strong influence on the establishment of the microbes that
322 will populate the explant culture. Future efforts at establishing an *ex vivo* mouse microbiome

323 should include additional measures geared towards stabilizing pH in order to avoid community
324 shifts related to physical changes in growth media.

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330 **References (Level 1)**

331 1. Luke K Ursell, Jessica L Metcalf, Laura Wegener Parfrey and RK. Definig the Human
332 Microbiome. NIH Manuscripts. 2013;70: 1–12. doi:10.1111/j.1753-
333 4887.2012.00493.x.Defining

334 2. Tourneur E, Chassin C. Neonatal immune adaptation of the gut and its role during
335 infections. Clin Dev Immunol. 2013;2013: 1–17. doi:10.1155/2013/270301

336 3. Schwabe R and JC. The microbiome and cancer. Nat Rev Cancer. 2013;13: 800–812.
337 doi:10.1038/nrc3610

338 4. Sommer F, Bäckhed F. The gut microbiota — masters of host development and
339 physiology. Nat Publ Gr. 2013;11. doi:10.1038/nrmicro2974

340 5. Cho I, Blaser MJ. The human microbiome: At the interface of health and disease. Nat Rev
341 Genet. 2012;13: 260–270. doi:10.1038/nrg3182

342 6. Li Q, Han Y, Dy ABC, Hagerman RJ. The Gut Microbiota and Autism Spectrum

343 Disorders. *Front Cell Neurosci.* 2017;11. doi:10.3389/fncel.2017.00120

344 7. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-Induced Obesity Is Linked to
345 Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host
346 Microbe.* 2008;3: 213–223. doi:10.1016/j.chom.2008.02.015

347 8. Ericsson AC, Franklin CL. Manipulating the gut microbiota: Methods and challenges.
348 *ILAR J.* 2015;56: 205–217. doi:10.1093/ilar/ilv021

349 9. Böhm L, Torsin S, Tint SH, Eckstein MT, Ludwig T, Pérez JC. The yeast form of the
350 fungus *Candida albicans* promotes persistence in the gut of gnotobiotic mice. *PLoS
351 Pathog.* 2017;13: 1–26. doi:10.1371/journal.ppat.1006699

352 10. Hart ML, Ericsson AC, Lloyd KCK, Grimsrud KN, Rogala AR, Godfrey VL, et al.
353 Development of outbred CD1 mouse colonies with distinct standardized gut microbiota
354 profiles for use in complex microbiota targeted studies. *Sci Rep.* 2018;8: 1–11.
355 doi:10.1038/s41598-018-28448-0

356 11. Roeselers G, Ponomarenko M, Lukovac S, Wortelboer HM. Ex vivo systems to study
357 host–microbiota interactions in the gastrointestinal tract. *Best Pract Res Clin
358 Gastroenterol.* 2013;27: 101–113. doi:<https://doi.org/10.1016/j.bpg.2013.03.018>

359 12. Lagier JC, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of
360 previously uncultured members of the human gut microbiota by culturomics. *Nat
361 Microbiol.* 2016;1. doi:10.1038/nmicrobiol.2016.203

362 13. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing
363 of “unculturable” human microbiota reveals novel taxa and extensive sporulation. *Nature.*

364 Nature Publishing Group; 2016;533: 543–546. doi:10.1038/nature17645

365 14. Rodriguez-Palacios A, Aladyshkina N, Ezeji JC, Erkkila HL, Conger M, Ward J, et al.
366 “Cyclical bias” in microbiome research revealed by a portable germ-free housing system
367 using nested isolation. *Sci Rep.* Springer US; 2018;8: 1–18. doi:10.1038/s41598-018-
368 20742-1

369 15. Trosvik P, de Muinck EJ. Ecology of bacteria in the human gastrointestinal tract--
370 identification of keystone and foundation taxa. *Microbiome.* 2015;3: 44.
371 doi:10.1186/s40168-015-0107-4

372 16. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, Dantas G, et al. Extensive
373 personal human gut microbiota culture collections characterized and manipulated in
374 gnotobiotic mice. *Proc Natl Acad Sci.* 2011;108: 6252–6257.
375 doi:10.1073/pnas.1102938108

376 17. Shokralla S, Spall JL, Gibson JF, Hajibabaei M. Next-generation sequencing technologies
377 for environmental DNA research. *Mol Ecol.* 2012;21: 1794–1805. doi:10.1111/j.1365-
378 294X.2012.05538.x

379 18. Zheng L, Kelly CJ, Colgan SP. Physiologic hypoxia and oxygen homeostasis in the
380 healthy intestine. A Review in the Theme: Cellular Responses to Hypoxia. *Am J Physiol -*
381 *Cell Physiol.* 2015;309: C350–C360. doi:10.1152/ajpcell.00191.2015

382 19. Brady JA, Faske JB, Castañeda-Gill JM, King JL, Mitchell FL. High-throughput DNA
383 isolation method for detection of *Xylella fastidiosa* in plant and insect samples. *J*
384 *Microbiol Methods.* Elsevier B.V.; 2011;86: 310–312. doi:10.1016/j.mimet.2011.06.007

385 20. Herlemann DPR, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF.
386 Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea.
387 ISME J. 2011;5: 1571–1579. doi:10.1038/ismej.2011.41

388 21. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of
389 general 16S ribosomal RNA gene PCR primers for classical and next-generation
390 sequencing-based diversity studies. Nucleic Acids Res. 2013;41: 1–11.
391 doi:10.1093/nar/gks808

392 22. Illumina. 16S Metagenomic Sequencing Library Preparation. Illumina.com. 2013; 1–28.
393 Available: http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

396 23. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et
397 al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample.
398 Proc Natl Acad Sci. 2011;108: 4516–4522. doi:10.1073/pnas.1000080107

399 24. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics.
400 2010;26: 2460–2461. doi:10.1093/bioinformatics/btq461

401 25. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a
402 chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl
403 Environ Microbiol. 2006;72: 5069–5072. doi:10.1128/AEM.03006-05

404 26. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database
405 Project: Improved alignments and new tools for rRNA analysis. Nucleic Acids Res.

406 2009;37: 141–145. doi:10.1093/nar/gkn879

407 27. Paulson JN, Colin Stine O, Bravo HC, Pop M. Differential abundance analysis for
408 microbial marker-gene surveys. *Nat Methods*. 2013;10: 1200–1202.
409 doi:10.1038/nmeth.2658

410 28. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al.
411 QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*.
412 Nature Publishing Group; 2010;7: 335. Available: <http://dx.doi.org/10.1038/nmeth.f.303>

413 29. R Core Team. R: A Language and Environment for Statistical Computing [Internet].
414 Vienna, Austria; 2018. Available: <https://www.r-project.org/>

415 30. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis
416 and Graphics of Microbiome Census Data. *PLoS One*. 2013;8.
417 doi:10.1371/journal.pone.0061217

418 31. Wickham H. ggplot2: Elegant Graphics for Data Analysis [Internet]. Springer-Verlag New
419 York; 2016. Available: <http://ggplot2.org>

420 32. Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Solymos P, et al. vegan:
421 Community Ecology Package [Internet]. 2008. Available: <http://cran.r-project.org/>,

422 33. Yap BW, Sim CH. Comparisons of various types of normality tests. *J Stat Comput Simul*.
423 2011;81: 2141–2155. doi:10.1080/00949655.2010.520163

424 34. Dinno A. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums [Internet].
425 2017. Available: <https://cran.r-project.org/package=dunn.test>

426 35. Clarke KR, Gorley RN. PRIMER v7. 2015;

427 36. Faust K, Raes J. CoNet app : inference of biological association networks using Cytoscape
428 [version 1 ; referees : 2 approved with reservations] Referee Status : F1000Research.
429 2017;1519: 1–14. doi:10.12688/f1000research.9050.1

430 37. Shannon P, Markiel A, Owen Ozier 2, Baliga NS, Wang JT, Ramage D, et al. Cytoscape:
431 a software environment for integrated models of biomolecular interaction networks.
432 Genome Res. 2003; 2498–2504. doi:10.1101/gr.1239303.metabolite

433 38. Barberán A, Bates ST, Casamayor EO, Fierer N. Using network analysis to explore co-
434 occurrence patterns in soil microbial communities. ISME J. 2012;6: 343–351.
435 doi:10.1038/ismej.2011.119

436 39. Faust K, Sathirapongsasuti JF, Izard J, Segata N, Gevers D, Raes J, et al. Microbial co-
437 occurrence relationships in the Human Microbiome. PLoS Comput Biol. 2012;8.
438 doi:10.1371/journal.pcbi.1002606

439 40. Roggenbuck M, Bærholm Schnell I, Blom N, Bærlum J, Bertelsen MF, Pontén TS, et al.
440 The microbiome of New World vultures. Nat Commun. 2014;5: 1–8.
441 doi:10.1038/ncomms6498

442 41. Weldon L, Abolins S, Lenzi L, Bourne C, Riley EM, Viney M. The gut microbiota of wild
443 mice. PLoS One. 2015;10: 1–15. doi:10.1371/journal.pone.0134643

444 42. Kreisinger J, Čížková D, Vohánka J, Piálek J. Gastrointestinal microbiota of wild and
445 inbred individuals of two house mouse subspecies assessed using high-throughput parallel
446 pyrosequencing. Mol Ecol. 2014;23: 5048–5060. doi:10.1111/mec.12909

447 43. Ormerod KL, Wood DLA, Lachner N, Gellatly SL, Daly JN, Parsons JD, et al. Genomic
448 characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of
449 homeothermic animals. *Microbiome*. *Microbiome*; 2016;4: 1–17. doi:10.1186/s40168-
450 016-0181-2

451 44. Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D. Current and
452 past strategies for bacterial culture in clinical microbiology. *Clin Microbiol Rev*. 2015;28:
453 208–236. doi:10.1128/CMR.00110-14

454 45. Khelaifia S, Raoult D, Drancourt M. A Versatile Medium for Cultivating Methanogenic
455 Archaea. *PLoS One*. 2013;8. doi:10.1371/journal.pone.0061563

456 46. Meng J, Xu J, Qin D, He Y, Xiao X, Wang F. Genetic and functional properties of
457 uncultivated MCG archaea assessed by metagenome and gene expression analyses. *ISME*
458 *J*. Nature Publishing Group; 2014;8: 650–659. doi:10.1038/ismej.2013.174

459 47. Gorlas A, Robert C, Gimenez G, Drancourt M, Raoult D. Complete genome sequence of
460 Methanomassiliicoccus luminyensis, the largest genome of a human-associated Archaea
461 species. *Journal of Bacteriology*. 2012. p. 4745. doi:10.1128/JB.00956-12

462 48. Peterfreund GL, Vandivier LE, Sinha R, Marozsan AJ, Olson WC, Zhu J, et al.
463 Succession in the Gut Microbiome following Antibiotic and Antibody Therapies for
464 Clostridium difficile. *PLoS One*. 2012;7. doi:10.1371/journal.pone.0046966

465 49. Zitomersky NL, Atkinson BJ, Franklin SW, Mitchell PD, Snapper SB, Comstock LE, et
466 al. Characterization of Adherent Bacteroidales from Intestinal Biopsies of Children and
467 Young Adults with Inflammatory Bowel Disease. *PLoS One*. 2013;8.

468 doi:10.1371/journal.pone.0063686

469 50. Fisher K, Phillips C. The ecology, epidemiology and virulence of *Enterococcus*.

470 Microbiology. 2009;155: 1749–1757. doi:10.1099/mic.0.026385-0

471 51. Winstedt L, Frankenberg L, Hederstedt L, Von Wachenfeldt C. *Enterococcus faecalis*

472 V583 contains a cytochrome bd-type respiratory oxidase. *J Bacteriol.* 2000;182: 3863–

473 3866. doi:10.1128/JB.182.13.3863-3866.2000

474 52. Ramsey M, Hartke A, Huycke MM. The Physiology and Metabolism of Enterococci -

475 PubMed - NCBI. Enterococci: From Commensals to Leading Causes of Drug Resistant

476 Infection. 2014. pp. 424–465. Available:

477 http://www.ncbi.nlm.nih.gov/pubmed/24649507%5Cnhttp://www.ncbi.nlm.nih.gov/books
478 /NBK190424/

479 53. Houghtelling PD. Why is initial bacterial colonization of the intestine important to the

480 infant's and child's health? *J Pediatr Gastroenterol Nutr.* 2016;60: 294–307.

481 doi:10.1097/MPG.0000000000000597.Why

482 54. Wampach L, Heintz-Buschart A, Hogan A, Muller EEL, Narayanasamy S, Laczny CC, et

483 al. Colonization and succession within the human gut microbiome by archaea, bacteria,

484 and microeukaryotes during the first year of life. *Front Microbiol.* 2017;8: 1–21.

485 doi:10.3389/fmicb.2017.00738

486 55. Smith EA, Macfarlane GT. Studies on amine production in the human colon: Enumeration

487 of amine forming bacteria and physiological effects of carbohydrate and pH. Anaerobe.

488 1996;2: 285-297. doi:10.1006/anae.1996.0037

489 56. Busse HJ. Polyamines. *Methods Microbiol.* 2011;38: 239–259. doi:10.1016/B978-0-12-
490 387730-7.00011-5

491 57. Doré J, Blottière H. The influence of diet on the gut microbiota and its consequences for
492 health. *Curr Opin Biotechnol.* Elsevier Ltd; 2015;32: 195–199.
493 doi:10.1016/j.copbio.2015.01.002

494 58. Gänzle MG, Follador R. Metabolism of oligosaccharides and starch in lactobacilli: A
495 review. *Frontiers in Microbiology.* 2012. pp. 1–15. doi:10.3389/fmicb.2012.00340

496 59. Ilhan ZE, Marcus AK, Kang D, Rittmann BE. pH-Mediated Microbial and Metabolic.
497 *mSphere.* 2017;2: 1–12. doi:10.1128/mSphere

498 60. Raymann K, Moeller AH, Goodman AL, Ochman H. Unexplored Archaeal Diversity in
499 the Great Ape Gut Microbiome. *mSphere.* 2017;2: e00026-17.
500 doi:10.1128/mSphere.00026-17

501 61. Ueda K, Tagami Y, Kamihara Y, Shiratori H, Takano H, Beppu T. Isolation of bacteria
502 whose growth is dependent on high levels of CO₂ and implications of their potential
503 diversity. *Appl Environ Microbiol.* 2008;74: 4535–4538. doi:10.1128/AEM.00491-08

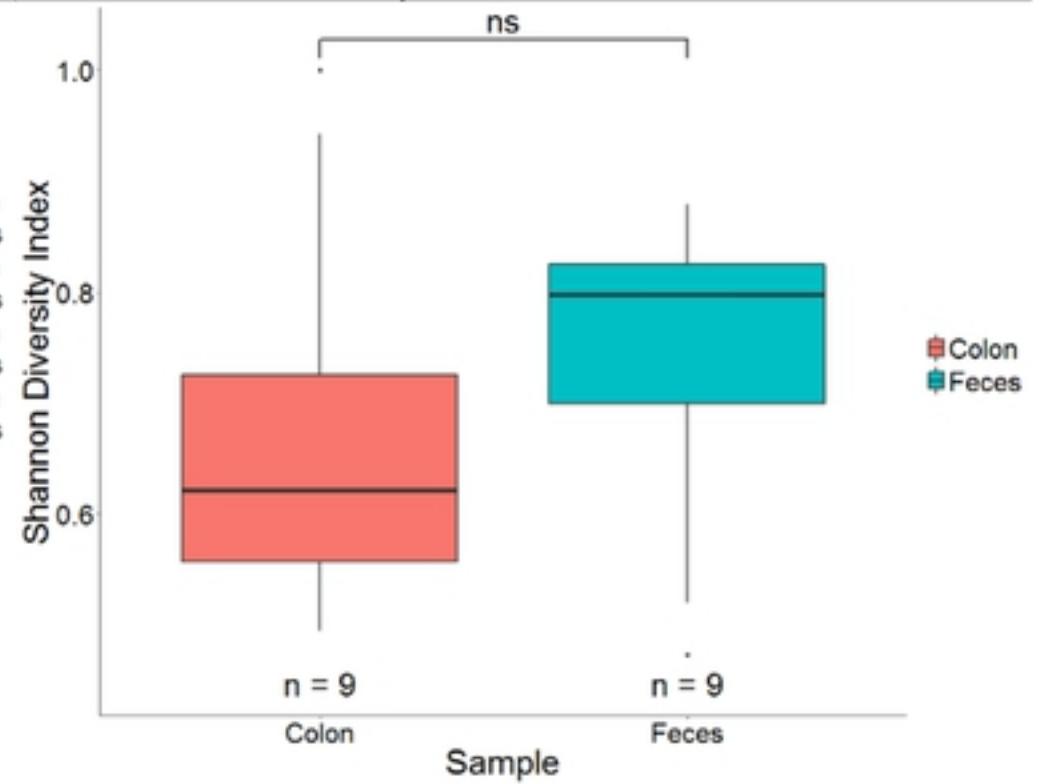
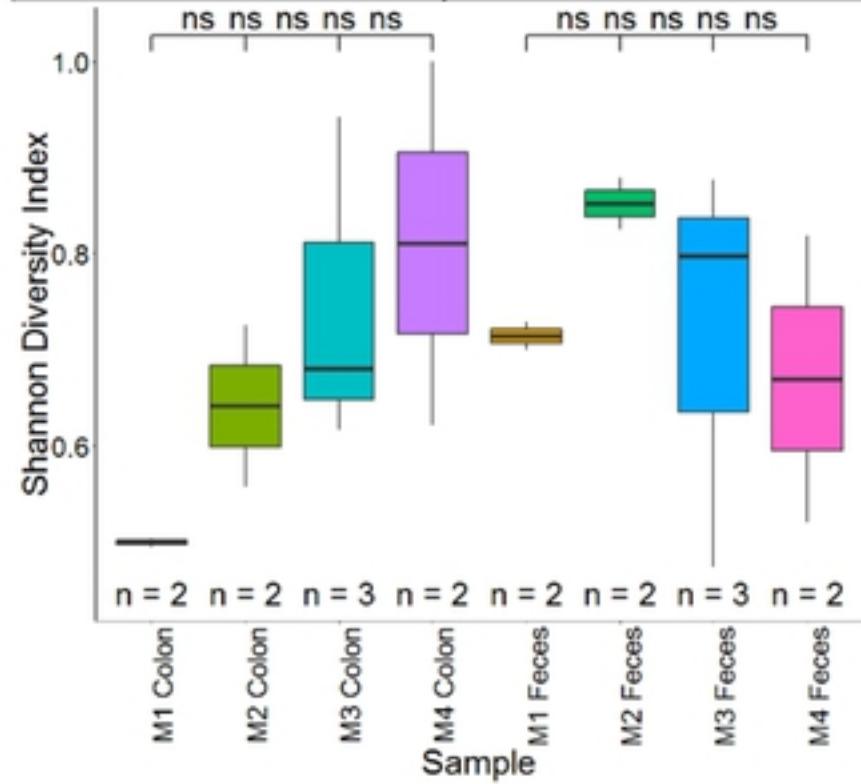
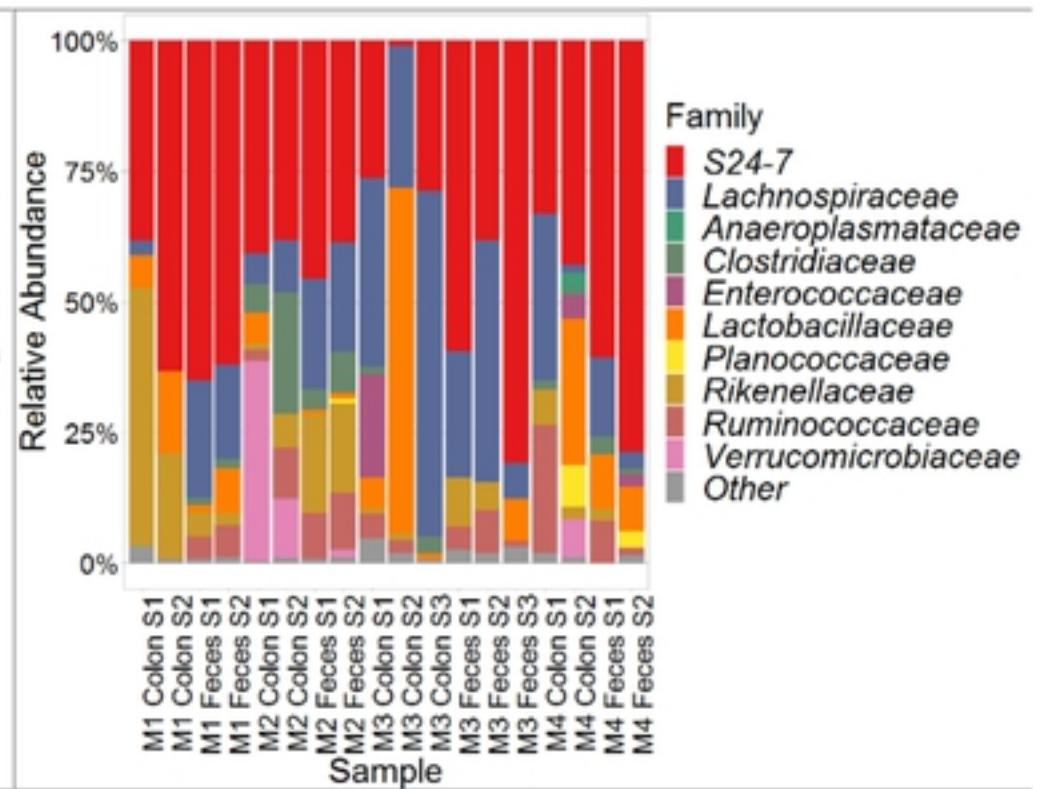
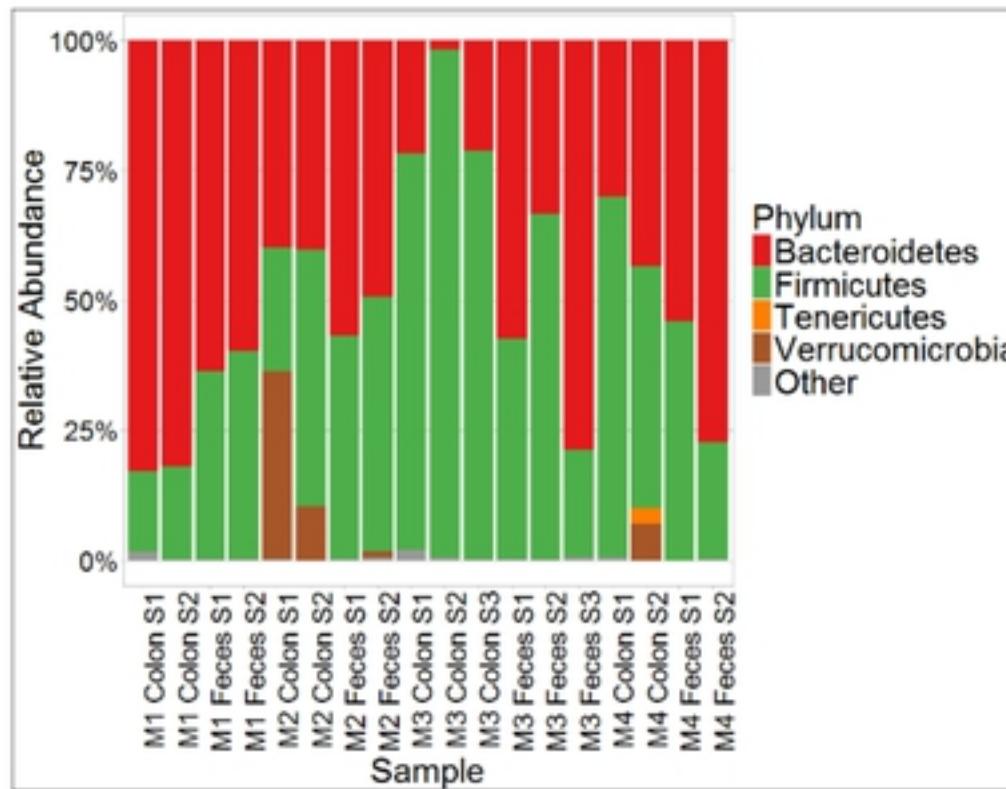
504 62. Mueller NT, Bakacs E, Combellick J, Grigoryan Z, Maria G. The infant microbiome
505 development: mom matters. *Trends Mol Med.* 2015;21: 109–117.
506 doi:10.1016/j.molmed.2014.12.002.The

507 63. Bian G, Ma S, Zhu Z, Su Y, Zoetendal EG, Mackie R, et al. Age, introduction of solid
508 feed and weaning are more important determinants of gut bacterial succession in piglets
509 than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environ*

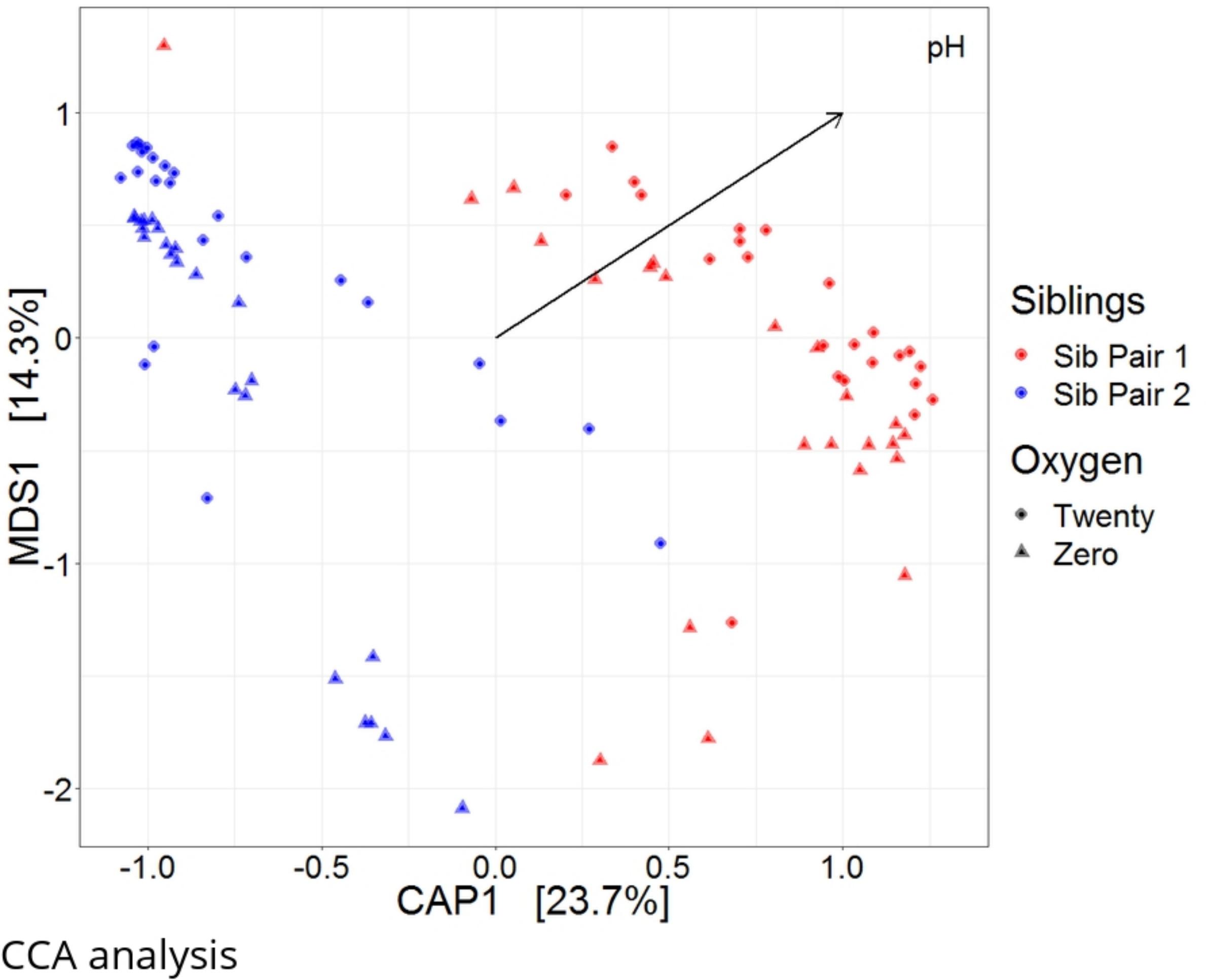
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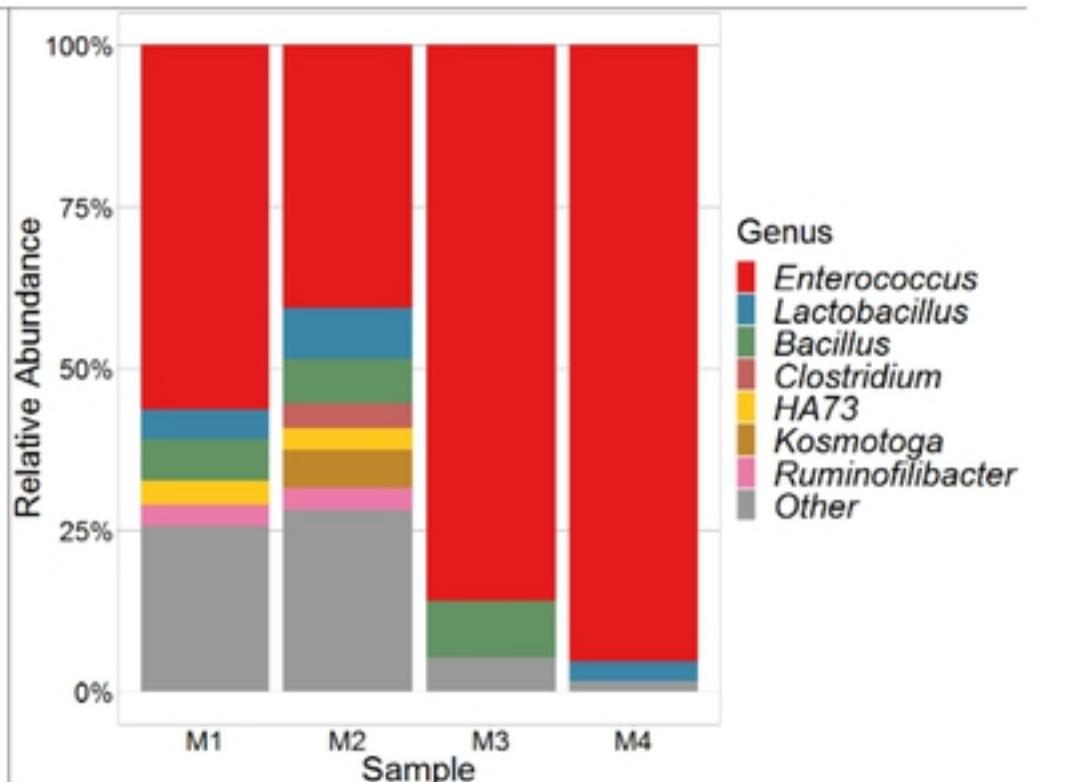
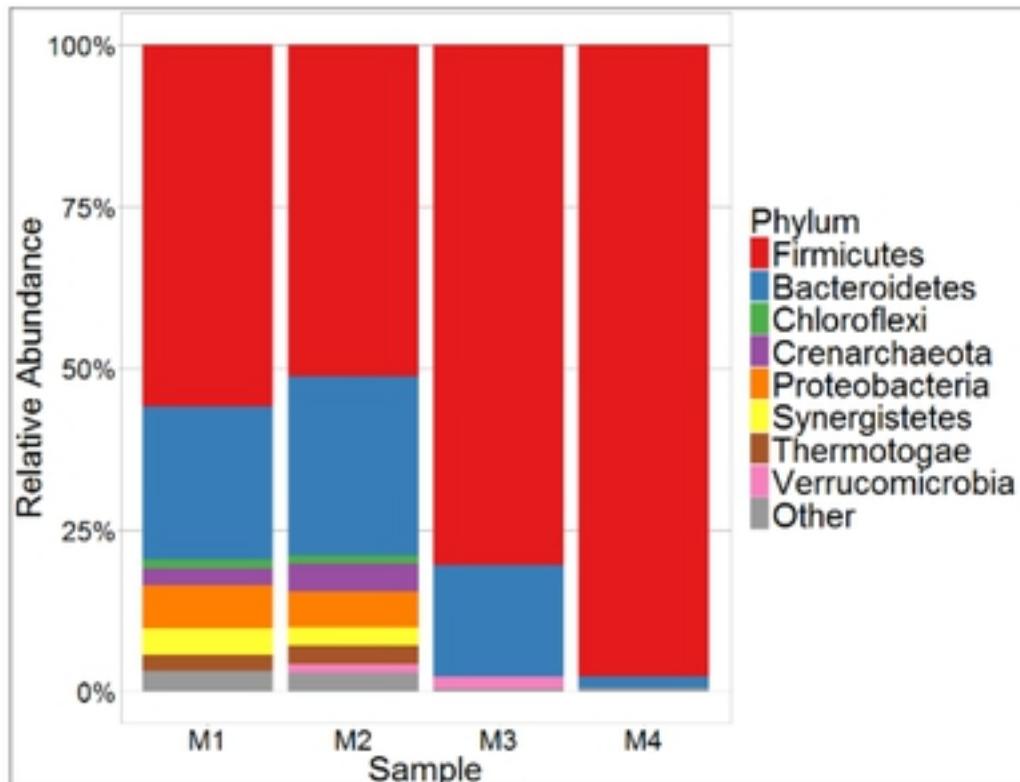
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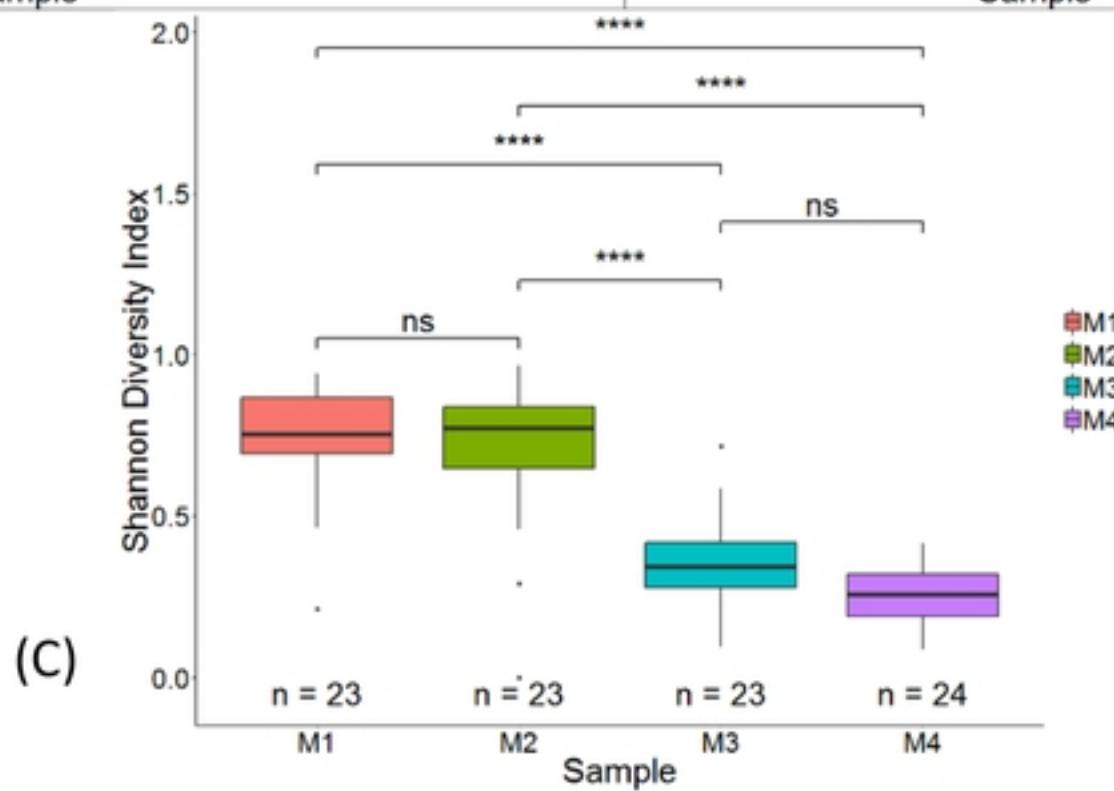
Microbiome inspection





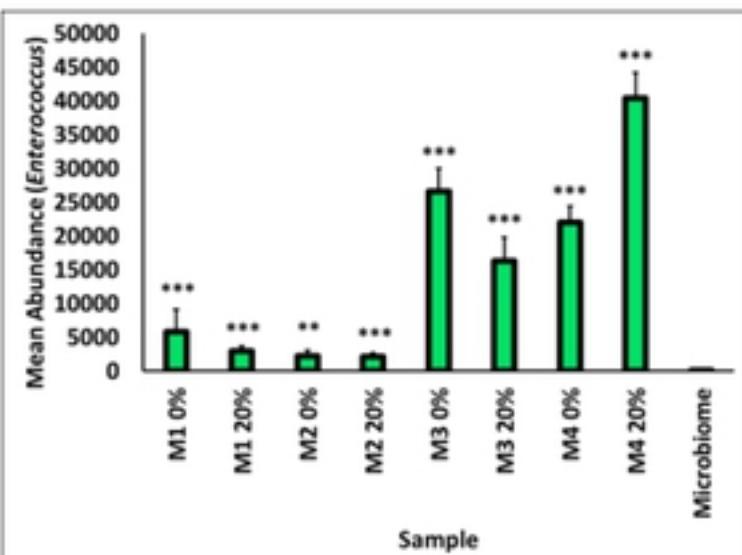
(A)

(B)

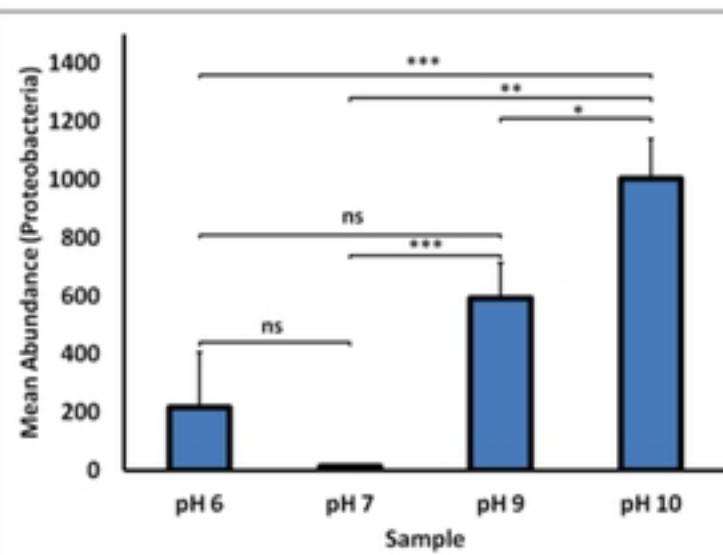


(C)

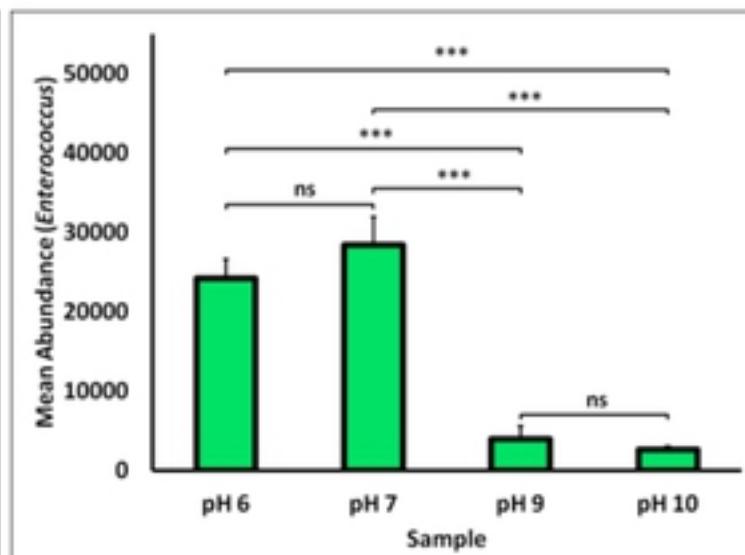
Mouse microbiome inspection



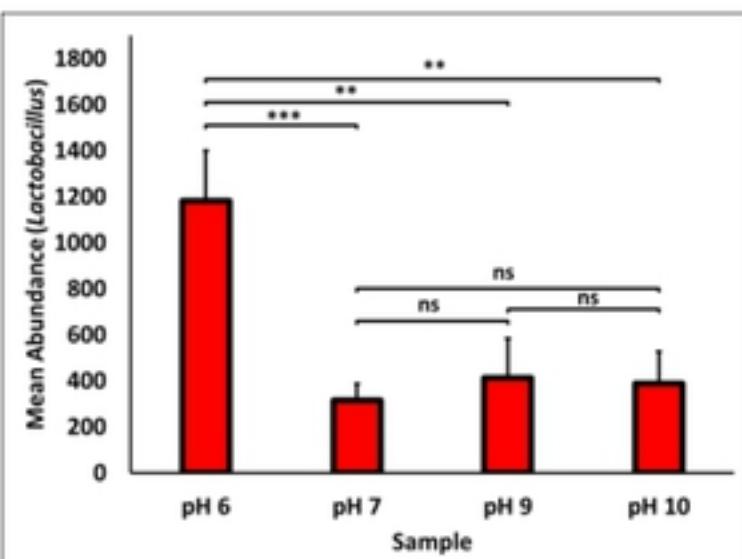
(A)



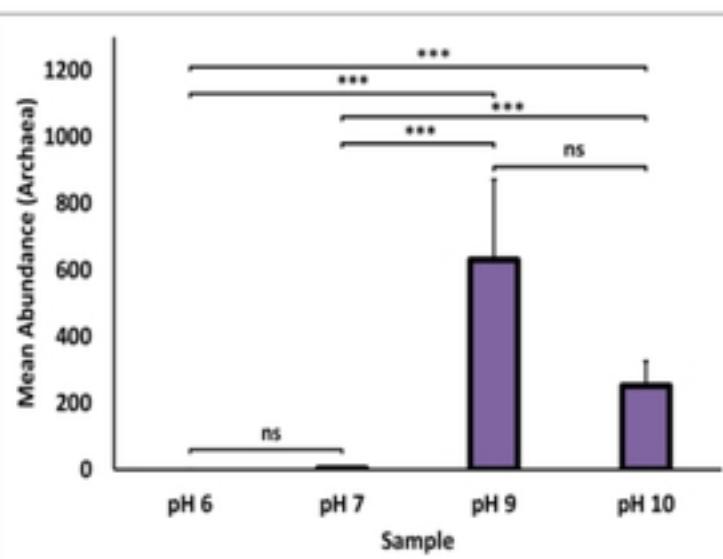
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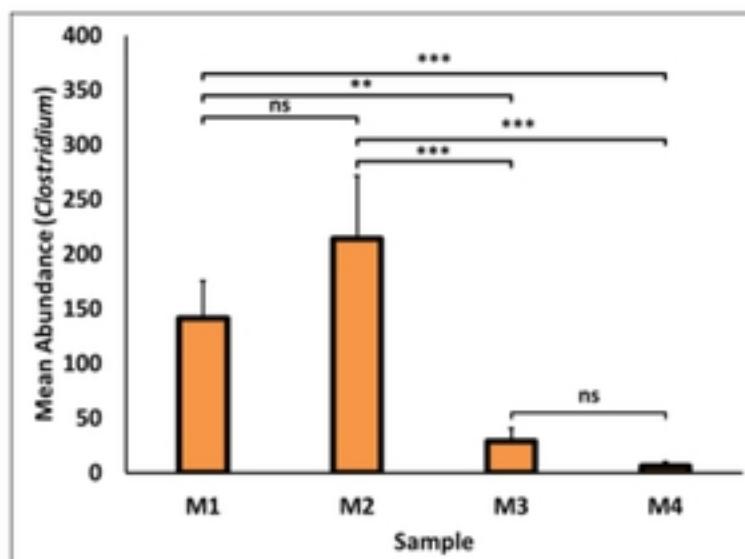
(C)



(D)

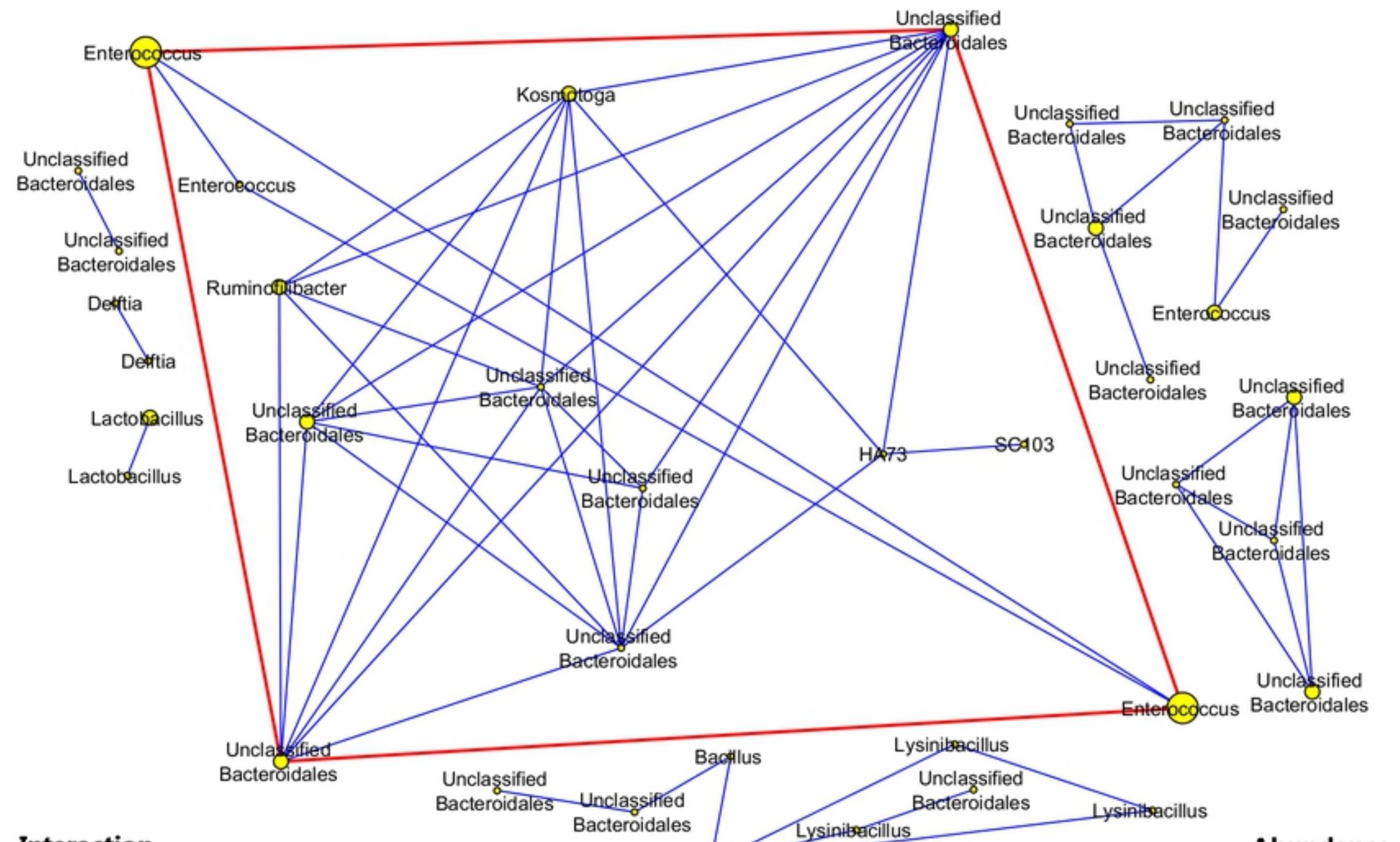


(E)



(F)

Barcharts of individual microbes



Interaction

Abundance

Mutual Exclusion

● > 10%

- $\geq 1\%$
- $< 1\%$

OTU network

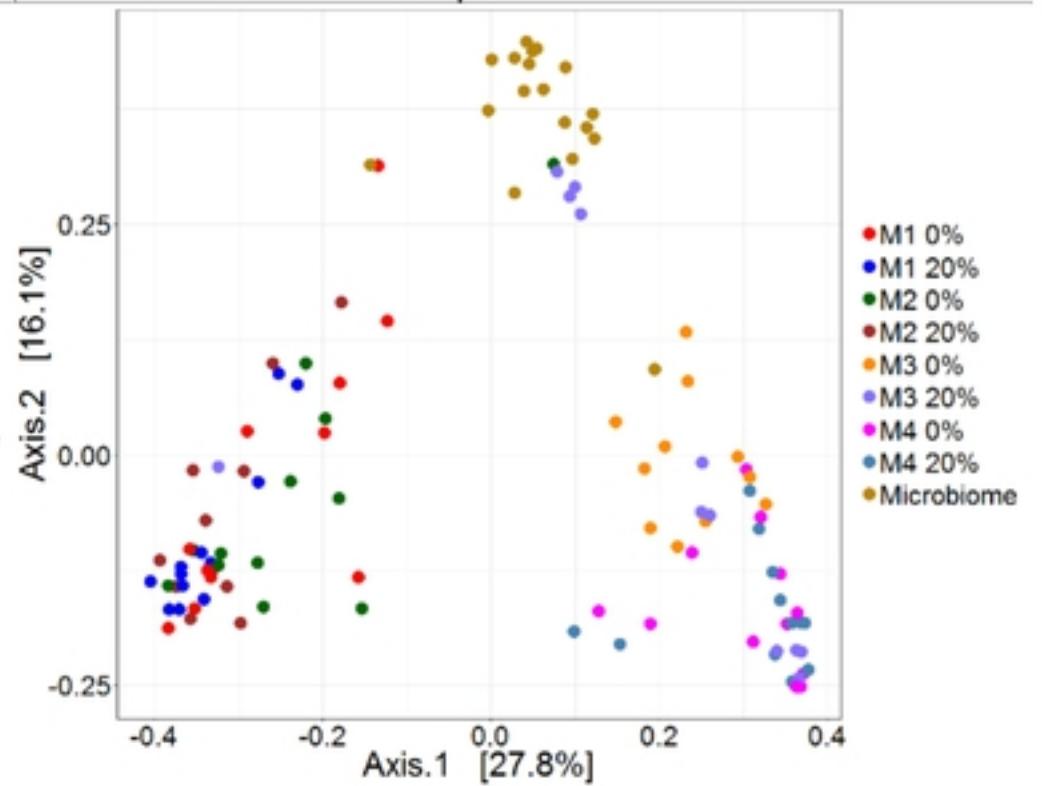
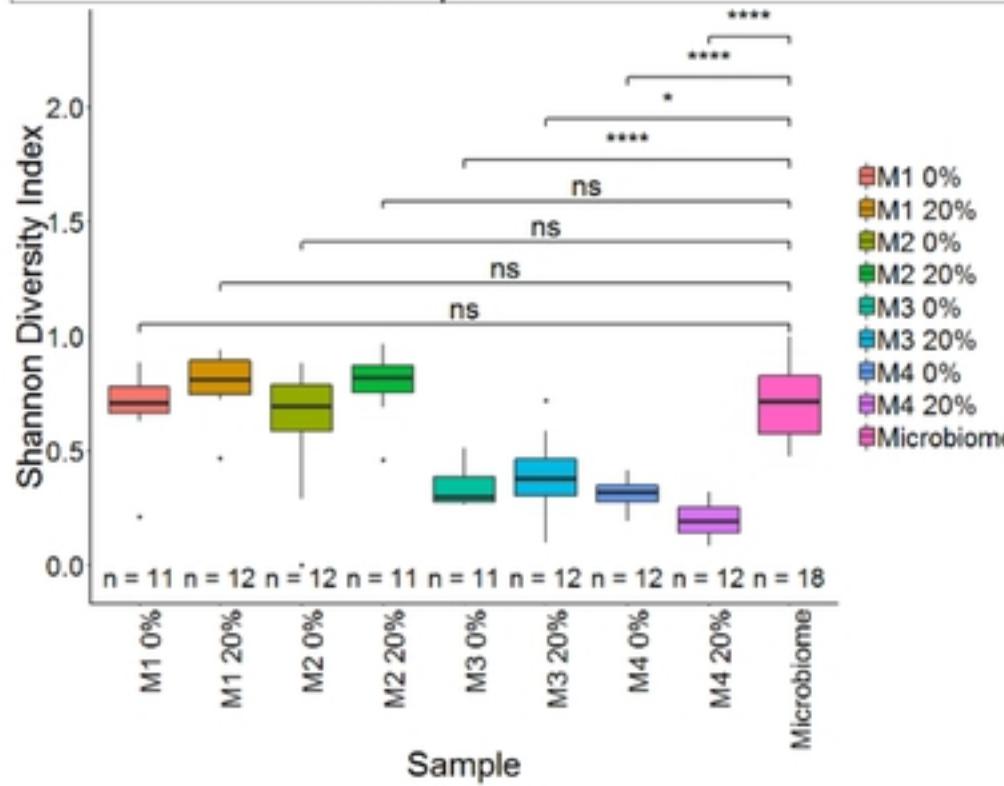
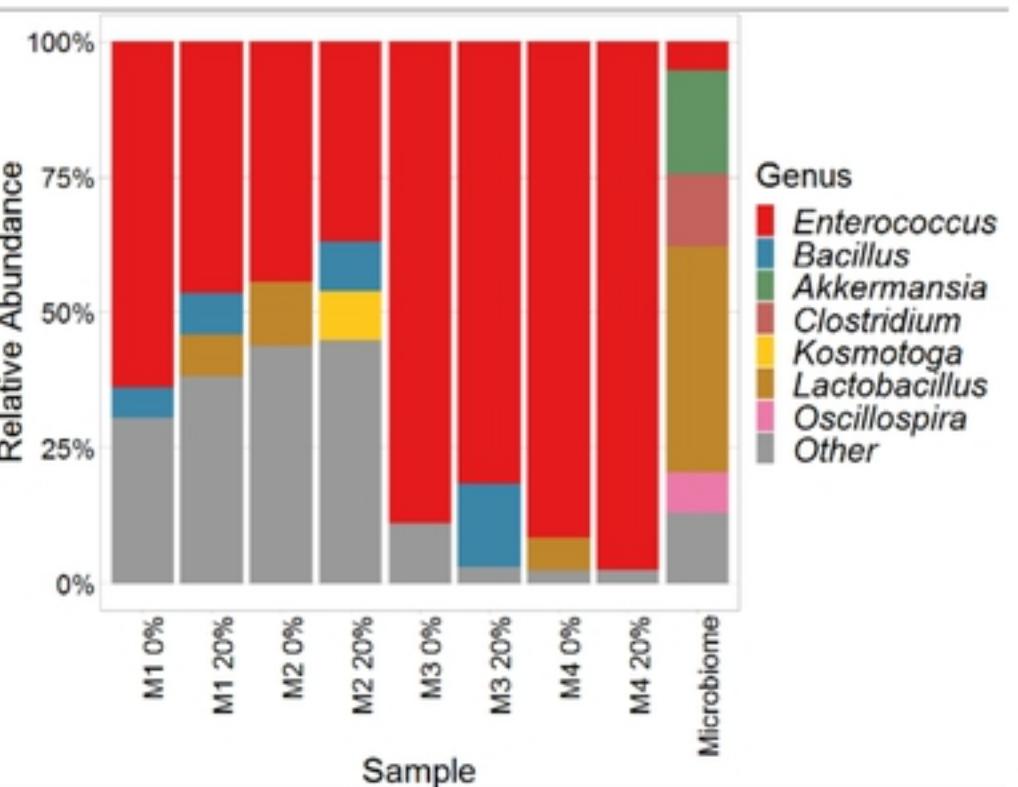
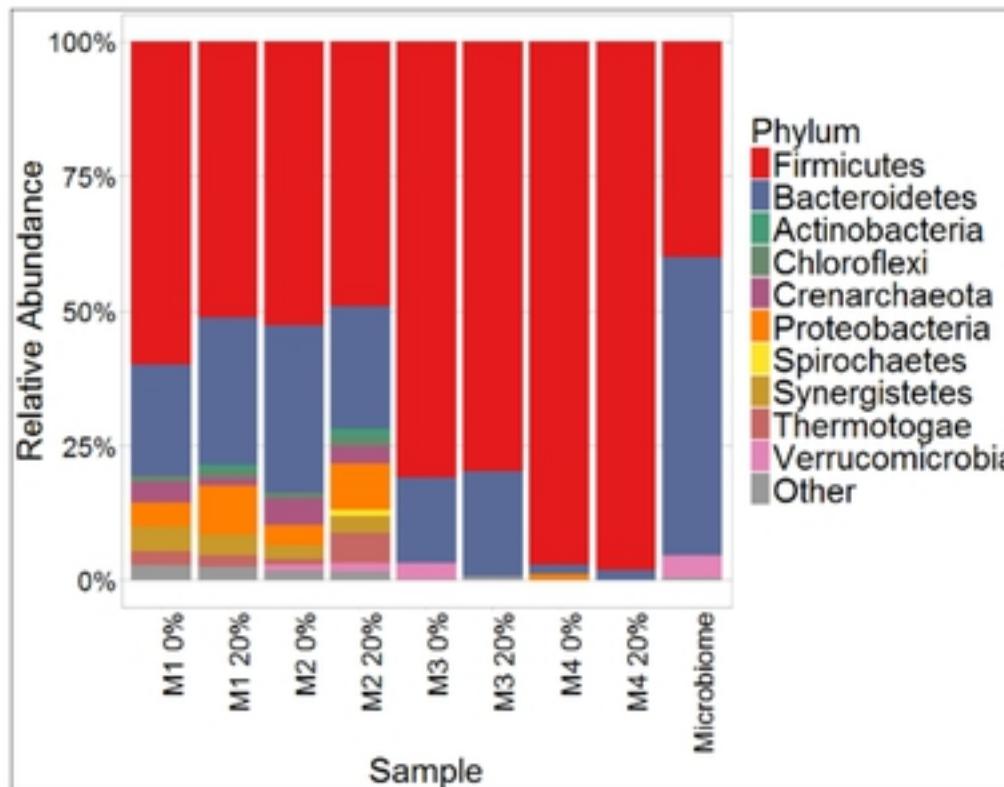
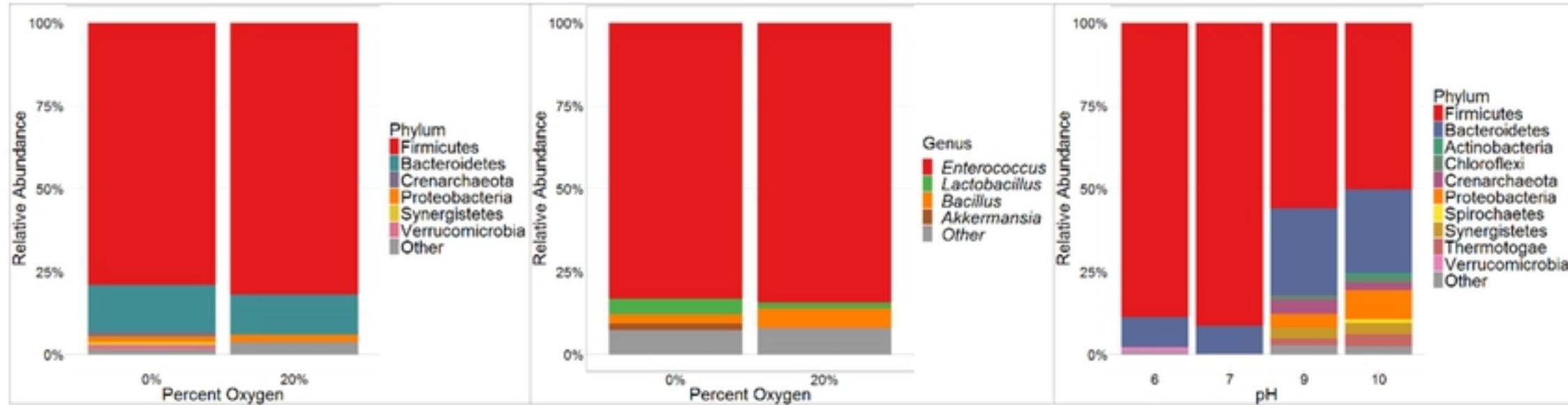


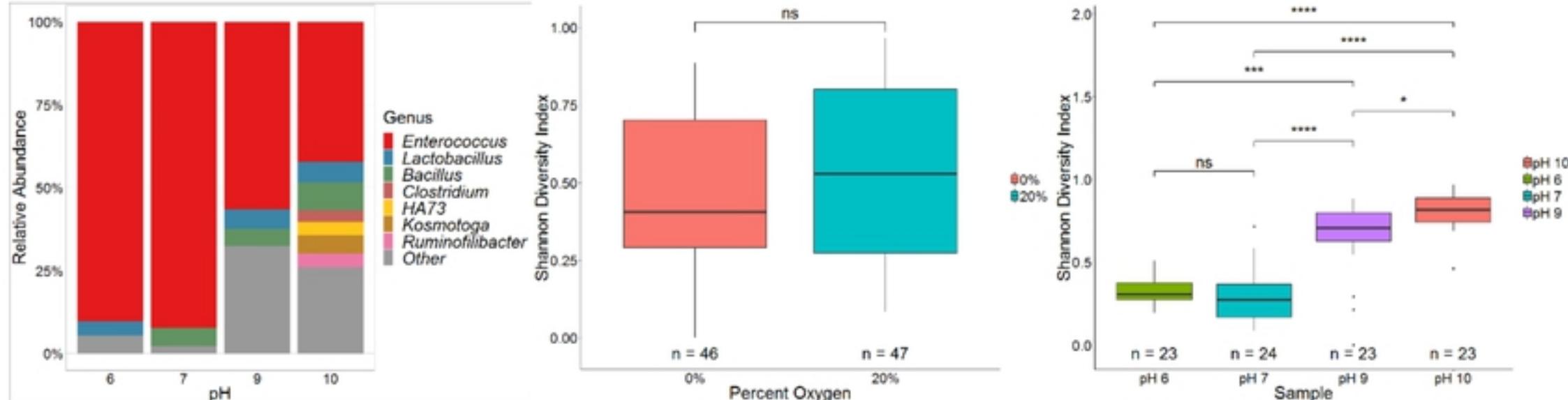
Plate microbiome inspection



(A)

(B)

(C)



(D)

(E)

(F)

Impact of environmental variables