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1 # Topical Application of Adult Caecal Contents to Eggs Transplants Spore-Forming Microbiota but Not Other
2 Members of the Microbiota to Chicks

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16 Running Title: Egg Treatment Transplants the Caecal Microbiota

17 ## ABSTRACT

18 The intestinal microbiota plays an essential role in the metabolism and immune competence of
19 chickens from the first day of hatch. In modern production systems, chicks are isolated from adult
20 chickens, instead hatching in a clean environment. As a result, chicks are colonised by environmental
21 bacteria including potential pathogens. There is a need to investigate methods by which chicks can be
22 exposed to the a more appropriate microbial community at hatch. Such methods must be easy to apply
23 in a hatchery and produce consistent results. The development of the intestinal microbiota of chicks
24 hatched from eggs sprayed with dilute adult caecal content during incubation was observed at 0, 3, 7
25 and 14 days post hatch (d.p.h) across two experiments. High-throughput Illumina sequencing was
26 performed for the V4 hypervariable region of the 16S rRNA gene. A topical treatment of dilute adult
27 caecal content was sufficient to transplant spore-forming bacteria such as *Lachnospiraceae* and
28 *Ruminococcaceae*. However, this treatment was not able to transplant other taxa that are considered to

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29 be core elements of the chicken caecal microbiota such as *Bacteroidaceae*, *Lactobacillaceae*,
30 *Bifidobacteriaceae* and *Burkholderiaceae*. The topical treatment significantly altered the microbiota of
31 chicks immediately post-hatch and accelerated the normal development of the microbiota with earlier
32 colonisation by *Ruminococcaceae* in the caecum and Candidatus *Arthromitus* in the ileum. The effect of
33 the treatment on caecal microbiota was maximal at 3 d.p.h but diminished over time.

34 **IMPORTANCE**

35 Over the last 60 years poultry production has intensified in response to increased demand for meat. In
36 modern systems, chicks hatch without contacting chickens and their gut bacteria. Consequently, they
37 are colonised by environmental bacteria that may cause disease. The normal bacteria that live in the
38 gut, or intestinal microbiota, play an important role in the development of the immune system.
39 Therefore, it's essential to find easy ways to expose chicks to the more appropriate bacteria at hatch.
40 This experiment investigated whether spraying eggs with adult caecal contents was sufficient to
41 transfer an adult microbiota to chicks. Our findings show that spore-forming bacteria were
42 transplanted but other members of the microbiota were not. In this respect, the spray application was
43 partially successful but the timing of the spray needs to be modified to ensure that more bacteria are
44 transferred.

45 **Introduction**

46 Between 1961 and 2001, global average annual meat consumption per capita nearly doubled from
47 23.1kg to 42.20kg (1). Much of this increase has been provided by a growing poultry industry which
48 has intensified and industrialised to meet demand. The broiler industry in the UK produced
49 approximately 82 million broilers per month in 2018 (2). The industrialisation of poultry production
50 has led to separation of mature adults, eggs and immature chickens at independent sites. Breeder

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51 flocks, constituting the genetic elite of the national chicken population, are kept exclusively to provide
52 fertile eggs. Eggs are transported to hatcheries, which may be on the same site, where they are
53 incubated in batches until hatch. After hatch, chicks are sold as 'day-old chicks' to finishers where they
54 remain until slaughter at around 42 days old. This separation of chicks from maternal contact delays
55 colonisation of the gut by normal commensals (3). Instead, chicks are first colonised by environmental
56 bacteria in hatcheries and during transport which may include possible pathogens such as *Clostridium*
57 *perfringens* or *Escherichia coli* (4-6)*.* Poultry flocks are exquisitely sensitive to the presence of enteric
58 pathogens (7,8). While many can be controlled using vaccination and biosecurity, pathogens such as
59 *Campylobacter* remain rife within the UK chicken population (9). Prophylactic use of antibiotics in the
60 poultry industry was widely adopted as a growth promoter with a secondary effect of facilitating
61 enteric pathogen control and reducing production losses (10). However, the indiscriminate use of
62 antibiotics has led to a rise in antimicrobial resistance. In order to combat this threat to public health,
63 the European Union enacted a ban on the use of antibiotics as growth promoters in 2006 (11). As a
64 result, this tool of the poultry industry must be replaced with alternatives.

65

66 Manipulation of the intestinal microbiota provides one such alternative. Many efforts to alter the
67 microbiota have focused on the introduction of probiotics via feed or water to growing and adult chick
68 (12). However, a growing body of evidence suggests that the ability to influence microbiota
69 composition decreases with age as a stable microbial community is established (13). Questions remain
70 over the optimal timing and delivery mechanism for microbiota interventions. Until recently, the
71 embryonic gut was thought to be sterile. With the advent of molecular techniques, this assumption of
72 sterility has been challenged with some evidence showing the presence of bacteria in the embryonic
73 gut. Molecular techniques have been used to visualise and detect bacteria in embryonic chick tissue.

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74 For example, viable bacteria were detected in the caecal tissue from embryos at 18 and 20 d.i using
75 fluoresence in situ hybridisation (14). Bacterial DNA from *Enterobacteriaceae*, *Actinomycetales*,
76 *Bifidobacteriales* and *Lachnospiraceae* was detected using T-RFLP of the entire gastrointestinal tract of
77 chicken embryos (15) raising the possibility that *in ovo* microbial colonisation occurs in proximal parts
78 of the gastrointestinal tract as well as the caecum. However, scepticism of such results is not
79 unwarranted as low microbial biomass samples are known to be prone to contamination leading to
80 false positive results and inflated microbial diversity (16). The presence of bacteria within embryos
81 and eggs would pose a question as to their origin. Vertical transmission is one possibility but
82 considered unlikely (17). Germ-free chicks can be derived by sterilising the eggshell immediately after
83 lay and rearing in an isolator indicating that vertical transmission would be an uncommon route of
84 colonisation for normal microbiota (18, 19). This suggests that the principal entry route for bacteria
85 would be penetration of the eggshell and subsequent egg defences. Most studies focus on the ability of
86 *Salmonella* and other bacteria of public health importance to translocate from the eggshell to the
87 embryo although one study does demonstrate that other bacterial taxa are able to penetrate the
88 eggshell (20). While these findings demonstrated that penetration of the eggshell is possible by certain
89 bacterial taxa, it cannot be taken as evidence that microbes on the egg surface are able to traverse the
90 albumen and successfully colonise the embryonic gut. An aim of this study was to detect bacteria
91 within the embryonic gut and to resolve whether a selection of commensal bacteria applied to the egg
92 surface during incubation would be detected in the embryonic gut.

93 This study also aimed to investigate the effect of a topical application of adult caecal content on the
94 development of the chicken intestinal microbiota and identify which bacterial taxa can be transplanted
95 to chicks. Altering the microbiota of chicks after hatch is not a new idea. Since the 1970s, research has
96 been conducted into the effectiveness of competitive exclusion cultures (CEC), usually anaerobically
97 cultured bacteria from adult caecal contents, in reducing *Salmonella* infection in chicks (21). With the

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98 observation that competitive exclusion was effective only when administered before *Salmonella*
99 challenge (22) the aim became to administer the probiotic as close to hatch as possible. The first report
100 of *in ovo* administration of a probiotic came from Cox *et al*. (23) who injected an undefined CEC into the
101 air cell of 17 d.i eggs. This treatment conferred a greater resistance to *Salmonella* Typhimurium (23).
102 Despite this early success, further results from injecting CECs into eggs have been variable with
103 reports of reduced hatchability and early mortality with increased disease resistance falling short of
104 antibiotic controls (14, 24-26). As such, it is worth questioning whether injection is the best delivery
105 method for CEC products. Prior to disinfection at hatcheries, which aims to reduce the abundance of
106 pathogenic bacteria which can reduce hatchability and chick performance, the egg has a surface
107 microbiota similar to the composition of the caecal microbiota (27). A topical application of adult
108 caecal bacteria may more accurately replicate the environment in which chickens and their
109 commensals co-evolved where a sitting hen would regularly replenish the surface bacteria of the egg.
110 Additionally, a spray application would remove the issue of hatchability caused by injecting probiotics
111 into eggs. A previous experiment has explored the ability of a topical application of diluted adult caecal
112 content to affect microbiota development but little analysis was conducted to determine which
113 amplicon sequence variants (ASVs) were successfully transplanted from the donor material to
114 recipient chicks (28). This is an important question in terms of developing interventions for
115 commercial use. Regulators are unlikely to approve a treatment of unclassified bacteria sourced
116 directly from adult chicken caecal content. Identifying bacterial taxa that are likely to be successfully
117 transplanted by topical application is the first step towards creating an effective topical probiotic
118 which is acceptable to regulators.

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119 ## Results

120 Two separate experiments, a pilot experiment and a repeat experiment, were conducted to observe the
121 effect of the topical application of adult caecal contents to eggs. Results from both experiments are
122 presented together. A summary of sampling time points and abbreviations can be found in Figure 1.
123 Briefly, sample groups are identified using abbreviations in which the first letter corresponds to the
124 experiment (P = pilot, R = repeat), the second letter corresponds to treatment (C = control, T = treated)
125 and the numbers correspond to the time point (0, 3, 7 or 14 d.p.h). Transplant material is identified by
126 the abbrevition TRPL.

127 ### Sequencing Effort

128 A total of 22,103,523 reads were obtained from 182 experimental samples submitted for sequencing.
129 After filtering, merging of paired reads and chimera removal, a total of 15,022,950 reads remained
130 (68% of the original total) giving a mean of 82,544 reads per sample. The median number of reads per
131 sample was 92,218.

132 ### Bacterial 16S rDNA was not detected in embryonic samples

133 Amplification of standard dilutions revealed that the PCR assay was able to clearly detect 10^3 bacterial
134 cells in a sample (Supplementary Figure 1). No positive amplification of bacterial 16S rRNA genes was
135 detected in any embryonic or egg sample at either 0 or 18 d.i. Amplicons were detected in positive
136 control samples and all spiked samples indicating that the absence of amplicons in other samples was
137 not due to PCR failure. This result indicates that no significant population of bacteria was present in
138 the embryonic gut at 18 d.i.

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139 **Treatment had no consistent effect on body weight**

140 The mean body weight of treated and control chicks in the repeat experiment was compared using
141 Student's t-test. No significant differences between groups were found at 0 (treated: M = 46g, SD =
142 5.83; control: M = 48.4g, SD = 6.65; conditions: t = -0.5, p = 0.6), 7 (treated: M = 135g, SD = 19.0;
143 control: M = 132g, SD = 17.1; conditions: t = 0.35, p = 0.7) and 14 (treated: M = 358.5g, SD = 44.8;
144 control: M = 322.5g, SD = 39.8; conditions: t = 1.8, p = 0.13) d.p.h. However, there was a significant
145 difference in body weight between groups at 3 d.p.h (treated: M = 77g, SD = 7.48; control: M = 65g, SD
146 = 5.0; conditions: t = 4.0, p = 0.002).

147 **Treated chicks had higher alpha diversity at early time points**

148 The alpha diversity of each sample group is displayed in Figure 2 with the significance of pairwise
149 Kruskal-Wallis tests comparing alpha diversity between sample groups displayed in Figure S2. Across
150 all experimental groups alpha diversity increased significantly with age with two exceptions. There
151 was no significant increase in alpha diversity in treated or control chicks between 0 and 3 d.p.h
152 (treated: H = 1.91, p = 0.19; control: H = 2.38, p = 0.15) or in treated chicks between 3 and 7 d.p.h
153 during the repeat experiment (H = 2.16, p = 0.17).

154 In general, treatment with an adult-derived microbiota resulted in a significantly higher alpha
155 diversity when compared to control chicks at 0 d.p.h (repeat: H = 6.82, p = 0.017) and 3 d.p.h (pilot: H =
156 9.02, p = 0.009; repeat: H = 9.8, p = 0.006) but not 7 and 14 d.p.h. There were significant differences in
157 alpha diversity between transplant material (TRPL) samples and samples taken at 0 and 3 d.p.h as well
158 as PT07 samples.

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159 Control chicks at 3 d.p.h had significantly higher alpha diversity in the repeat experiment compared to
160 the pilot experiment (H = 8.27, p = 0.01). There were no further significant differences in alpha
161 diversity between equivalent groups from the pilot and repeat experiments.

162 **Treatment significantly affected beta diversity**

163 When measured with an unweighted UniFrac metric, the factor 'Age' had the largest effect on beta
164 diversity (ANOSIM test statistic = 0.78, p = 0.001), followed by 'Treatment' (ANOSIM test statistic =
165 0.13, p = 0.001) and 'Experiment' (ANOSIM test statistic = 0.10, p = 0.012). The average unweighted
166 UniFrac distance between groups is displayed in Figure S3. A PCoA analysis showed clustering of
167 samples by group (Figure 3A). When measured with a weighted UniFrac metric, the factor 'Age' had
168 the largest effect on beta diversity (ANOSIM test statistic = 0.40, p = 0.001), followed by 'Experiment'
169 (ANOSIM test statistic = 0.16, p = 0.002) and 'Treatment' (ANOSIM test statistic = 0.13, p = 0.001). A
170 PCoA analysis showed clustering of samples by group (Figure 3B).

171 In plots of unweighted UniFrac distance RC0 and RT0 tend to cluster together in the PCoA plot with the
172 exception of one RT0 sample. PC3 and RC3 samples clustered together along with three PT3 samples.
173 The remaining PT3 samples and all RT3 samples clustered togther and were closer to samples from
174 later time points that PC3 and RC3 samples. At 7 d.p.h, PC7, PT7, RC7 and RT7 samples cluster together
175 although there is a tendency for treated samples from both experiments to cluster closer to samples
176 from 14 d.p.h. RC14 and RT14 samples formed separate clusters to each other. A similar pattern of
177 clustering was present in plots of weighted UniFrac distance although there was no separate clustering
178 of RC14 and RT14 samples. Instead, all samples from 7 and 14 d.p.h tended to cluster together along
179 with RT3 samples and one PT3 sample. Additionally, PC3 and RC3 samples formed distinct clusters
180 compared to the unweighted UniFrac distance plot.

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181 Distance between sample groups and TRPL samples gives some indication of transplant success as the
182 unweighted UniFrac distance between similar samples is lower reflecting closer clustering of samples..
183 At 3, 7 and 14 d.p.h treated samples were significantly closer to TRPL samples than controls (3 d.p.h: t
184 = 16.6, p < 0.001 ; 7 d.p.h: t = 7.2, p < 0.001 and 14 d.p.h: t = 4.8 , p < 0.001). This pattern of increased
185 similarity of treated samples to TRPL samples in both experiments suggests that bacteria from adult
186 caecal content successfully colonised chicks by those time points. However, the success of the
187 treatment was not uniform between experiments. PT3 samples are further from TRPL samples than
188 RT3 samples (0.76 c.f. 0.66).

189 ASVs were differentially abundant between treated and control chicks

190 For ease of interpretation, results from the pilot and repeat experiments were interpreted separately.
191 Gneiss analysis was used to identify differentially abundant ASVs between treated and control chicks
192 since it accounts for the compositional nature of microbiome data. Firstly, a dendrogram of ASVs is
193 prepared. Each node is termed a 'balance' with taxa on one side of the balance designated as
194 numerators and on the other, denominators. The log ratio of abundances between numerator and
195 denominator taxa for each balance is calculated. This value can be compared between sample groups
196 to determine differences in microbiome composition. A significant difference between samples
197 indicates that one of five hypotheses is true: i) The numerator taxa are increased in the group with a
198 higher log ratio; ii) The denominator taxa are decreased; iii) A combination of hypotheses i) and ii); iv)
199 Both numerator and denominator taxa are increased but numerator taxa have increased more; v) Both
200 numerator and denominator taxa are decreased but denominator taxa have decreased more.
201 Quantitative PCR is required to discern which hypothesis is correct as changes in relative abundance
202 are not always reflective of absolute abundance (29).

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203 **Pilot Experiment**

204 Gneiss analysis revealed differential ASV abundance between treated and control chicks at 3 and 7
205 d.p.h. The ASV table was filtered to exclude ASVs with a total frequency of less than 39 (a justification
206 for filtering thresholds is provided in the Materials and Methods section) reducing the number of ASVs
207 in the analysis from 408 to 306. The overall linear regression model fit was R2 = 0.34 with covariate
208 "Treatment" accounting for 17.1% of variance. Log ratio balances y0 ($\beta = -19.8$, $p < 0.001$), y2 ($\beta = 9.62$,
209 $p < 0.001$), y5 ($\beta = -3.97$, $p = 0.003$), y12 ($\beta = -5.42$, $p < 0.001$), y14 ($\beta = 6.56$, $p < 0.001$) and y27 ($\beta =$
210 7.20, $p = 0.006$) were significant predictors for the covariate of "Treatment". On review of the heatmap,
211 balance y6 was considered to describe ASVs differentially present in treated chicks at 3 d.p.h. Figure 4
212 show the log abundance of ASVs at 3 and 7 d.p.h between treated and control chicks along with a
213 summary of balances created by Gneiss analysis. Individual log ratios by group for significant balances
214 and balance taxonomy are available in Figure S4. The taxonomy of ASVs identified as differentially
215 abundant between treated and control samples is presented in Table 1A with relative abundance of
216 bacterial families in each sample displayed in Figure S5. A higher number of ASVs assigned to
217 *Lachnospiraceae*, *Bacillaceae*, *Ruminococcaceae*, and *Lactobacillaceae* were found to have a higher
218 relative abundance in treated samples compared to control samples. Some ASVs were found to have a
219 higher abundance in control compared to treated samples and were assigned to *Enterobacteriaceae,*
220 *Erysipelotrichaceae* and *Peptostreptococcaceae*.

221 **Repeat Experiment**

222 Gneiss analysis revealed differential ASV abundance between treated and control chicks at 0, 3, 7 and
223 14 d.p.h. The ASV table was filtered to exclude ASVs with a total frequency of less than 30 reducing the
224 number of ASVs in the analysis from 633 to 475. The overall linear regression model fit was R2 = 0.31
225 with covariated "Treatment" accounting for 9.65% of variance. Log ratio balances y0 ($\beta = 14.2$, $p <$

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226 0.001), y5 (β = -6.0, p = 0.001), y10 (β = -5.7, p = 0.009), y14 (β = -7.8, p < 0.001), y27 (β = 4.1, p = 0.01)
227 and y28 (β = 2.9, p < 0.001) were significant predictors for the covariate of "Treatment". On review of
228 the heatmap, balance y4 was considered to describe ASVs differentially present in control chicks at 3
229 d.p.h and balance $y1_{denominator}$ ASVs were considered to be equally abundant between treated and
230 control samples. Balances y5, y14, y27 and y28 contained ASVs already identified as differentially
231 abundant in treated or control samples by other balances. Figure 5 shows the log abundance of ASVs at
232 0, 3, 7 and 14 d.p.h between treated and control chicks along with a summary of balances created by
233 Gneiss analysis. Individual log ratios for significant balances and balance taxonomy are available in
234 Figure S6. The taxonomy of ASVs identified as differentially abundant between treated and control
235 samples is presented in Table 1B with relative abundance of bacterial families in each sample
236 displayed in Figure S5. A higher number of ASVs assigned to *Lachnospiraceae*, *Ruminococcaceae*,
237 *Clostridiales* vadin BB60 group, *Bacillaceae*, *Peptostreptococcaceae* and *Mollicutes* RF39 were found to
238 have a higher relative abundance in treated samples compared to control samples. Some ASVs were
239 found to have a higher abundance in control compared to treated samples and were assigned to
240 *Clostridiaceae* 1, *Enterobacteriaceae* and *Enterococcaceae*.

241 12% of ASVs present in the transplant material were identified as successfully transplanted in the Pilot 242 Experiment and 20% in the Repeat

243 445 ASVs were defined as present in the transplant. ASVs present in the transplant material that were not
244 subsequently identified in any samples from the pilot and repeat experiments were removed from the analysis (n =
245 274). Most of these ASVs were assigned to *Ruminococcaceae* (n = 125), *Clostridiales* vadin BB60 group
246 (n = 38), *Lachnospiraceae* (n = 33), *Christensenellaceae* (n = 12) and *Peptococcaceae* (n = 9). ASVs
247 assigned to *Bacteroidaceae*, *Lactobacillaceae*, *Coriobacteriaceae*, *Bifidobacteriaceae*, *Burkholderiaceae*

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248 and *Eggerthellaceae* had a high relative abundance in the transplant material (Figure S5). However,
249 none of these ASVs were successfully transplanted in either the pilot or repeat experiments.

250 **Pilot Experiment**

251 A total of 56 ASVs were categorised as successfully transplanted (Table 2 and Figure S7). The
252 taxonomy assignment of ASVs is shown in Table 3A. Only ASVs assigned to *Lachnospiraceae*,
253 *Ruminococcaceae*, *Erysipelotrichaceae* and *Mollicutes* RF39 (uncultured rumen bacteria) were defined
254 as successfully transplanted. A further 49 ASVs were categorised as possibly transplanted of which the
255 majority were assigned to the families *Ruminococcaceae* and *Lachnospiraceae*. Other ASVs categorised
256 as possibly transplanted were assigned to *Clostridiaceae* 1, *Erysipelotrichaceae*, *Bacillaceae*,
257 *Peptostreptococcaceae*, *Enterobacteriaceae*, *Enterococcaceae* and *Christensenellaceae*. At the genus
258 level, the two ASVs assigned to *Clostridiaceae* 1 were identified as Candidatus *Arthromitus.* The
259 remaining 201 ASVs were categorised as environmental. Some taxa were almost exclusively
260 categorised as environmental including *Clostridiaceae* 1, *Enterobacteriaceae*, *Peptostreptococcaceae*,
261 *Bacillaceae*, *Clostridiales* vadin BB60 group, *Enterococcaceae, Paenibacillaceae* and *Lactobacillaceae*. A
262 Hybrid Sankey diagram showed how the taxonomy of transplanted and environmental ASVs relates to
263 that of ASVs identified as differentially abundant between treated and control samples (Figure S8A).

264 **Repeat Experiment**

265 A total of 89 ASVs were categorised as successfully transplanted (Table 2 and Figure S7B). The
266 taxonomy assignment of ASVs is shown in Table 3B. The majority were assigned to the families
267 *Lachnospiraceae* and *Ruminococcaceae*. One ASV that was categorised as successfully transplanted and
268 assigned to *Clostridiaceae* 1 was identified at the genus level as Candidatus *Arthromitus*. A further 37
269 ASVs were categorised as possibly transplanted of which the majority were assigned to the family
270 *Ruminococcaceae*. The remaining 349 ASVs were categorised as environmental. As for the pilot

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271 experiment, some taxa were mainly categorised as environmental including *Clostridiaceae* 1,
272 *Clostridiales* vadin BB60 group, *Erysipelotrichaceae*, *Peptostreptococcaceae*, *Enterobacteriaceae*,
273 *Bacillaceae*, *Enterococcaceae*, *Paenibacillaceae* and *Lactobacillaceae*. A Hybrid Sankey diagram showed
274 how the taxonomy of transplanted and environmental ASVs relates to that of ASVs identified as
275 differentially abundant between treated and control samples. A Hybrid Sankey diagram showed how
276 the taxonomy of transplanted and environmental ASVs relates to that of ASVs identified as
277 differentially abundant between treated and control samples (Figure S7B).

278 A contingency table (Table 2) shows the overlap between ASVs identified as differentially abundant
279 and their classification in terms of transplant success in Experiments One and Two. The relationship
280 between group assignment was significant in the pilot, χ^2 (4) = 29.2, p < 0.001, and repeat, χ^2 (4) = 72.8,
281 p < 0.001, experiments. ASVs identified as differentially abundant in treated chicks were more likely to
282 be defined as successfully transplanted or possibly transplanted.

283 **Quantitative PCR confirmed differentially abundant taxa between treated and control samples**

284 **Caecum**

285 Genera within *Lachnospiraceae* have generally been placed in *Clostridium* Cluster XIVa while genera
286 within *Ruminococcaceae* have generally been placed in *Clostridium* Cluster IV (30, 31). As such, primers
287 for *Clostridium* Cluster XIV were used to estimate the abundance of *Lachnospiraceae* and primers for
288 *Clostridium* Cluster IV were used to estimate the abundance of *Ruminococcaceae*. Results are presented
289 in Figure 6.

290 The abundance of *Clostridium* Cluster XIV was significantly different in RT0 samples (t = 22.14, p <
291 0.001) as no DNA was amplified using this primer in RC0 samples. In both experiments, treated chicks
292 had significantly more *Clostridium* Cluster XIV at 3 d.p.h (pilot: t = 7.24, p < 0.001; repeat: t = 11.3, p <

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293 0.001). The difference between treated and control chicks continued to be significant in the repeat
294 experiment at 7 d.p.h ($t = 4.0$, $p < 0.001$) but not in the pilot experiment. There was no significant
295 difference in *Clostridium* Cluster XIV abundance between groups at 14 d.p.h.

296 There was no significant difference in *Clostridium* Cluster IV abundance between RC0 and RT0
297 samples, as DNA from this taxa was amplified in only one RT0 sample. The abundance of *Clostridium*
298 Cluster IV was significantly higher in RT3 samples compared to RC3 samples ($t = 4.9$, $p < 0.001$) but the
299 result was not quite significant when comparing PT3 and PC3 samples ($t = 1.95$, $p = 0.07$). By 7 and 14
300 d.p.h there was no significant difference in *Clostridium* Cluster IV abundance between treated and
301 control chicks.

302 There were differences in abundance of *Clostridium* Cluster IV between treated chicks from the pilot
303 and repeat experiments. RT3 samples had a higher abundance of *Clostridium* Cluster IV compared to
304 PT3 samples ($t = 3.63$, $p = 0.002$).

305 No *Enterobacteriaceae* were detected in either RT0 or RC0 samples. There was a significantly lower
306 abundance of *Enterobacteriaceae* RT3 samples compared to RC3 samples ($t = -5.42$, $p < 0.001$).
307 However, in the pilot experiment, the opposite result was obtained with a significantly higher
308 abundance of *Enterobacteriaceae* in PT3 samples compared to PC3 samples ($t = 3.54$, $p = 0.005$). At 7
309 d.p.h, there was a significantly lower abundance of *Enterobacteriaceae* in treated chicks in both
310 experiments (pilot: $t = -5.24$, $p < 0.001$; repeat: $t = -2.85$, $p = 0.01$). On average, the abundance of
311 *Enterobacteriaceae* was lower in RT14 samples compared to RC14 samples but the difference was not
312 significant ($t = -1.95$, $p = 0.07$). There was a large inter-experiment variation in *Enterobacteriaceae*
313 abundance with higher abundance detected at 3 d.p.h in the repeat experiment.

314 High levels of *Clostridium* were detected in RT0 and RC0 samples were no significant difference
315 between the groups. The abundance of *Clostridium* was significantly lower in RT3 samples compared

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316 to RC3 samples (t = -7.78, $p < 0.001$) but was significantly higher in RT7 samples compared to RC7
317 samples (t = 3.5, p = 0.002). There were no significant differences in *Clostridium* abundance between
318 treated and control chicks in the pilot experiment or in the repeat experiment at 14 d.p.h.

319 **Ileum**

320 No *Enterobacteriaceae* were detected in treated or control chicks in the repeat experiment at 0 d.p.h
321 (Figure 7). There was a significantly lower abundance of *Enterobacteriaceae* in treated chicks
322 compared to control chicks at 3 d.p.h in the repeat experiment (t = -4.78, $p < 0.001$) and 7 d.p.h in the
323 pilot experiment (t = -9.27, $p < 0.001$). There were no significant differences between treated and
324 control chicks at other time points.

325 There was no significant difference in *Clostridium* abundance between treated and control chicks at
326 any time point except at 3 d.p.h in the repeat experiment where the abundance was lower in treated
327 chicks (t = -4.33, $p < 0.001$).

328 In both experiments, no Candidatus *Arthromitus* was present in the ileum until 7 d.p.h. At 7 d.p.h, the
329 abundance of Candidatus *Arthromitus* was significantly higher in treated chicks in the pilot and repeat
330 experiments (t = 4.35, $p < 0.001$ and t = 2.97, p = 0.008 respectively). Although the average abundance
331 of Candidatus *Arthromitus* was higher in treated chicks from the repeat experiment at 14 d.p.h the
332 difference was not significant (t = 1.88, p = 0.08).

333 **Treatment did not alter intestinal morphology**

334 Histological examination of ileal and caecal tonsil tissues from the repeat experiment at 0, 3, 7 and 14
335 d.p.h was conducted to observe morphological parameters associated with intestinal development
336 such as villus height and width, epithelial cell height and crypt mitotic figure counts.

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337 Significantly more mitotic figures (Figures S9A and S9B) were recorded in the crypts of control chicks
338 than treated chicks at 3 d.p.h in the ileum ($t = 3.53$, $p = 0.008$) and caecal tonsil ($t = 2.81$, $p = 0.03$).
339 There was no statistically significant difference in mitotic figure counts between age groups in the
340 ileum ($F = 3.1$, $p = 0.09$) and caecal tonsil ($F = 0.18$, $p = 0.94$).

341 There were no significant differences in epithelial cell height, villus height or villus width between
342 treated and control chicks at 0, 3, 7 or 14 d.p.h (Figures S9C, S9D and S9E). There was a statistically
343 significant difference in villus height ($F = 41.6$, $p < 0.001$), villus width ($F = 18.1$, $p < 0.001$) and
344 epithelial cell height ($F = 58.9$, $p < 0.001$) between age groups. Post hoc comparisons revealed that
345 villus height was significantly different between 0 and 3 d.p.h ($p = 0.001$), 3 and 7 d.p.h ($p = 0.001$) and
346 7 and 14 d.p.h ($p = 0.001$). Villus width was significantly different between 0 and 3 d.p.h ($p = 0.01$) and
347 7 and 14 d.p.h ($p = 0.001$) but not between 3 and 7 d.p.h ($p = 0.75$). Epithelial cell height was
348 significantly different between 0 and 3 d.p.h ($p = 0.001$) and 3 and 7 d.p.h ($p = 0.001$) but not between
349 7 and 14 d.p.h ($p = 0.66$).

350 Segmented filamentous bacteria, were observed in the ileum from 7 d.p.h with presence on ileal
351 histology correlating with presence of Canidadatus *Arthromitus* detected by qPCR. In the repeat
352 experiment, segmented filamentous bacteria were also seen in the caecal tonsil in close approximation
353 to epithelial cells and in the lumen (Figure S10A).

354 No bacteria were found in the caecal crypts of any chicks at 0 d.p.h or control chicks at 3 d.p.h,
355 however, bacteria were identified in the caecal crypts of four treated chicks (Figure S10B). At 7 d.p.h,
356 four treated chicks and six control chicks were positive. At 14 d.p.h, no treated or control chicks were
357 positive, although occasional bacteria were noted in caecal crypts.

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358 **Treatment did not affect immune cell populations in the caecal tonsil**

359 Tissue from chicks at 3 d.p.h was examined as this was the time point when most differences were
360 found between the microbiota of treated and control chicks. No significant differences in counts of
361 CD4, CD8α, CD8β, γδ TCR and Bu1 cells were found between treated and control caecal tonsils at 3 d.p.h.

362 **Discussion**

363 This study demonstrated that there were no detectable bacteria present in the embryonic gut at 18 d.i.
364 It could be argued that detection of PCR amplicons using gel electrophoresis was not sensitive enough
365 to detect small numbers of bacteria within the embryonic gut. It could be expected that a bacterial
366 population too small to be detected using PCR would also be too small to have an impact in the face of
367 overwhelming colonisation by other bacterial taxa at hatch. It is likely that the chicken gut remains
368 sterile until hatch when bacteria present on the egg surface are the first to colonise.

369 Inoculation of the egg surface with an adult-derived microbiota was sufficient to transfer elements of
370 the microbiota to chicks with the result of accelerating caecal microbiota development. In treatment
371 and control groups across both experiments, microbial succession followed the same pattern. The
372 microbiota of day-old chicks was poorly diverse and composed of environmental bacteria, a pattern
373 well described in current literature (13, 28, 32). The order of succession whereby environmental
374 bacteria were replaced first by *Lachnospiraceae* and then *Ruminococcaceae* and other *Clostridiales* was
375 common across treated and control chicks. However, the speed of succession was faster in treated
376 chicks in both experiments with an initial strong colonisation by *Lachnospiraceae* followed by an
377 increase in *Ruminococcaceae*. Many *Ruminococcaceae* ASVs were classified as successfully or possibly
378 transplanted suggesting that these ASVs were present at 0 and 3 d.p.h but were unable to colonise the
379 caecum initially. This suggests that alterations to caecal conditions by *Lachnospiraceae* or some other

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380 unknown host factor are a prerequisite for colonisation by *Ruminococcaceae*. These results are in
381 contrast to those of Donaldson *et al*. (28) who did not observe significant differences in alpha diversity
382 or the pattern of bacterial colonisation between treated and control birds. These differing results can
383 be explained by different techniques used to apply the transplant material. Donaldson *et al*. (28)
384 swabbed the egg surface with diluted adult caecal content once during incubation which may have
385 resulted in the application of lower numbers of spores and vegetative cells to the eggshell than a spray.
386 Additionally, multiple treatments may have allowed for an accumulation of viable bacterial spores
387 more akin to the effect of close contact with the hen during incubation.

388 The presence of ASVs common to the transplant material from as early as a few hours post hatch
389 shows that the caecal microbiota can be successfully transplanted to chicks by topical application to
390 the egg surface. Most of the successfully transplanted ASVs were assigned to *Lachnospiraceae* and
391 *Ruminococcaceae* which differs from previously published results. Pedroso *et al*. (14) found only one
392 operational taxonomic unit assigned to *Lachnospiraceae* was transferred to treated chicks after
393 inoculating eggs with an *in ovo* injection of a commercial probiotic competitive exclusion (CPCE)
394 product. In contrast, we found that the majority of transferred features were assigned to
395 *Lachnospiraceae*, *Ruminococcaceae* and other *Clostridiales*. CPCE products are collections of culturable
396 bacteria but the results of our study show that the ASVs most likely to successfully colonise and persist
397 within the chicken caecum belonged to taxa that are challenging to culture in the laboratory such as
398 *Lachnsopiraceae*, *Ruminococcaceae* and, to a lesser degree, *Clostridiales* vadin BB60 and *Mollicutes*
399 RF39. It's unlikely that current CPCE products have an optimal bacterial composition for long term
400 colonisation of chicks. Development of CPCE products should focus on including the aforementioned
401 bacterial taxa as these have been shown to readily colonise newly hatched chicks and persist within
402 the caecum. However, topical application of caecal contents was unable to transplant several
403 important taxa such as *Bacteroidaceae*, *Lactobacillaceae* and *Bifidobacteriaceae*. A recent study of

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404 whole genome sequences from caecal bacteria revealed that genes enabling sporulation were found
405 within most Gram-positive *Firmicutes*, such as *Lachnospiraceae* and *Ruminococcaceae*, with the
406 exception of *Lactobacillaceae* (33). No *Bacteroidetes* isolates were spore-forming, however, 73% of
407 *Bacteroidetes* isolates were microaerotolerant and able to survive air exposures of 24 hours (33).
408 Similarly, the Bifidobacteria isolated from the chicken gut are non-spore forming (34). This difference
409 in environmental survival strategy explains the pattern of transplanted features observed in this study.
410 Spores would be able to survive on the egg surface and colonise the chick at hatch whereas non-spore
411 forming members of the caecal microbiota would not survive the 72 hours from the last treatment to
412 hatch. Alternatively, since bacterial viability was not assessed in the transplant material, the storage
413 and handling of the caecal content may have negatively impacted the survival of taxa that were not
414 transplanted. The inability of a topical treatment of diluted adult caecal content at 18 days of
415 incubation to transfer *Bacteroidaceae*, *Lactobacillaceae* and *Bifidobacteriaceae* exposes a major
416 weakness of the technique whether that is due to oxygen exposure during treatment or reduced
417 viability due to storage. *Bacteroidaceae* is considered a core member of the chicken caecal microbiota
418 (12, 35, 36) while members of *Bifidobacteriaceae* have been positively correlated with increased bird
419 weight (35). Any future experiments aiming to transplant an adult caecal microbiota would need to
420 take these taxa into account by delivering treatments immediately after hatch, either directly to the
421 chick or into the environment. Exploring different methods of bacterial preservation by using more
422 appropriate storage media to improve bacterial viability provides another avenue for future
423 investigation.

424 The abundance of Candidatus *Arthromitus*, also known as Candidatus *Savagella* or segmented
425 filamentous bacteria was studied in the ileum due to its importance as an immunostimulatory
426 bacterium (37-39). Consistent with previous experiments, Candidatus *Arthromitus* was absent from
427 the ileum until 7 d.p.h (40) after which a higher abundance was present in treated chicks. The

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428 environmental factors influencing Candidatus *Arthromitus* colonisation have not been explored
429 although increased abundance was noted in the ilea of chicks housed on reused litter (41). The
430 transplant material may have contained Candidatus *Arthromitus* spores that transferred to treated
431 chicks resulting in a higher abundance once ileal conditions were suitable for colonisation.
432 Alternatively, the presence of transplanted bacteria in treated chicks may have created favourable
433 metabolic or immunological conditions allowing earlier and greater colonisation by Candidatus
434 *Arthromitus*. Treatments which result in early colonisation by Candidatus *Arthromitus* should be of
435 interest to poultry producers as earlier colonisation by Candidatus *Arthromitus* has been positively
436 correlated with body weight (35). Segmented filamentous bacteria were found in the caecal tonsil on
437 histology. The presence of segmented filamentous bacteria in close approximation to the caecal tonsil
438 epithelium was previously reported in 1978 (42). While previous studies have been conducted to
439 investigate the role of segmented filamentous bacteria on immune development in the ileum of mice
440 (38,43), no studies focus on similar effects in either the ileum or caecal tonsil of chicken.

441 The transplant was more successful in the repeat experiment as evidenced by improved early
442 transplant uptake and presistence of significant differences in alpha diversity until 7 d.p.h. The reason
443 for this variability is hard to assess. Since the storage and application of transplant material was
444 uniform across both experiments, uncontrolled variables such as the initial microbiota or other
445 environmental bacteria may have affected transplant success.

446 A potential use for caecal microbiota transplants in chicks is the competitive exclusion of potential
447 pathogens such as *Enterobacteriaceae* and *Clostridium* during the first week post-hatch. In the repeat
448 experiment there was a significant difference in the colonisation of *Enterobacteriaceae* with a
449 consistently lower abundance in treated chicks. The role of some *Enterobacteriaceae* in the chicken
450 caecal microbiota is unclear. While *Escherichia coli* has the potential for pathogenicity, it is often found

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451 in the caeca of healthy chickens. As such, the higher abundance of *Enterobacteriaceae* in control chicks
452 may not be a cause for concern. However, large blooms of *Enterobacteriaceae* unopposed by other taxa,
453 such as that in control chicks from the repeat experiment, are unlikely to be beneficial to the host. In
454 this regard, the transplant was successful as a similar overgrowth of *Enterobacteriaceae* was avoided
455 in treated chicks. The lower abundance of *Enterobacteriaceae* in treated chicks is likely due to the
456 presence of short chain fatty acid (SCFA) producing bacteria such as *Lachnospiraceae* and
457 *Ruminococcaceae*. Previous studies have found an inhibitory effect of SCFAs on *Enterobacteriaceae*
458 growth both *in vitro* and *in vivo* (44). The treatment had less of an impact on the abundance of
459 *Clostridium*. The most significant species of *Clostridium* in terms of chicken health is *Clostridium*
460 *perfringens* which has been linked to necrotic enteritis in chicks. Direct challenges using this species
461 and other more significant pathogens such as *Campylobacter* and *Salmonella* are required to further
462 explore how caecal microbiota transplants can affect pathogen abundance in the caecum.

463 This study did not find statistically significant differences in intestinal morphology between treated
464 and control chicks, except that the mitotic figure count was higher in both the ileum and the caecum of
465 control chicks at 3 d.p.h. The caecal microbiota of treated and control chicks at this time point was
466 markedly different with control chicks mainly colonised by *Enterobacteriaceae*. If mitotic figure count
467 is reflective of epithelial cell replacement rates, this could imply that the presence of
468 *Enterobacteriaceae* increased epithelial cell replacement. Equally, it could be argued that the lack of
469 *Lachnospiraceae* and *Ruminococcaceae* may have induced higher epithelial cell turnover in control
470 chicks since the bacterial metabolite butyrate decreases apoptosis of normal enterocytes (45). Body
471 weight was also significantly different between treated and control chicks at 3 d.p.h. As with ileal
472 epithelial turnover, if this difference were attributable to the microbiota it is not possible to distinguish
473 if the cause was a negative effect of *Enterobacteriaceae* or positive effect of *Lachnospiraceae* and
474 *Ruminococcaceae*.

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475 No significant differences in immune cell populations were found between treated and control chicks
476 at 3 d.p.h despite large differences in caecal microbiota. While these differences did not have an impact
477 on the number of immune cells in the caecal tonsil, it remains possible that the presence of different
478 bacterial species stimulates differential gene expression in immune cells since a role of SCFA
479 producing bacteria in immune development has been studied in other species (46,47).

480 The presence of bacteria in caecal tonsil crypts has not previously been reported in chickens. This
481 observation was age dependent with sparse bacteria observed at 14 d.p.h compared to 3 and 7 d.p.h.
482 This raises the prospect that the presence of bacteria in the caecal tonsil crypts has some role in
483 immune development which subsequently excludes them from this niche. It was not possible to
484 determine the taxonomy of these bacteria, however, it's likely that they were *Lachnospiraceae* or
485 *Ruminococcaceae* due to the absence of bacteria in the caecal tonsil crypts of all chicks at 0 d.p.h and
486 control chicks at 3 d.p.h. Additionally, a previous study found that these taxa have a higher relative
487 abundance in caecal mucus compared to lumen contents (48).

488 In summary, three topical applications of dilute adult caecal content to the eggshell was sufficient to
489 transplant elements of the caecal microbiota to newly hatched chicks resulting in accelerated
490 development of the caecal microbiota. However, while important members of the caecal microbiota
491 such as *Lachnospiraceae* and *Ruminococcaceae* were successfully transplanted, topical application
492 failed to transplant *Bacteroidaceae* or *Lactobacillaceae*. Topical application of characterised bacterial
493 communities to the eggshell during incubation provides a mechanism to transfer a desirable
494 intetestinal microbiota to chicks and reduce colonisation by possible pathogens. However, treatment
495 ending at 18 d.i only successfully transferred spore-forming bacteria with further experiments
496 required to determine whether non spore-forming microbiota can be transplanted by topical
497 treatments in the hours before or after hatch.

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498 ## Materials and Methods

499 ### Animals and Housing

500 #### Pilot Experiment

501 61 Ross 308 eggs were purchased from a local hatchery (Annyalla Chicks, Wrexham). Hatchery eggs
502 were disinfected daily during storage using a fog application of Virocid® (Cid Lines), a disinfectant
503 based on quaternary ammonium, glutaraldehyde and isopropanol. Eggs at the hatchery are disinfected
504 further using formaldehyde fumigation before being set. On arrival at the experimental housing, 5 eggs
505 were selected for sampling at 0 d.i. The remaining 56 eggs were divided into a treatment group and a
506 control group of 28 eggs each. Each group was housed in different incubators in different rooms. A
507 biosecurity protocol was implemented whereby the control group was handled first to avoid transfer
508 of environmental bacteria from the treatment to the control group. Eggs were incubated at 37.5°C for
509 21 days. The eggs were candled at 7 d.i to assess viability. In both groups, 8 eggs were removed as no
510 embryonic development had occurred. 5 eggs from each group were removed for sampling at 18 d.i.
511 The remaining 15 eggs in each group were left to hatch. 15 and 14 chicks hatched from the treatment
512 and control groups respectively. Chicks were left in the incubators until dry before being transferred to
513 brooder pens with a wood shaving substrate. Water and feed were provided *ad libitum* by a drinker
514 and feeder present in each brooder. Chicks were fed a vegetable protein based starter diet for the
515 duration of the experiment (Table 4). Seven chicks from each group were sampled at 3 days post hatch
516 (d.p.h) with the remaining 8 treated and 7 control chicks sampled at 7 d.p.h. No unexpected deaths
517 occurred in either group over the course of the experiment.

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518 **Repeat Experiment**

519 56 Ross 308 eggs were purchased from a local hatchery (Annyalla Chicks, Wrexham). Eggs underwent
520 the same disinfection procedure at the hatchery as described for the Pilot Experiment. On arrival at the
521 experimental housing, the 56 eggs were divided between two incubators of 28 eggs each. The day that
522 incubation started was defined as 0 d.i. Both incubators were housed in the same room. Eggs were
523 incubated at 37.5°C for 22 days. The eggs were candled at 7 d.i to assess viability. Eggs began to hatch
524 at 20 d.i with 6 chicks hatching on 20 d.i, 21 on 21 d.i and 17 on 22 d.i giving a total of 44 chicks. After
525 hatch, chicks were left in the incubators until dry before being transferred to brooder pens with a
526 wood shaving substrate. On the 22 d.i, 40 day old chicks were purchased from the same hatchery.
527 These chicks were the control group and were housed separately from treated chicks. Water and feed
528 were provided *ad libitum* by a drinker and feeder present in each brooder. Chicks were fed a vegetable
529 protein based starter diet for the duration of the experiment. Five chicks from each group were
530 sampled on the same day the control chicks were brought to the housing (defined as 0 d.p.h). Ten
531 chicks from each group were sampled at 3, 7 and 14 d.p.h. Two chicks from the treatment group died
532 unexpectedly during the experiment, one at 1 d.p.h and another at 6 d.p.h. The cause of death was not
533 determined although a preliminary gross post mortem examination revealed peritonitis and
534 perihepatitis consistent with early opportunistic bacterial infection.

535 **Treatment**

536 Entire caecal contents were collected from healthy 42 day old chickens from three different breeds
537 (Ross 308, Hubbard JA87 and Cobb 500) as part of an experiment to observe the normal development
538 of the caecal microbiota (48). 200mg of caecal contents from five individuals of each breed were
539 pooled and DNA extracted for sequencing. The remaining caecal contents were stored at -20°C for 14
540 months. Before experimental work began, caecal contents from Ross, Cobb and Hubbard birds were

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541 defrosted, mixed and diluted 1:20 in sterile phosphate buffered saline. Aliquots of 5ml of diluted caecal
542 content were prepared and frozen at -20°C for use as treatments. Treatment group eggs were sprayed
543 at 2, 7, 14 and 18 d.i in both trials. The diluted caecal contents was defrosted at room temperature and
544 loaded into a 10ml spray bottle. Eggs were sprayed evenly at a distance of 10cm ensuring all eggs
545 received at least two sprays until the 5ml of diluted caecal content had been used.

546 **Sample Collection**

547 **Pilot Experiment**

548 Samples were taken from eggs at 0 d.i. To minimise the risk of contamination, eggs were sprayed with
549 70% ethanol and left for 10 minutes before being wiped clean. Samples were taken in as sterile an
550 environment as possible. All samples were taken inside an exclusion cabinet and sterile gloves were
551 worn and changed between eggs. An electric rotary tool (Dremel 3000) was used to cut through the
552 egg shell without penetrating the shell membranes. A sterile scalpel was used to cut the shell
553 membrane to remove the top of the egg shell and reveal the yolk. Sterile needles and syringes were
554 used to sample from the albumen and the yolk.

555 At 18 d.i, samples were taken from five embryos from each group. Embryos were killed by
556 refrigerating the egg at 3°C for four hours. The egg shell was opened as previously described. A sample
557 of amniotic fluid was taken using a sterile needle and syringe. The embryo was removed from the egg,
558 placed in a sterile petri dish and placed under a stereomicroscope for dissection. Using a sterile scalpel
559 and forceps, the coelom was opened to reveal the gastrointestinal tract which was removed. The
560 duodenum, jejunum and ileum were stored together with both caeca stored in a separate container.
561 Finally, the brain was removed using a new sterile scalpel to be used as a control for contamination
562 should bacterial DNA be recovered from the gastrointestinal tract.

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563 Further samples were taken at 3 and 7 d.p.h. Chicks were euthanased by cervical dislocation. To
564 sample chicks, the abdomen was sprayed with 70% ethanol. Skin incisions were made to expose the
565 sternum which was then reflected to give good access to the coelom. The gastrointestinal tract was
566 removed carefully to avoid external contamination. The ileum, defined as the intestinal segment from
567 Meckel's diverticulum to the ileocaecocolic junction; and both caeca were removed and stored in
568 separate containers. Samples for DNA extraction were snap frozen in liquid nitrogen and stored at -
569 20°C.

570 **Repeat Experiment**

571 The same sampling protocol was used as for the pilot experiment with chicks sampled at 0, 3, 7 and 14
572 d.p.h. After euthanasia chicks were weighed and their body weight recorded in grams. Additionally,
573 tissue samples from the caecal tonsils, identified as the proximal section of the caecum, and the ileum
574 were taken. One caecal tonsil and a section of ileum were fixed in 4% paraformaldehyde solution for
575 histological examination. The other caecal tonsil was fixed in OCT (CellPath, UK) on a cork plate and
576 snap frozen in liquid nitrogen. Samples fixed in paraformaldehyde were stored at 4°C, samples for
577 DNA extraction were stored at -20°C and samples fixed in OCT were stored at -80°C.

578 **DNA Extraction**

579 DNA was extracted from each sample using Zymobiomics DNA MiniKits (Cambridge Bioscience, UK)
580 according to the manufacturer's instructions. DNA was extracted from 250µl of liquid samples
581 (albumen and yolk). For tissue samples (ileum and caecum), a 200mg section of intestinal tissue along
582 with content was used for DNA extraction. This section was cut longitudinally and transversely using a
583 sterile scalpel blade to expose the mucosa and luminal contents to beat-beating. Both liquid and tissue
584 samples underwent a bead-beating step using a Qiagen TissueLyser at 30Hz for 10 minutes. At each
585 extraction, two controls were included: a blank extraction kit to control for contamination and 75µl of

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586 Zymobiomics Standard Bacterial Community (Cambridge Bioscience, UK) to control for variations in
587 DNA extraction efficacy. Extracted DNA was quantified using a NanoDrop 2000 spectrophotometer
588 (NanoDrop Technologies).

589 **PCR to Detect Bacterial DNA**

590 The detection of bacterial DNA in egg and embryonic samples was performed by PCR detection of
591 bacterial 16S rRNA gene. Purified DNA from egg and embryonic samples from the pilot experiment
592 was used as the template in a PCR mixture composed of 5µl of 5x FIREPol Master Mix Ready to Load
593 (Solis BioDyne, Estonia), 1µl of each primer, 17µl of purified water and 1µl of DNA template. A primer
594 pair spanning the V4 region of the 16S rRNA gene (515F: TGCCAGCMGCCGCGGTAA, R806:
595 GGACTACHVGGGTWTCTAAT) was used (49). DNA extracted from the Zymobiomics Standard Bacterial
596 Community (ZSBC), which contains approximately 1.4×10^{10} cells/ml, was used as a positive control.
597 Thermal cycling consisted of an initial cycle of 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 45 s
598 and 72°C for 40 s followed by a final cycle of 72°C for 40 s. The presence of PCR products was
599 confirmed by electrophoresis using a 1.0% agarose gel containing ethidium bromide. To exclude the
600 possibility that negative results were due to PCR inhibitors present within samples, 9µl of each sample
601 was spiked with 1µl of DNA extracted from the ZSBC and submitted for PCR amplification. To
602 determine the sensitivity of the PCR assay, DNA extracted from the ZSBC was diluted to include the
603 equivalent of DNA extracted from 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 bacterial cells.

604 **Illumina MiSeq Sequencing**

605 Extracted DNA from between five and eight caecal samples in each treatment group at each time point
606 was sent for paired-end sequencing of the 16S rRNA gene at the Centre for Genomic Research
607 (University of Liverpool) using an Illumina MiSeq run. The V4 hypervariable region (515F/R806) was
608 amplified for 25 cycles to yield an amplicon of 254 base pairs (50). Library preparation was performed

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609 using a universal tailed tag design with subsequent amplification performed using a two-step PCR with
610 a HiFi Hot Start polymerase (Kapa) (51). The first round of PCR was performed using the primers 5'-
611 ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3' (forward)
612 and 5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3' (47). The raw
613 Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1.
614 The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of
615 20. Reads shorter than 10 base pairs after trimming were removed. Raw sequence reads are available
616 in the NCBI Sequence Repository Archive under BioProject PRJNA517619.

617 **Data Analysis**

618 QIIME2 version 2019.1.0 was used for analysis of the Illumina data (52). Amplicon sequence variant
619 (ASV) assignment was completed using the dada2 plugin (53) and an ASV table produced using the
620 ASV-table plugin (https://github.com/qiime2/q2-ASV-table) to produce a BIOM format table (54). The
621 resulting ASV table was divided into three individual tables: one containing all samples including
622 transplant samples for use in diversity analyses and one each for samples from the pilot and repeat
623 experiments to identify differentially abundant ASVs between control and treatment groups.
624 Taxonomy was assigned using the q2-feature-classifier plugin (55) with a pre-trained NaiveBayes
625 classifier based on the SILVA 132 database of the 515F/R806 region of the 16S rRNA gene (56)
626 available for download at https://docs.qiime2.org/2018.11/data-resources/.

627 Alpha and beta diversity analyses were performed at a sampling depth of 5,000 using the alignment
628 (57), phylogeny (58) and diversity (https://github.com/qiime2/q2-diversity) plugins. Alpha diversity,
629 a metric used to assess species richness and evenness, was measured using a Shannon diversity index.
630 Taxa plots were produced using the q2-taxa plugin (https://github.com/qiime2/q2-taxa). Beta

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631 diversity, a metric used to compare species diversity and abundance between samples, was calculated
632 with an unweighted and weighted UniFrac metric

633

634 **Statistics**

635 For statistical analysis, samples were grouped according to age, treatment group and experiment
636 creating 13 different groups for comparison: samples from the pilot experiment from control (C) and
637 treated (T) chicks at 3 and 7 d.p.h (PC3, n=7; PT3, n=7; PC7, n=7; PT7, n=8), samples from the repeat
638 experiment from control (C) and treated (T) chicks at 0, 3, 7 and 14 d.p.h (RC0, n=5; RT0, n=5; RC3,
639 n=7; RT3, n=7; RC7, n=7; RT7, n=7; RC14, n=7; RT14, n=7) and transplant material (TRPL, n=3). Alpha
640 diversity was compared between groups using a pairwise Kruskal-Wallis test with a False Discovery
641 Rate correction. An ANOSIM test was used to identify metadata categories which significantly affected
642 beta diversity. The average distance from samples in each group to TRPL samples was compared using
643 an independent Student's t-test to find which group was closest to TRPL samples. Gneiss analysis (29)
644 was used to identify taxa which were differentially abundant between treatment and control groups in
645 the pilot and repeat experiments separately. First, the ASV table was filtered to exclude transplant
646 samples and low abundance ASVs. The count threshold for exclusion of ASVs was set at the first
647 quartile to exclude the lowest 25% of ASVs by total frequency across all samples. Principal balances for
648 use in Gneiss were obtained via Ward's hierarchical clustering using the correlation-clustering
649 command. Log ratios for each balance were calculated using the ilr-transform command. A
650 multivariate response linear regression model of log ratios balances was constructed with treatment
651 and days post hatch as covariates using the ols-regression command. Results were visualised through a
652 regression summary, dendrogram heatmaps and balance taxonomies to identify ASVs which were
653 differentially abundant in treated and control groups. Based on this analysis ASVs were divided into

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654 three groups: ASVs with a higher relative abundance in treated samples, ASVs with a higher relative
655 abundance in control samples and ASVs with no differential abundance between groups. The results of
656 this analysis were used to select taxa for further analysis using quantitative PCR.

657 **Identifying ASVs Transplanted from the Treatment**

658 ASVs present in an unfiltered ASV table of TRPL samples were defined as being present in the
659 transplant material. The same ASV table used for Gneiss analysis was used to compile a list of ASVs
660 present in each sample group. Once lists of ASVs were compiled for the transplant and sample groups,
661 intersections between sets of ASVs were visualised using UpSet (59). ASVs which were present only in
662 the transplant were removed to facilitate visualisation of other intersections. Based on their presence
663 in intersections ASVs were classified as 'successfully transplanted' , 'possibly transplanted' or
664 environmental . ASVs were classified as successfully transplanted if they were present in the
665 transplant and in treated chicks at least one time point before control chicks. ASVs were classified as
666 possibly transplanted if they were present in the transplant and in both treated and control chicks at
667 the same time point. ASVs were classified as environmental if they were present in the transplant and
668 present in only control chicks or present in control chicks before treated chicks. Any ASV not present
669 in the transplant was classified as environmental.

670 A chi-square test of independence was performed to examine the relationship between ASVs identified
671 as differentially abundant between treatment groups and those defined as successfully transplanted,
672 possibly transplanted or environmental using Python's scipy module. The taxonomy of ASVs classified
673 as succcessfully transplanted, possibly transplanted and environmental was compared to that of ASVs
674 identified as more abundant in treated chicks, more abundant in control chicks and not differentially
675 abundant with a Hybrid Sankey diagram created using sankeyview (version 1.7.7) (60).

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676 ## Quantitative PCR

677 Taxa were selected for further testing using quantitative PCR based on results from Gneiss analysis. A
678 literature search was conducted to find suitable primers. Where suitable primers were not available,
679 the sequences retrieved from Illumina sequencing were used to produce taxa specific primers. The
680 sequence was input into Primer-BLAST and a suitable primer pair was chosen. To test specificity of
681 primers, each primer pair was input into TestPrime for comparison against the SILVA database SSU-
682 r132. Further testing of primers was conducted using PCR. The primers were tested against known
683 positive and negative samples to check for the correct amplicon size and non-specific amplification. A
684 gradient PCR was conducted to establish the correct annealing temperature for quantitative PCR.
685 Primers used are displayed in Table 5.

686 The real-time quantitative PCR assay was conducted on a 1:10 solution of extracted DNA using a Rotor-
687 Gene Q PCR machine (Qiagen) and PrecisionPLUS qPCR master mix (Primer Design, UK). The V4 region
688 of the 16S rRNA gene was used as a reference gene. Rotor-Gene Q software (version 2.3.1.49) was used
689 to produce melting curves and identify the cycle threshold (Ct), the point at which fluorescence above
690 the background level is detectable. Each sample was run in triplicate with an averaged Ct used in
691 further analysis. The ΔCt, defined as the difference between the Ct value for taxa specific primers and
692 the Ct value for the reference gene, was calculated for each sample. Results were expressed as 40 –
693 ΔCt. Amplification of DNA in one PC3 sample failed in all reactions. As a result, this sample was
694 excluded from quantitative PCR analysis.

695 ## Haematoxylin and eosin staining

696 Tissue fixed in 4% paraformaldehyde solution was examined histologically to identify differences in
697 morphological development of the ileum and caecal tonsil between treated and control chicks. Four
698 sections of ileum and four sections of caecal tonsil from each chick each chick underwent tissue

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699 processing using a Tissue-Tek VIP (vacuum infiltration processor) overnight before being
700 embedded in paraffin (Ultraplast premium embedding medium, Solmedia). 4µm paraffin
701 sections were cut on a Leica RM2125 RT microtome, floated on a waterbath and placed on
702 colour slides (Solmedia, MSS54511YW). For H&E staining slides were dewaxed in xylene
703 and rehydrated through descending grades of ethanol (100%, 96%, 85%, 70%) to distilled
704 water before being stained in haematoxylin (5mins), "blued" in tap water for 5 mins and
705 stained in eosin (2mins). Slides were then dehydrated through 96% and 100% ethanol to
706 xylene and cover slipped using DPX (Thermo Scientific, Lamb/DPX). Haematoxylin (Atom
707 Scientific, RRBD61-X) and Eosin (TCS, HS250) solutions made up in house.

708 Haematoxylin and eosin stained tissue sections were examined by light microscopy (Nikon Eclipse 80i)
709 with a Leica DMC 4500 digital camera attachment (Leica Microsystems, Switzerland). Images were
710 viewed and measurements taken using Leica Application Suite X software.

711 Sections were assessed for suitability based on orientation of tissue samples. Villus height, villus width
712 and epithelial cell height were recorded in transverse ileal sections where entire villi could be
713 visualised to the lamina propria. In such sections, the height and width of five villi with an intact
714 lamina propria was measured. Villus height was defined as the distance from the villus tip to the villus-
715 crypt junction. Villus width was measured at the widest section of the villus. Epithelial cell height was
716 measured at the villus tip and was defined as the distance from the distal point of the microvilli to the
717 basement membrane. Measurements were expressed as a mean for each bird.

718 Mitotic figure counts in the ileum and caecal tonsil were used as an indication of intestinal villus
719 development (65, 66). All orientations of tissue were included for mitotic counts where crypts were
720 visible adjacent to the lamina propria and muscular layers. Mitotic figures in crypts within one high

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721 power field (400x) of the lamina propria were counted. Cells were identified as mitotic if their nuclei
722 were strongly basophilic and homogenous with care taken to count cells in the late stages of division as
723 a single mitotic figure. The length of lamina propria over which mitotic figures were counted was
724 measured and results expressed as number of mitotic figures per 100μm. Results were expressed as a
725 mean for each bird.

726 Results were compared between treatment groups using Student's t-test implemented in the scipy
727 (version 1.1.0) Python module (67). A Benjamini-Hochberg false discovery rate correction
728 implemented in the statsmodels (version 0.9.0) Python module (68) was applied to account for
729 multiple tests. Results were compared between age groups using a one-way analysis of variance
730 (ANOVA) test with a post-hoc Tukey HSD test when significant differences were identified.

731 During the analysis, it was noted that some samples had large aggregates of bacterial cells within the
732 crypts of the caecal tonsil. In order to ascertain whether the presence of bacteria in the caecal tonsil
733 crypts was associated with age or treatment group, slides were re-examined. Samples were classified
734 as positive if bacteria were observed in more than one crypt and in at least two sections.

735 **Immunostaining**

736 Serial 7.5μm thick sections of caecal tonsil tissue frozen in OCT were cut using a cryostatic microtome.
737 Four sections of caecal tonsil from each bird were mounted on poly-l-lysine coated slides (VWR
738 International, UK) and fixed in acetone for 10 min. Immunostaining was performed on a Dako
739 Autostainer Link 48 using Envision™ FLEX reagents. Following a buffer rinse tissue sections
740 underwent a peroxidase block for 5mins (Agilent, SM801) before being incubated for 20mins with
741 mouse monoclonal antibodies against chicken CD4, CD8α, CD8β, γδ TCR and Bu1 (B cells and subsets
742 of monocytes and macrophages), antigens (Cambridge Bioscience Ltd, 8210-01, 8220-01, 8280-01,
743 8230-01 and 8395-01 respectively). The antibodies CD4 (1:200), CD8α (1:200), CD8β (1:1000), γδ TCR

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744 (1:100) and Bu1 (1:400) were diluted in Envision™ FLEX Antibody Diluent (Agilent, K8006). Antibody
745 binding was detected using the labelled polymer Envision™ FLEX/HRP (Agilent, SM802) for 20mins
746 and the reaction visualised using the substrate-chromogen FLEX DAB+Sub Chromo (Agilent, DM827 &
747 SM802). Tissue sections were counterstained for 5mins in Envision™ FLEX Haematoxylin (Agilent,
748 K8008), washed in deionized water and dehydrated through increasing grades of ethanol (85%, 96%,
749 3x 100%) before clearing in xylene and mounted as per H&E staining above. All intermediate buffer
750 washes between reagents used Envision™ FLEX Wash Buffer (K8007).

751 Stained tissue sections were examined using the same apparatus as described for haematoxylin and
752 eosin stained tissue. Quantification of cell abundance between treated and control chicks was
753 performed by counting cells in photographs taken at a magnification of ×200 with each field of view
754 representing an area of 142,000μm^2. Five photographs for each bird taken randomly from serial
755 sections were used. Results were expressed as a mean for each bird. Student's t test was used to
756 identify significant differences in cell abundance between treatment groups.

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766 ## Conflict of Interest Statement

767 The authors declare that this study received funding from DuPont Industrial Biosciences. The funder
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948 ## Tables

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Table 1. A taxonomy summary at the family level of ASVs identified as higher abundance in treated and control samples from the pilot (A) and repeat experiments (B) by Gneiss analysis. ASVs identified as NDA were not differentially abundant between treatment groups.

A) Pilot Experiment

Taxonomy	Total	Number of Higher Abundance ASVs		NDA
		Treated	Control	
Lachnospiraceae	119	80	18	21
Ruminococcaceae	104	46	7	51
Clostridiaceae 1	21	10	10	1
Erysipelotrichaceae	16	4	9	3
Enterobacteriaceae	10	3	3	4
Peptostreptococcaceae	9	4	5	0
Bacillaceae	7	5	0	2
Clostridiales vadinBB60 group	6	2	0	4
Paenibacillaceae	4	3	1	0
Enterococcaceae	3	1	0	2
uncultured rumen bacterium	2	2	0	0
Lactobacillaceae	2	2	0	0
Christensenellaceae	1	0	0	1
Bacillales	1	1	0	0
Microbacteriaceae	1	1	0	0

B) Repeat Experiment

Taxonomy	Total	Number of Higher Abundance ASVs		NDA
		Treated	Control	
Lachnospiraceae	193	90	20	83
Ruminococcaceae	155	79	17	59
Clostridiaceae 1	39	1	5	33
Clostridiales vadinBB60 group	15	7	1	7
Erysipelotrichaceae	12	2	0	10
Peptostreptococcaceae	11	3	0	8
Enterobacteriaceae	8	0	4	4
Enterococcaceae	7	0	3	4
Bacillaceae	7	4	0	3
Lactobacillaceae	5	2	1	2
Christensenellaceae	3	1	0	2
Paenibacillaceae	3	0	0	3
Microbacteriaceae	2	0	0	2
Staphylococcaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Thermaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1
Streptococcaceae	1	1	0	0
Hydrogenophilaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Alicyclobacillaceae	1	0	0	1

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Table 2. A contingency table showing observed frequencies of ASV classification by differential abundance and transplant success in the pilot (A) and repeat experiments (B). Expected frequencies calculated using a chi squared test of independence are displayed in brackets.

A) Pilot Experiment

	Hihger Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	40 (31)	63 (58)	98 (112)	201
Possibly Transplanted	3 (8)	21 (14)	25 (27)	49
Successfully Transplanted	4 (9)	5 (16)	47 (31)	56
Total	47	89	170	

B) Repeat Experiment

	Higher Abundance in Control	Not Differentially Abundant	Higher Abundance in Treated	Total
Environmental	41 (37)	206 (171)	102 (141)	349
Possibly Transplanted	5 (4)	9 (18)	23 (15)	37
Successfully Transplanted	5 (10)	17 (43)	67 (36)	89
Total	51	232	192	

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Table 3. The taxonomy at the family level of ASVs defined as "Successfully Transplanted", "Possibly Transplanted" and "Environmental" in pilot (A) and repeat experiments (B).

A) Pilot Experiment

Taxonomy	Number of ASVs			
	Total	Successfully Transplanted	Possibly Transplanted	Environmental
Lachnospiraceae	119	37	20	62
Ruminococcaceae	104	13	21	70
Clostridiaceae 1	21	0	2	19
Erysipelotrichaceae	16	4	1	11
Enterobacteriaceae	10	0	1	9
Peptostreptococcaceae	9	0	1	8
Bacillaceae	7	0	1	6
Clostridiales vadinBB60 group	6	0	0	6
Paenibacillaceae	4	0	0	4
Enterococcaceae	3	0	1	2
Lactobacillaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Bacillales	1	0	0	1
Christensenellaceae	1	0	1	0
Microbacteriaceae	1	0	0	1

B) Repeat Experiment

Taxonomy	Number of ASVs			
	Total	Successfully Transplanted	Possibly Transplanted	Environmental
Lachnospiraceae	193	45	8	140
Ruminococcaceae	155	36	22	97
Clostridiaceae 1	39	1	1	37
Clostridiales vadinBB60 group	15	1	2	12
Erysipelotrichaceae	12	3	0	9
Peptostreptococcaceae	11	1	0	10
Enterobacteriaceae	8	1	0	7
Bacillaceae	7	1	0	6
Enterococcaceae	7	0	0	7
Lactobacillaceae	5	0	0	5
Christensenellaceae	3	0	2	1
Paenibacillaceae	3	0	0	3
uncultured rumen bacterium	2	0	2	0
Staphylococcaceae	2	0	0	2
Microbacteriaceae	2	0	0	2
Alicyclobacillaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Hydrogenophilaceae	1	0	0	1
Thermaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Streptococcaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1

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Table 4. Composition of starter and grower diets

Analytical Constituents (%)	Diet	
	Starter	Grower
Crude Fat	2.7	2.4
Crude Protein	18.9	15.6
Crude Fibre	3.8	4.1
Crude Ash	6.6	5.6
Lysine	0.99	0.69
Methionine	0.44	0.27
Calcium	1.05	0.89
Phosphorus	0.7	0.62
Sodium	0.15	0.15
Magnesium	0.17	0.22
Copper	15mg/kg	16 mg/kg

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Table 5. Primer pairs used for quantitative PCR

Target Taxa	Primers	Amplicon Size (b.p.)	Reference
Domain *Bacteria* (targets V4 region)	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	254	(49)
Clostridium	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	131	(61)
Enterobacteriaceae	F: GTGCCAGCMGCCGCGGTAA R: GCCTCAAGGGCACAACCTCCAAG	429	(62)
Candidatus *Savagella*	F: GATGCGTAGGCGGTTGAGTA R: GGGTTTCTAATCCTGTTTGCTCC	90	This study
Clostridium cluster IV	F: TTACTGGGTGTAAAGGG R: TAGAGTGCTCTTGCGTA	580	(63)
Clostridium cluster XIV a&b	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	438-441	(64)

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961 ## Figures

962 **Figure 1**

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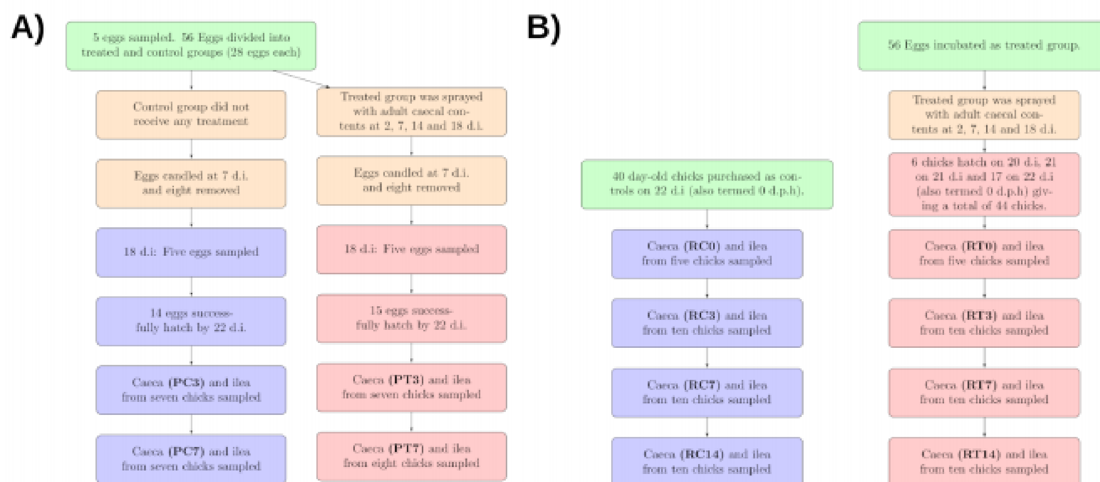

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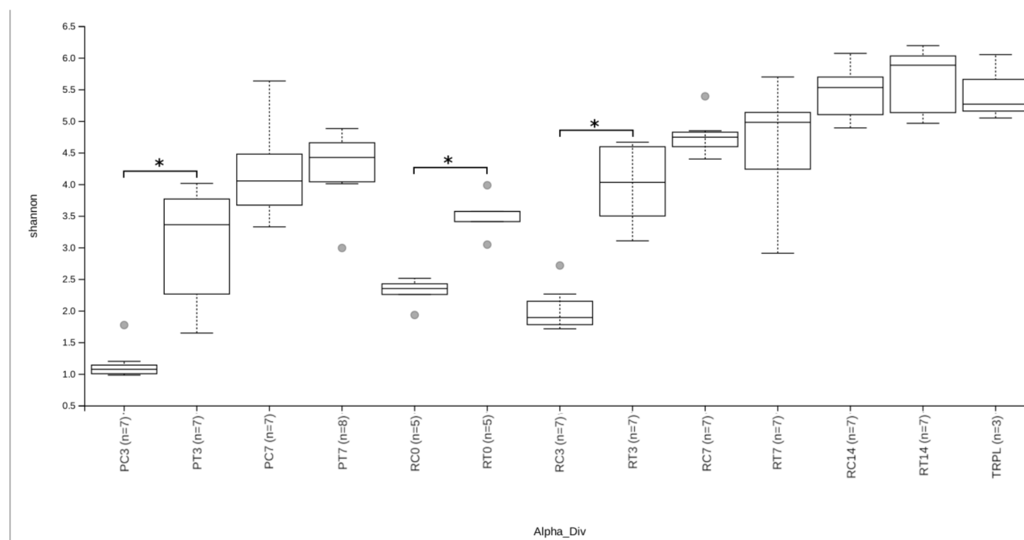
964 Sampling regimes for the Pilot **(A)** and Repeat **(B)** Experiments including abbreviations for sample

965 groups used when discussing the results.

966 **Figure 2**



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974 Alpha diversity measured by a Shannon index at a sequencing depth of 5000.Sample groups divided by
975 experiment (P = pilot, R = repeat), treatment (C = control, T = treated) and age. Shannon diversity of
976 transplant material (TRPL) is also shown. The alpha diversity of treated chicks was significantly higher
977 than that of control chicks at 3 d.p.h (both experiments) and 0 d.p.h (repeat experiment). * $p < 0.05$.

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979 **Figure 3**

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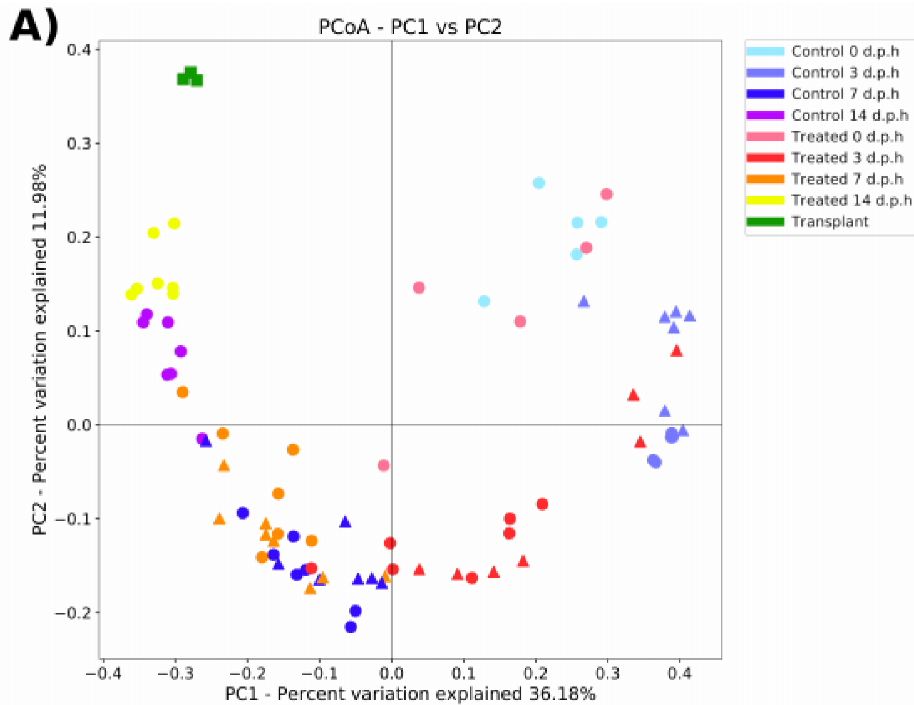

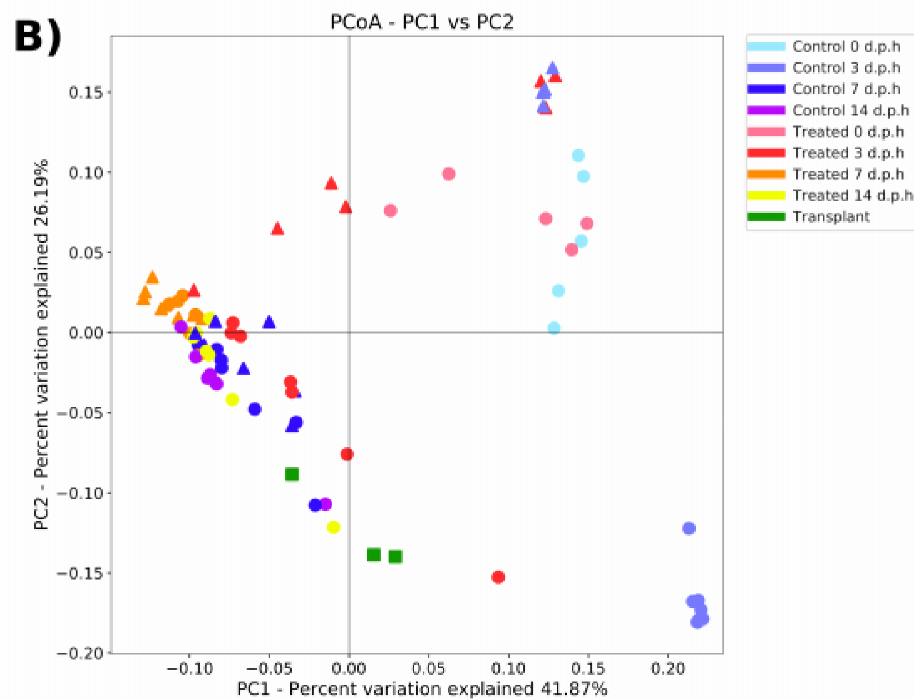

981 Principal
982 coordinat
983 es
984 analysis
985 (PCoA)
986 plot
987 showing
988 differenc
989 es in
990 unweight
991 ed **(A)**
992 and
993 weighted
994 **(B)**
995 UniFrac
996 beta
997 diversity
998 between
999 sample
1000 groups
1001 and

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1002 treatment groups in pilot (triangle), repeat experiments (circle) and transplant samples (square). Each

1003 point represents an individual sample with distance between points representative of differences in

1004 microbiota composition.

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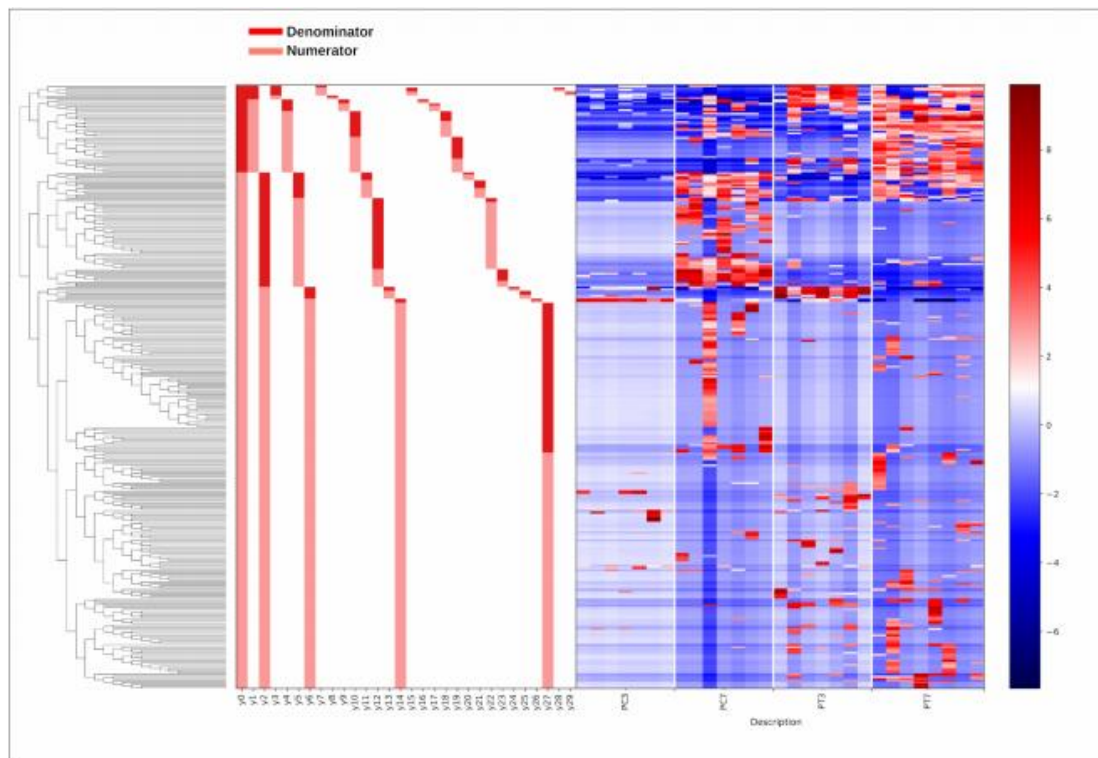

1007 **Figure 4**

1008

1009 A dendrogram heatmap of ASV log abundance in the caecal microbiota of control and treated chicks at
1010 3 and 7 d.p.h in the pilot experiment. The dendrogram represents the organisation of ASVs within the
1011 system of balances created by hierarchical clustering. Each node on the dendrogram is a balance with
1012 the first node designated balance y0. Each terminal branch represents a ASV present within the
1013 analysis. The bar charts visualise which ASVs are denominator (dark red) and which are numerator
1014 (light red) ASVs for each balance. The heatmap shows log abundance of each ASV in samples organised

1015 by group. Low abundance ASVs are represented by blue while higher abundance ASVs are represented

1016 by red.

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Figure 5

A dendrogram heatmap showing log abundance of ASVs in the caecal microbiota of control and treated chicks at 0, 3, 7 and 14 d.p.h in the repeat experiment. The dendrogram represents the organisation of ASVs within the system of balances created by hierarchical clustering. Each node on the dendrogram is a balance with the first node designated balance y0. Each terminal branch represents a ASV present within the analysis. The bar charts visualise which ASVs are denominator (dark red) and which are numerator (light red) ASVs for each balance. The heatmap shows log abundance of each ASV in samples organised by group. Low abundance ASVs are represented by blue while higher abundance ASVs are represented by red.

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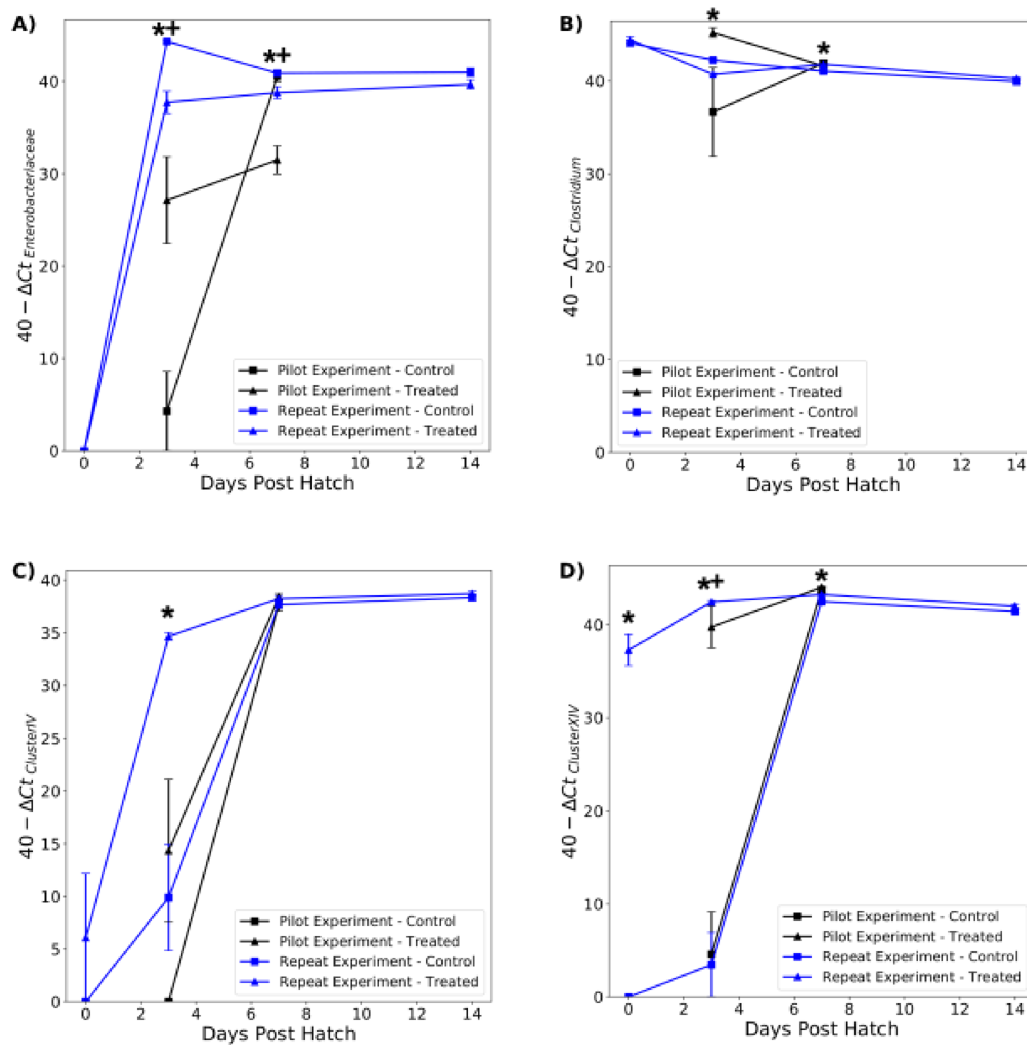
1028 **Figure 6**



1029 Relative abundance of *Enterobacteriaceae* **(A)**, *Clostridium* **(B)**, *Clostridium* Cluster IV **(C)** and

1030 *Clostridium* Cluster XIVa&b **(D)** in the caeca of treated and control chicks between 0 and 14 d.p.h.

1031 Significant differences between treated and control chicks in the pilot (+) and repeat (*) experiments

1032 are indicated.

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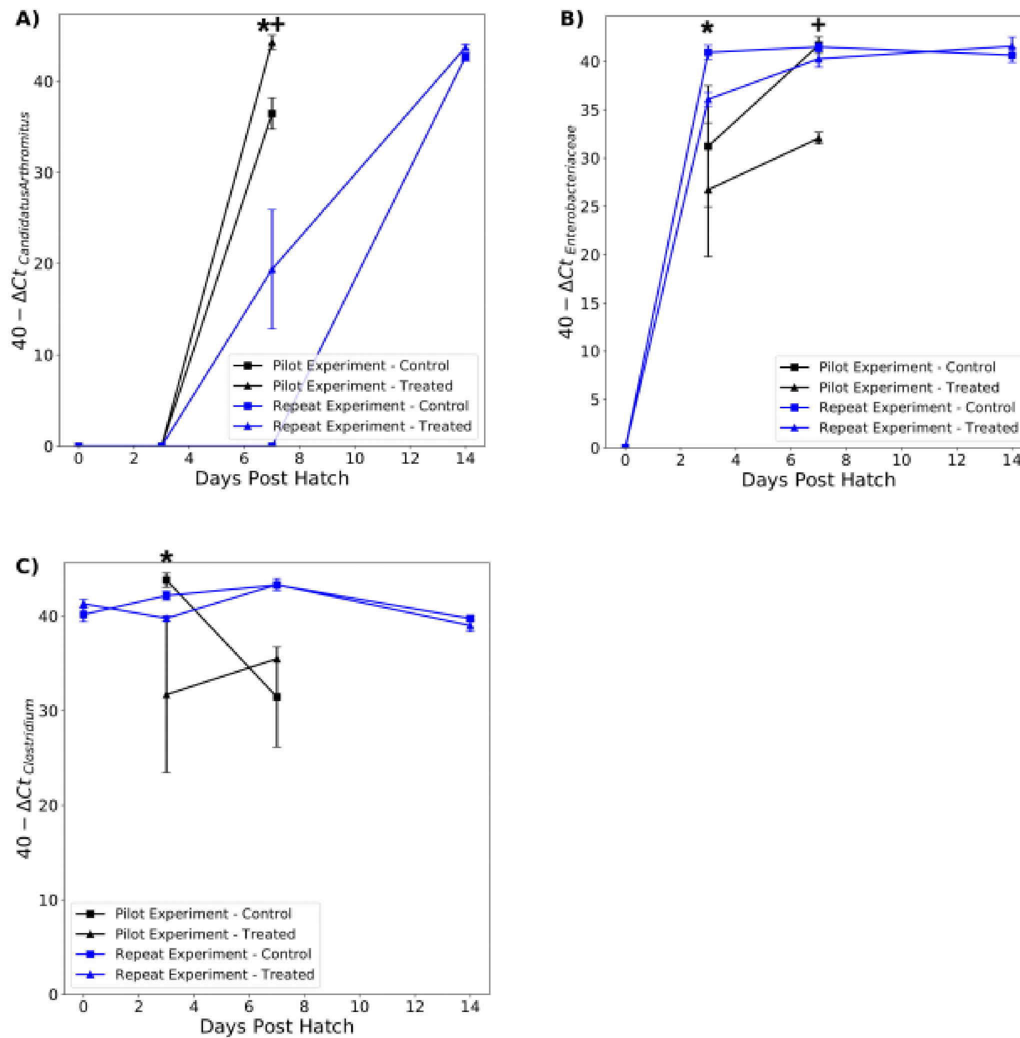

1033 **Figure 7**

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1035 Relative abundance of Candidatus *Arthromitus* **(A)**, *Enterobacteriaceae* **(B)** and *Clostridium* **(C)** in the

1036 ilea of treated and control chicks between 0 and 14 d.p.h. Significant differences between treated and

1037 control chicks in the pilot (+) and repeat (*) experiments are indicated.

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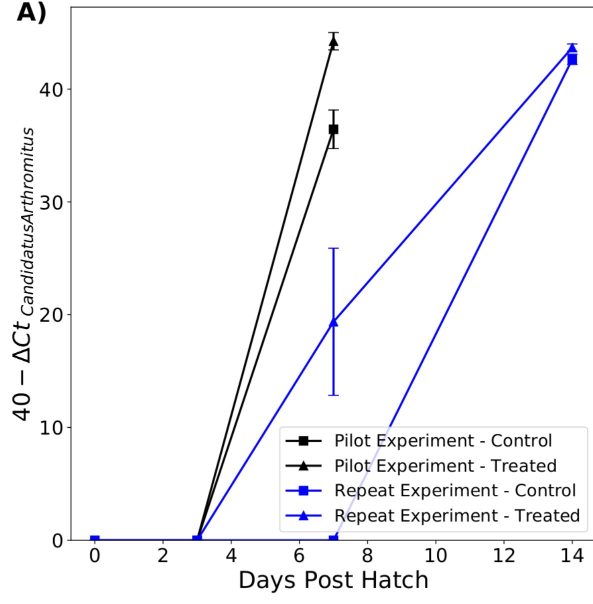

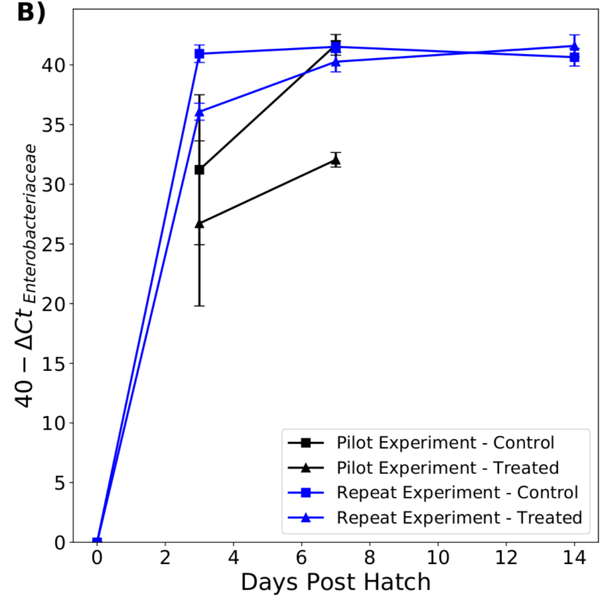

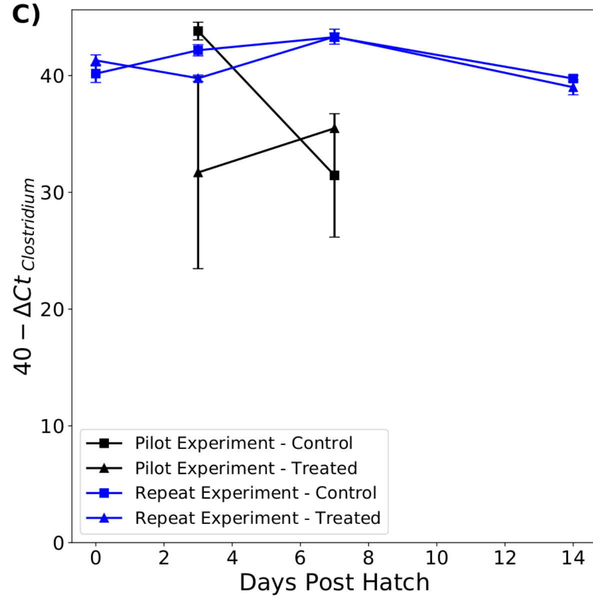

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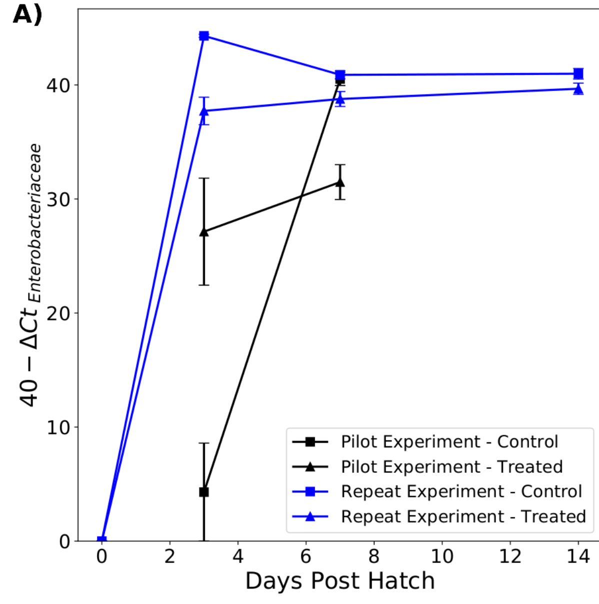

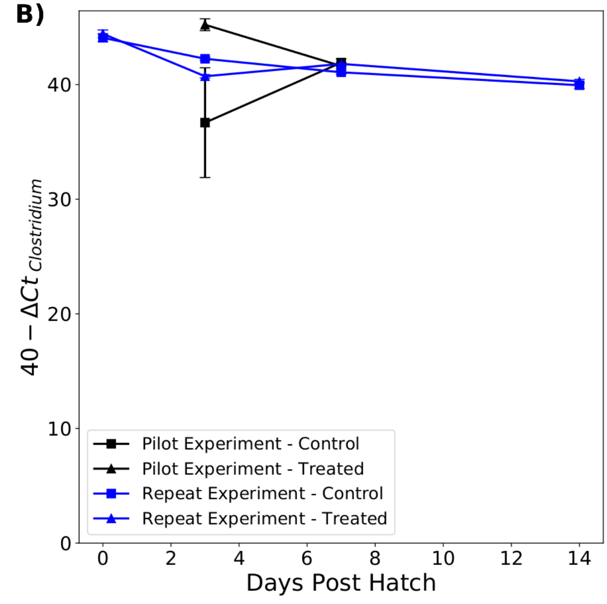

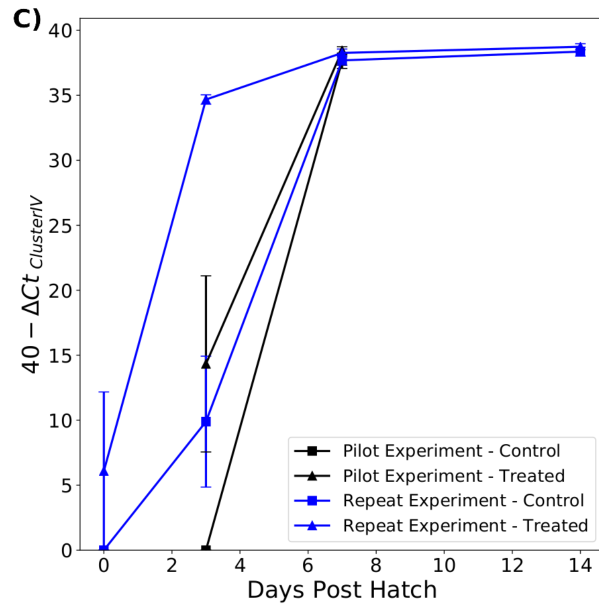

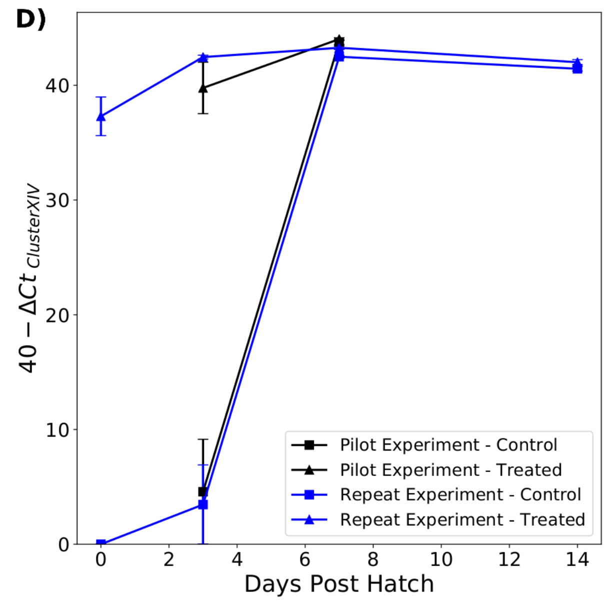

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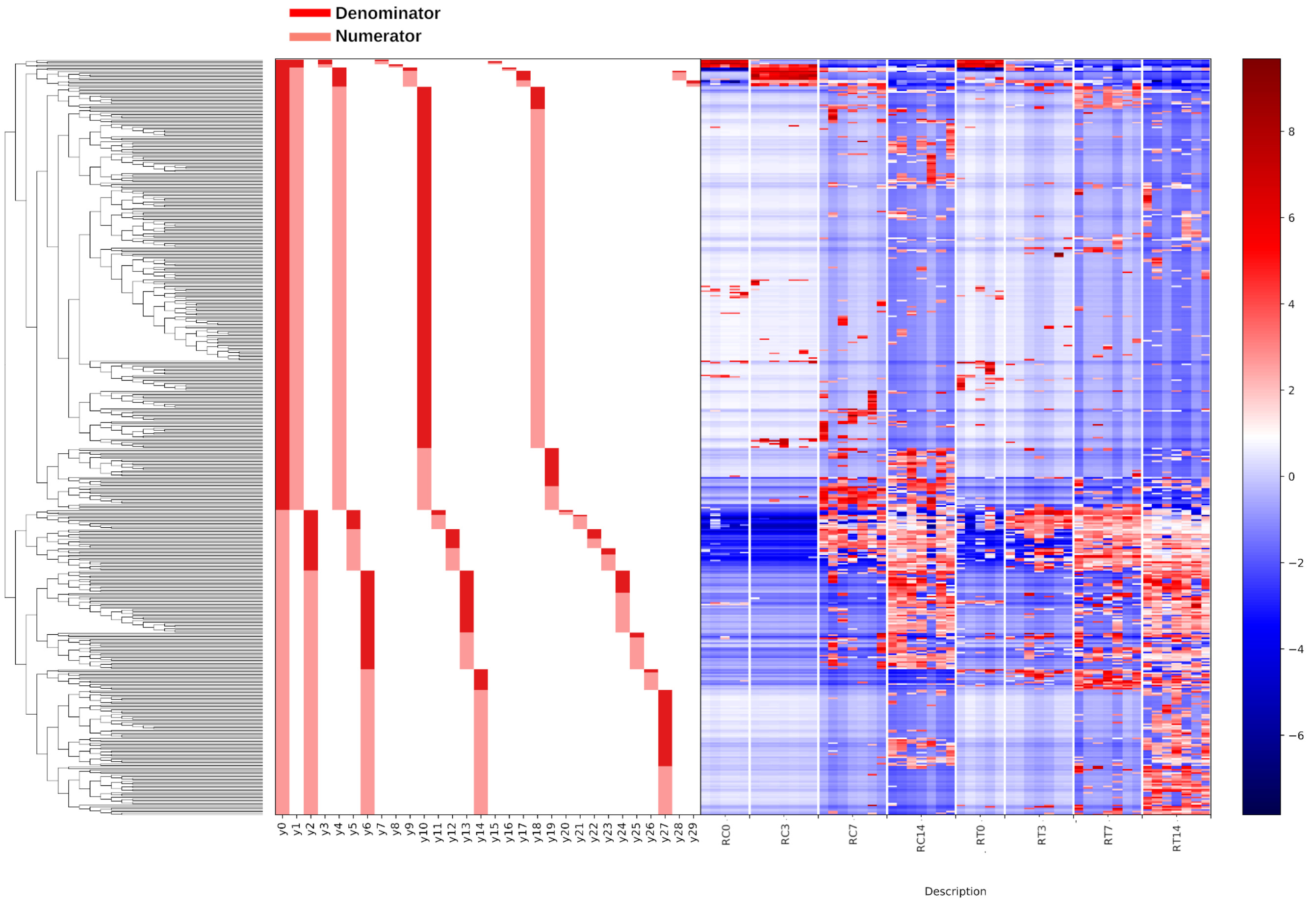

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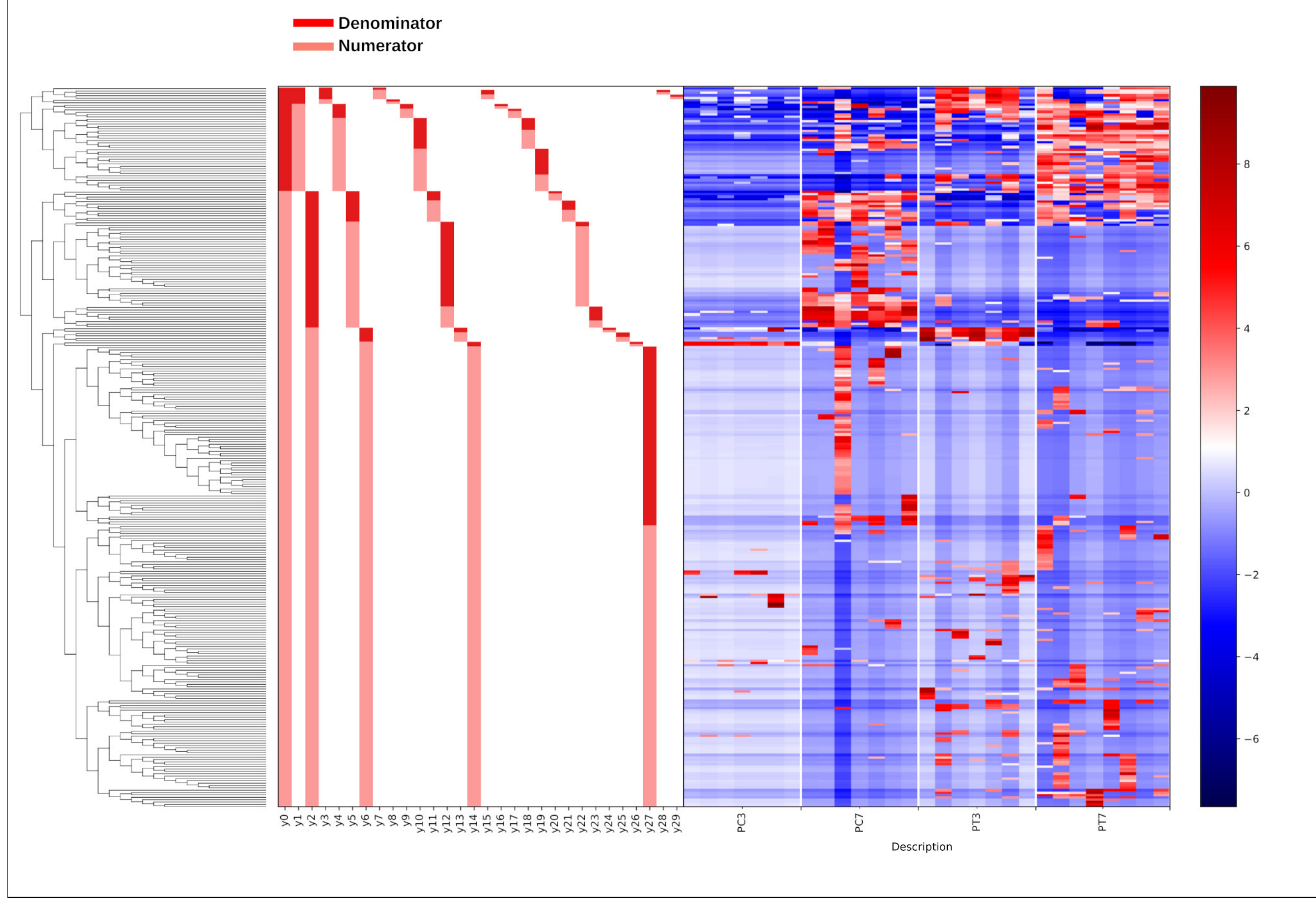

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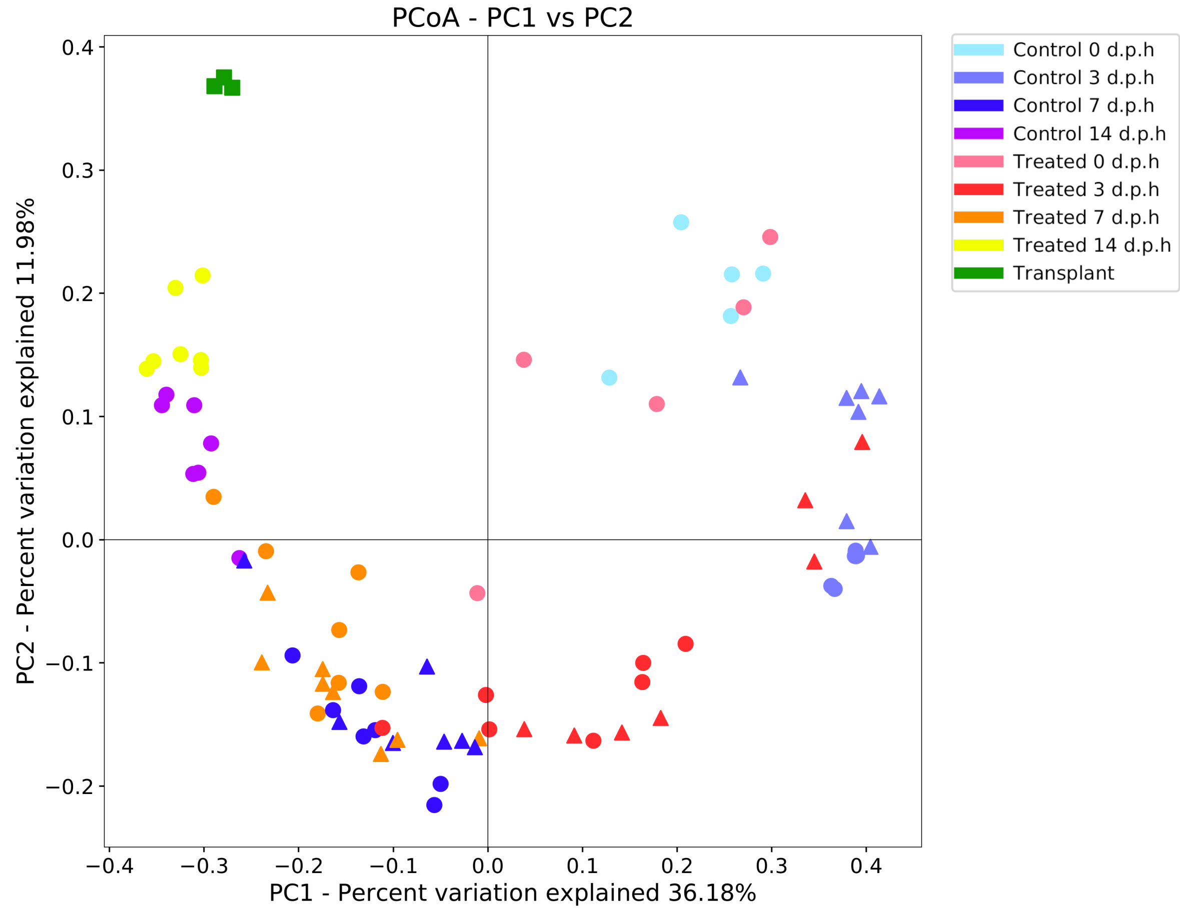

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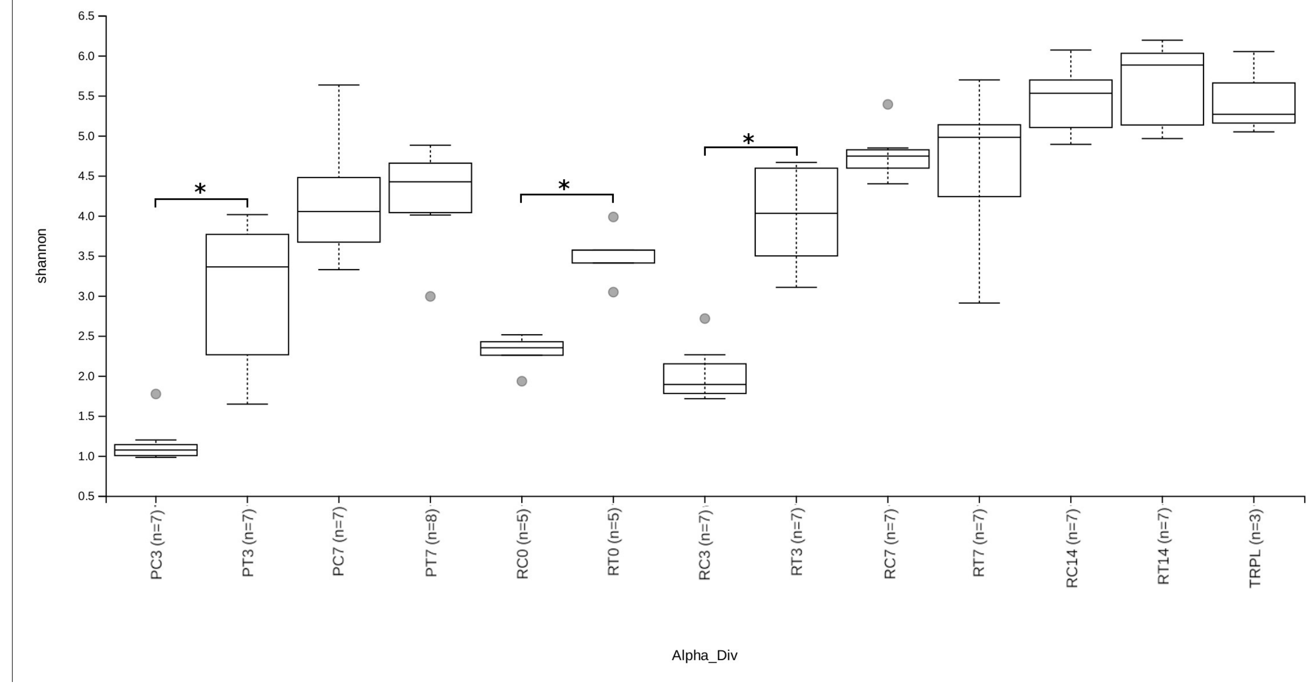

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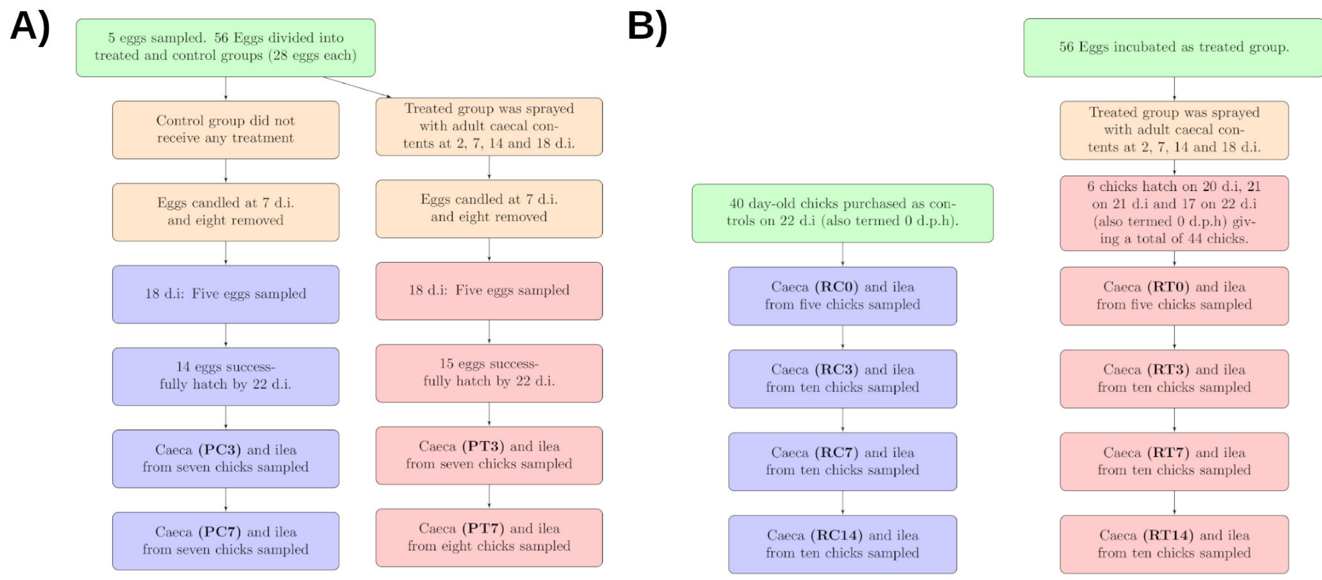

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Table 4. Primer pairs used for quantitative PCR

Target Taxa	Primers	Amplicon Size (b.p.)	Reference
Domain *Bacteria* (targets V4 region)	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	254	(57)
Clostridium	F: TGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT	131	(58)
Enterobacteriaceae	F: GTGCCAGCMGCCGCGGTAA R: GCCTCAAGGGCACAACCTCCAAG	429	(59)
Candidatus *Savagella*	F: GATGCGTAGGCGGTTGAGTA R: GGGTTTCTAATCCTGTTTGCTCC	90	This study
Clostridium cluster IV	F: TTACTGGGTGTAAAGGG R: TAGAGTGCTCTTGCGTA	580	(60)
Clostridium cluster XIV a&b	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	438-441	(61)

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Table 3. The taxonomy of features defined as "Successfully Transplanted", "Possibly Transplanted" and "Environmental" in pilot (A) and repeat experiments (B).

A) Pilot Experiment

	Number of ASVs			
Taxonomy	**Total**	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	119	37	20	62
Ruminococcaceae	104	13	21	70
Clostridiaceae 1	21	0	2	19
Erysipelotrichaceae	16	4	1	11
Enterobacteriaceae	10	0	1	9
Peptostreptococcaceae	9	0	1	8
Bacillaceae	7	0	1	6
Clostridiales vadinBB60 group	6	0	0	6
Paenibacillaceae	4	0	0	4
Enterococcaceae	3	0	1	2
Lactobacillaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Bacillales	1	0	0	1
Christensenellaceae	1	0	1	0
Microbacteriaceae	1	0	0	1

B) Repeat Experiment

	Number of ASVs			
Taxonomy	**Total**	**Successfully Transplanted**	**Possibly Transplanted**	**Environmental**
Lachnospiraceae	193	45	8	140
Ruminococcaceae	155	36	22	97
Clostridiaceae 1	39	1	1	37
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Erysipelotrichaceae	12	3	0	9
Peptostreptococcaceae	11	1	0	10
Enterobacteriaceae	8	1	0	7
Bacillaceae	7	1	0	6
Enterococcaceae	7	0	0	7
Lactobacillaceae	5	0	0	5
Christensenellaceae	3	0	2	1
Paenibacillaceae	3	0	0	3
uncultured rumen bacterium	2	0	2	0
Staphylococcaceae	2	0	0	2
Microbacteriaceae	2	0	0	2
Alicyclobacillaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Hydrogenophilaceae	1	0	0	1
Thermaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Streptococcaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1

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Table 2. A contingency table showing observed frequencies of feature classification by differential abundance and transplant success in the pilot (A) and repeat experiments (B). Expected frequencies calculated using a chi squared test of independence are displayed in brackets.

A) Pilot Experiment

	Differentially Abundant in Control	Not Differentially Abundant	Differentially Abundant in Treated	Total
Environmental	40 (31)	63 (58)	98 (112)	201
Possibly Transplanted	3 (8)	21 (14)	25 (27)	49
Successfully Transplanted	4 (9)	5 (16)	47 (31)	56
Total	47	89	170	

B) Repeat Experiment

	Differentially Abundant in Control	Not Differentially Abundant	Differentially Abundant in Treated	Total
Environmental	41 (37)	206 (171)	102 (141)	349
Possibly Transplanted	5 (4)	9 (18)	23 (15)	37
Successfully Transplanted	5 (10)	17 (43)	67 (36)	89
Total	51	232	192	

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Table 1. A taxonomy summary of ASVs identified as differentially abundant in treated and control samples from the pilot (A) and repeat experiments (B) by Gneiss analysis. ASVs identified as NDA were not differentially abundant between treatment groups.

A) Pilot Experiment

	Number of Differentially Abundant ASVs			
Taxonomy	**Total**	**Treated**	**Control**	**NDA**
Lachnospiraceae	119	80	18	21
Ruminococcaceae	104	46	7	51
Clostridiaceae 1	21	10	10	1
Erysipelotrichaceae	16	4	9	3
Enterobacteriaceae	10	3	3	4
Peptostreptococcaceae	9	4	5	0
Bacillaceae	7	5	0	2
Clostridiales vadinBB60 group	6	2	0	4
Paenibacillaceae	4	3	1	0
Enterococcaceae	3	1	0	2
uncultured rumen bacterium	2	2	0	0
Lactobacillaceae	2	2	0	0
Christensenellaceae	1	0	0	1
Bacillales	1	1	0	0
Microbacteriaceae	1	1	0	0

B) Repeat Experiment

	Number of Differentially Abundant ASVs			
Taxonomy	**Total**	**Treated**	**Control**	**NDA**
Lachnospiraceae	193	90	20	83
Ruminococcaceae	155	79	17	59
Clostridiaceae 1	39	1	5	33
Clostridiales vadinBB60 group	15	7	1	7
Erysipelotrichaceae	12	2	0	10
Peptostreptococcaceae	11	3	0	8
Enterobacteriaceae	8	0	4	4
Enterococcaceae	7	0	3	4
Bacillaceae	7	4	0	3
Lactobacillaceae	5	2	1	2
Christensenellaceae	3	1	0	2
Paenibacillaceae	3	0	0	3
Microbacteriaceae	2	0	0	2
Staphylococcaceae	2	0	0	2
uncultured rumen bacterium	2	2	0	0
Thermaceae	1	0	0	1
Sanguibacteraceae	1	0	0	1
Streptococcaceae	1	1	0	0
Hydrogenophilaceae	1	0	0	1
Burkholderiaceae	1	0	0	1
Propionibacteriaceae	1	0	0	1
Leuconostocaceae	1	0	0	1
Nocardiaceae	1	0	0	1
Peptococcaceae	1	0	0	1
Moraxellaceae	1	0	0	1
Alicyclobacillaceae	1	0	0	1