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1 **Impact of industrial production system parameters on chicken microbiomes: mechanisms**
2 **to improve performance and reduce *Campylobacter***
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23 **Keywords**
24 Chicken
25 Microbiome
26 *Campylobacter*
27 Production Systems
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29 Phylogenetic Signal

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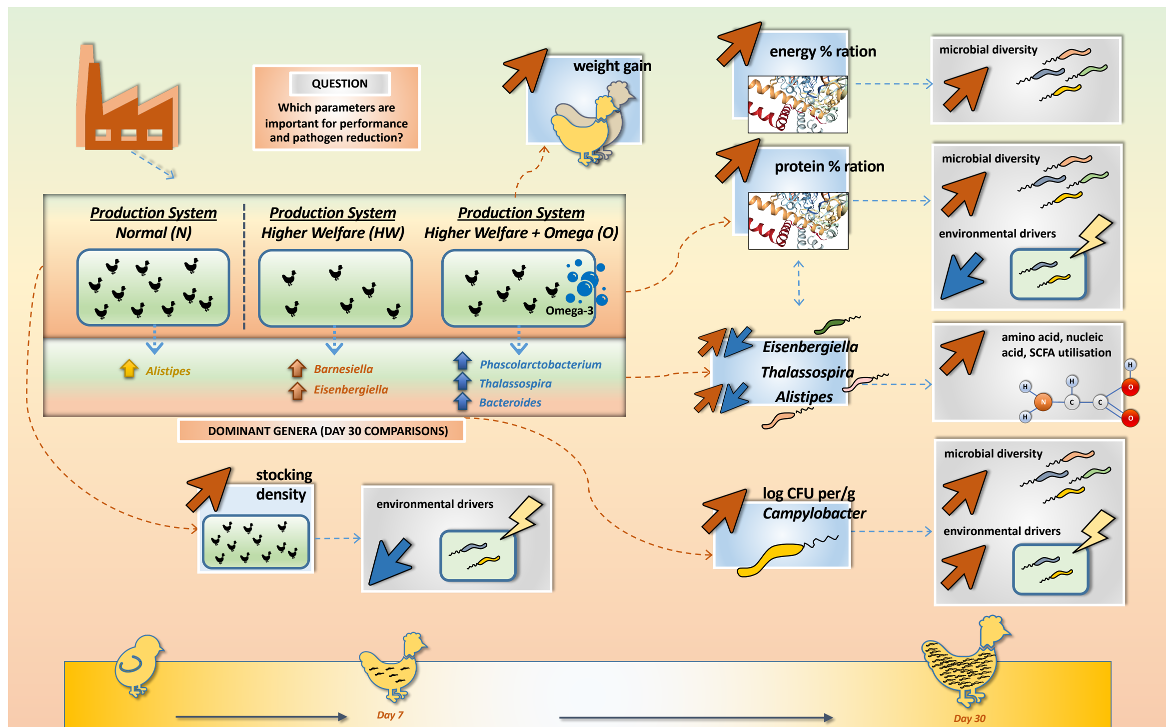
32

33 **Graphical Abstract:**



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45 **Abstract**

46 Background

47 The factors affecting host-pathogen ecology in terms of the microbiome remain poorly studied.
48 Chickens are a key source of protein with gut health heavily dependent on the complex microbiome
49 which has key roles in nutrient assimilation and vitamin and amino acid biosynthesis. The chicken
50 gut microbiome may be influenced by extrinsic production system parameters such as *Placement*
51 *Birds/m^2* (stocking density), feed type and additives. Such parameters, in addition to on-farm
52 biosecurity may influence performance and also pathogenic bacterial numbers such as
53 *Campylobacter*. In this study, three different production systems 'Normal' (N), 'Higher Welfare'
54 (HW) and 'Omega-3 Higher Welfare' (O) were investigated "in a natural environment" at day 7 and
55 day 30 with a range of extrinsic parameters assessing performance in correlation with microbial
56 dynamics and *Campylobacter* presence.

57 Results

58 Our data identified production system N as significantly dissimilar from production systems HW
59 and O when comparing the prevalence of genera. An increase in *Placement Birds/m^2* density led
60 to a decrease in environmental pressure influencing the microbial community structure. Prevalence
61 of genera such as *Eisenbergiella* within HW and O, and likewise *Alistipes* within N were representative.
62 These genera have roles directly relating to energy metabolism, amino acid, nucleotide and short chain
63 fatty acid (SCFA) utilisation. Thus, an association exists between consistent and differentiating
64 parameters of the production systems, that affect feed utilisation, advance our knowledge of mechanistic
65 underpinnings, leading to competitive exclusion of genera based on competition for nutrients and
66 other factors. *Campylobacter* was identified within specific production system and presence was linked
67 with the increased diversity and increased environmental pressure on microbial community structure.
68 Addition of Omega-3 though did alter prevalence of specific genera, in our analysis did not differentiate
69 itself from HW production system. However, Omega-3 was linked with a positive impact on weight gain.

70 Conclusions

71 Overall, our results show that microbial communities in different industrial production systems are
72 deterministic in elucidating the underlying biological confounders, and these recommendations are

73 transferable to farm practices and diet manipulation leading to improved performance and better
74 intervention strategies against *Campylobacter* within the food chain.

75 Background

76 Chickens are a key source of protein for humans where poultry production is predicted to produce
77 approximately 130 million tons of chicken meat in 2020 [1, 2]. Sustainable poultry practices are
78 needed to help maintain an adequate supply of poultry products for the increasing human
79 population without compromising the chicken or human health [3]. Selective breeding programmes
80 has resulted in chickens that efficiently convert food into body mass, as defined by feed conversion
81 ratios (FCR) [4]. The fundamental component needed to ensure an efficient poultry production is
82 highly dependent in having an optimised nutrition and production set up [1].
83 Production systems vary immensely between countries, businesses and at farm level. Certain
84 parameters such as *Placement Birds/m*^{2} (stocking density), *Protein_perc_ration* (protein percentage
85 within ration) and *Energy_of_ration* (energy content) in relation to the feed are key determinants that if
86 varied, may directly influence chicken microbial community structure. Thus, impacting performance and
87 potentially reduction of pathogenic bacteria. Chicken diets are typically formulated to enhance
88 production efficiency. However, some diets are formulated to enhance human health, such as diets
89 containing Omega-3 polyunsaturated fatty acids (PUFA's) [5]. There is anecdotal evidence at farm
90 level to suggest that this enrichment has potential to improve chicken gut health and performance
91 or reduce pathogen colonisation.
92 Many bacterial species found within the microbiome of farmed animals can potentially be
93 considered pathogens and detrimental to human health. One of these pathogens is *Campylobacter*
94 which is the leading cause of human foodborne bacterial gastroenteritis causing bloody diarrhoea,
95 fever and abdominal pains in humans and can also cause post infectious sequelae such as
96 Guillain-Barré syndrome which is a potentially fatal paralytic autoimmune illness [6].
97 *Campylobacter jejuni* (predominant species causing infection to humans) colonises chicken ceca
98 with relatively high numbers (>10^{9} CFU per gram) and can be pathogenic to the chicken, with this
99 dependent on the genetics of host and strain of infection [7-10]. Although there exists many
100 intervention studies, in an industrial farm environment, we currently do not understand why we

101 typically see *Campylobacter* at approximately two weeks into the chicken life cycle [11-13]. The
102 natural growth and flux of the gut microbiome may have a role to play [14]. This is further
103 convoluted by the fact that there are very few studies on how chicken diet impacts *Campylobacter*
104 presence within the chicken gut microbiome, particularly from within an industrial farm
105 environment.

106 Chicken performance and gut health is heavily dependent on the largely unexplored complex gut
107 microbial community which plays a role in nutrient assimilation, vitamin and amino acid
108 biosynthesis and prevention of pathogen colonization [15-18]. The microbiota is responsible for
109 hydrolysing indigestible carbohydrates and polysaccharides allowing further fermentation by other
110 members of the gut ecosystem that produce SCFA, in turn allowing utilisation by the host [1]. The
111 relationship between gut microbiota, chicken health and performance represents a tripartite that
112 has been under the scrutiny of the research community with the prospect of improving the
113 efficiency of current microbiome manipulation strategies [19, 20]. As an example, a xylanase gene
114 from chicken cecum has been isolated and overexpressed, potentially leading to development of
115 new feed additives for industrial application [21]. Our understanding of diet and its impact on the
116 intestinal microbiota is still nascent and requires further exploration.

117 In the present study, we aim to build on our previous research where we performed a
118 comprehensive analysis of the chicken cecal microbiome from days 3 to 35 investigating the driving
119 forces of bacterial dynamics over time, and how this relates to *Campylobacter* appearance within
120 a natural habitat setting [14]. Microbial variation over time was heavily influenced by the diet, where
121 significant shifts in bacterial composition were observed [14]. The factors affecting host-pathogen
122 ecology in terms of microbial community structure remain poorly studied at an industrial farm level.
123 Therefore, extending our previous work and in view of the above ambitions, in this study, three
124 different industrial production systems, namely, ‘Normal’ (N), ‘Higher Welfare’ (HW) and ‘Omega-
125 3 Higher Welfare’ (O) were investigated at day 7 and day 30, along with extrinsic parameters (which
126 were not available in our previous study) to ascertain mechanisms on improving the overall
127 performance of chickens, and also elucidating the role of microbial community dynamics on
128 revealing *Campylobacter* pathogenesis.

129 **Materials and Methods**

130 **Ethics Statement**

131 Euthanasia of birds was carried out under veterinary supervision alongside routine veterinary
132 diagnostic inspection and after consultation and approval from the ethical committee within Moy
133 Park.

134 **Experimental design, broilers and sample collection**

135 Chickens reared under three different industrial growing regimes, ‘Normal’ (N), ‘Higher Welfare’
136 (HW) and ‘Omega-3 Higher Welfare’ (O), were sourced from three different contract farms
137 supplying chicken to Moy Park (39 Seagoe Industrial Estate, Portadown, Craigavon, Co. Armagh,
138 BT63 5QE, UK).

139 Although the three production systems differ in receiving chicks from multiple flocks, our analysis
140 suggested that they had no bearing on microbial community structure at finer level (Fig. 3 and
141 discussion later) although some parameters were implicated as significant in PERMANOVA
142 analysis. All chicks were Ross 308 as hatched (AH) and were supplied from the same Moy Park
143 hatchery to all three farms on 11/10/2018. All birds were grown in typical industrial poultry houses
144 and were raised on a four-stage diet made up of a starter, grower, finisher and withdrawal ration,
145 however composition of these diets differed across the three growing regimes. Farm N – Birds
146 were offered standard starter ration from days 0 to 11, standard grower ration from days 11 to 22
147 and standard finisher from day 22 to day 34 before moving to a standard withdrawal ration prior to
148 slaughter. Farm HW - Birds were offered higher welfare starter ration from days 0 to 11, higher
149 welfare grower ration from days 11 to 23 and higher welfare finisher from day 23 to day 31 before
150 moving to a higher welfare withdrawal ration prior to slaughter. Farm O - Birds were offered higher
151 welfare starter ration from days 0 to 11, higher welfare grower ration from days 11 to 20 and
152 Omega-3 finisher ration from day 20 to day 30 before moving to an Omega-3 withdrawal ration
153 prior to slaughter. Both Omega-3 finisher rations are identical to HW equivalent ration, but with
154 addition of an Omega-3 premix produced by Devenish Nutrition Ltd (Belfast, UK) and added at the
155 feed mill. The change in dietary ration has influenced microbial community structure, as can be

156 seen later in the analysis. At day 7, 19 chickens and at day 30, 10 chickens were randomly
157 removed from the same single poultry house on each of the three farms.

158 **Campylobacter isolation and identification**

159 The contents of a pair of ceca were transferred into a sterile stomacher bag (~10 g ± 1 g), diluted
160 with maximum recovery diluent (MRD) buffer to make a 1/10 dilution and stomached at 260 rpm
161 for 1 minute. Further decimal dilutions were carried out in MRD to give a range of dilutions suitable
162 to achieve countable plates. 100 µl of the original suspension (10^{-1} dilution) was inoculated onto
163 duplicate plates of mCCDA (Oxoid, UK). The inoculum was spread uniformly over the surface of
164 the agar plate with a sterile spreader until fully absorbed. This spread plating was repeated for all
165 the other decimal dilutions. When low numbers of *Campylobacter* were expected, the limit of
166 detection was increased by also spread plating 1 ml of the original suspension. The 1 ml inoculum
167 was inoculated over three plates. The plates were incubated in a microaerophilic atmosphere (5%
168 oxygen, 10% carbon dioxide and 85% nitrogen) at 41.5ºC and examined after 44 ± 4 hours for
169 typical colonies of *Campylobacter* spp. Plates containing less than 300 colonies were counted.
170 Colonies were considered typical if they were greyish (often with a metallic sheen), flat and moist,
171 with a tendency to spread. *Campylobacter* numbers were expressed as CFU/g cecal content.
172 Genomic DNA (gDNA) was also extracted from chicken ceca for 16S metagenomics experiments.

173 **Poultry Growth and Performance Measurements**

174 Performance parameters on per flock basis were recorded in line with typical industrial practices.
175 *Weight_Gain_per_Day*, *Feed_Conversion_Ratio*, *Total_Mortality_percentage*, *Energy_of_Ration*,
176 *Protein_perc_ration*, *Water_Consumption_per_Bird*, *Age_At_Thin*, *Age_at_Total_Depopulation*,
177 *PMI_Rejects_percentage*, *Hockmark_percentage*, *Pododermatitis_percentage*,
178 log_CFU_per_g_Campylobacter, EPEF were all captured. These variables were then correlated
179 with the microbial community's composition in various statistical analyses (Supplementary S13).

180 **DNA extraction, 16S rRNA amplification and sequencing**

181 Cecal gDNA was extracted using QIAamp DNA Stool Mini Kit according to the manufacturer's
182 instructions and stored at -20°C. 16S metagenomic sequencing library construction was performed
183 using Illumina guidelines (Illumina, U.S.A). The 16S ribosomal primers used were V3

184 (tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag) and V4
185 (gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaatcc) [22, 23]. A second PCR step was
186 performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit.
187 Sequencing was performed on Illumina MiSeq at LSHTM using a v3 300 bp paired-end kit.

188 **Bioinformatics and Statistical Analysis**

189 Details are described in the supplementary methods (Supplementary S12).

190 **Results**

191 ***Diversity patterns representative of the production systems***

192 At alpha-diversity level, to investigate how diversity within the samples were influenced by the
193 production system, both richness and Shannon entropy were calculated for day 7 and day 30
194 samples from production systems N, HW and O. In the host microbiome, time has a significant
195 effect on microbial richness with all production systems significantly increasing from day 7 to day
196 30 (Fig. 1a). For day 7, production system N displayed the highest microbial richness when
197 compared to HW and O. At day 30, no statistical difference in terms of richness was identified
198 between the three production systems. Beta diversity using *Bray-Curtis* was measured, along with
199 these OTUs collated together at genera level with only top-25 most abundant genera shown next
200 to the PCoA diagram (Fig. 1b). At day 7, in terms of abundance counts, samples from production
201 system O are far off from the clusters that contain samples from N and HW, respectively. On the
202 other hand, at day 30, N seems to have a different community structure as compared to N and HW
203 production systems, respectively. The breakdowns of taxa going from finer (OTUs), up coarser
204 levels (family, class, phylum etc), are shown in Supplementary S3. Note, we are only considering
205 the top-25 most abundant taxa identified at different taxonomic levels. Of interest, clear differences
206 were observed using visual cues when comparing production system N at day 30 to HW and O,
207 where genera *Bacteroides* and *Alistipes* were present in production system N at higher
208 abundances.

209 Ecological drivers of microbial community were explored observing the clustering in the
210 phylogenetic tree of the OTUs and utilising phylogenetic alpha diversity measures such as
211 NRI/NTI. Positive NRI/NTI values indicate strong phylogenetic clustering (driven by environmental

212 filtering), whereas reduced values represent phylogenetic overdispersion (environment has little or
213 no role to play). Here, using NRI and NTI we can observe an increase in N, HW and O samples
214 respectively from day 7 to day 30. Since chicken ceca are already a constrained environment to
215 begin with (as opposed to real environmental datasets), the values <0[a] (traditionally this implies
216 stochasticity) may not be feasible to ascertain randomness/stochasticity/competitive exclusion
217 principle, and hence values should be taken relatively with an increasing value implying increasing
218 host environmental pressure (in the immediate case it is the environment within a chicken, whilst
219 extrinsic environmental factors such as surroundings where chicken are confined and their diet
220 may also have a role to play). Fig. 1a corroborates our findings from our previous longitudinal study
221 [14].

222 ***Key drivers of microbial community structure variation in terms of beta diversity***

223 Next, we wanted to explore what drives the beta diversity amongst different categories (production
224 system) and time. In this regard, we employed the "BVSTEP" routine [24] which was used to search
225 for the highest correlation, in a Mantel test, by imploding the abundance table at genera level to
226 the absolute minimal set of genera that preserve the beta diversity between samples in reduced
227 feature space as compared to the complete feature space. This subset analysis was performed by
228 considering all samples and then correlating the subset of genera for these samples that explain
229 approximately the same beta diversity distances through permutation analysis (Supplementary
230 S1). At the same time, after imploding to the subset of genera, we wanted to see if the resulting
231 subset still has discriminatory power (in terms of grouping) through PERMANOVA analysis. In
232 addition to PERMANOVA analysis, we employed several discriminatory algorithms such as i)
233 DESeq2 with adjusted p-value significance cut-off of 0.05 and log2 fold change cut-off of 2 to find
234 upregulated genera and vice versa (Supplementary S11), ii) Heat Tree analysis to find clades that
235 were differentially expressed (Fig. 2), and iii) MINT analysis to consolidate the genera that have

[a] It should be noted that NRI reflects phylogenetic clustering in a broad sense (whole phylogenetic tree) with lower values representing evenly spread community. On the other hand, NTI focuses more on the tips of the tree with positive values of NTI indicating that species co-occur with more closely related species than expected, and lower values indicating that closely related species do not co-occur by chance.

236 simultaneous discriminatory power at both spatial (production system N, HW and O) and temporal
237 scales (day 7 and day 30)(Supplementary S2). To aid interpretation, all up/downregulated taxa are
238 annotated with up and down arrows in Figure 1 according to which analysis they were selected in
239 and with taxa from subset analysis as the seeding point. Redundancy in these analyses was to
240 ensure there was no biases associated with the mathematical algorithms to find discriminating
241 terms. In some cases, direction of the discrimination was contradictory, for example, there were a
242 few cases where DESeq2 and Heat Tree agreed, whilst MINT analysis showed a reciprocal trend.
243 This latter disagreement may possibly be because MINT consolidates all possible sources of
244 variation. Nonetheless, we wanted to be comprehensive and therefore discuss only those taxa
245 which achieve a majority consensus on multiple analyses albeit MINT underperforming in most
246 cases.
247 For production system HW, *Alistipes*, *Ruminococcaceae UCG-014* and *Escherichia-Shigella* were
248 significant genera increasing from day 7 to day 30*.* *Bacteroides* displayed significant increase at
249 day 7 (decreasing at day 30) using DESeq2 and Heat Tree. For production system O,
250 *Thalassospira*, *Alistipes* and *Bacteroides* increased at day 30 (*Bacteroides* directionality was only
251 observed with MINT analysis). *Lachnoclostridium*, *Eisenbergiella*, *Escherichia-Shigella* all
252 increased at day 7 (decreasing at day 30). For production system N, *Lachnoclostridium*,
253 *Eisenbergiella* increased at day 7 (decreasing at day 30). *Bacteroides* was identified as decreasing
254 at day 7 (increasing at day 30; directionality was only observed with MINT analysis). In general,
255 *Alistipes* was observed to increase consistently at day 30 for all production systems, whilst all other
256 genera showed mixed trends. Although it should be noted that *Alistipes* was present at higher
257 abundance at production system N as compared to others.
258 Key genera were identified when comparing production systems at day 7. For HW vs O
259 comparison, *Lachnoclostridium* was identified as increased for HW. *Escherichia-Shigella* was
260 identified increased for production system O. For O vs N comparison, *Bacteroides* was increased
261 for O. This was also replicated for HW when comparing to N. Key genera were identified when
262 comparing production systems at day 30. For HW vs O comparison, *Phascolarctobacterium*,
263 *Thalassospira* and *Bacteroides* were identified as increased for O, whereas *Barnesiella* was

264 increased for HW when comparing to O. *Subdoligranulum*, *Eisenbergiella*, *Alistipes*,
265 *Ruminiclostridium* 5 and *Ruminococcaceae UCG-014* were all identified as part of the subsets that
266 explain beta diversity, although they were not implicated as discriminating in differential analyses.
267 For N vs HW comparison at day 30, *Eisenbergiella* was increased for HW, and *Alistipes* was
268 increased for N. For O vs N comparison at day 30, *Bacteroides* was observed with subset analysis
269 alone. *Thalassospira* was observed, yet its discriminatory power was inconclusive.
270 Core microbiome where genera persist in 85% of the samples (something that is traditionally used
271 to define the prevalence of core microbiome) for different production systems (day 7 and day 30)
272 was assessed (Fig. 2). Genera identified include *Bacteroides*, *Lachnoclostridium*, *Eisenbergiella*,
273 *Ruminoclostridium 9*, *Lactobacillus*, *Ruminococcaceae*, *Shigella flexneri K−671*, *Flavonifractor*,
274 *Ruminococcaceae*, *Lachnospiraceae*, *Ruminiclostridium 5*, *Ruminiclostridium*, *Coprococcus 1*,
275 *Ruminiclostridium 5* at varying level of abundance. In Fig. 2, OTUs are sorted by their abundances
276 with those on the top being low abundant prevalent OTUs, whereas those at the bottom are highly
277 abundant prevalent OTUs.

278 ***Parameters deriving microbial community structure***

279 Analysis of parameters that had a significant effect on microbial diversity were assessed using
280 PERMANOVA against performance parameters when using different dissimilarity measures on
281 microbiome data (Table 1). Using R^2, if significant, to represent the variability explained in the
282 community structure for Bray-Curtis distance, the parameter with the greatest impact was days,
283 explaining 21.2% of the variation. Next, key parameters of interest were:
284 *log_CFU_per_g_Campylobacter* (4.1% variability), *Energy_of_Ration* (4.0% variability) and
285 *Protein_perc_ration* (1.2% variability). Using both unweighted and weighted *UniFrac* as a beta
286 diversity measure, the pattern was more or less similar with the same trends with days having the
287 greatest impact on microbial diversity (32.7% and 43.0% respectively). All other parameters had
288 R^2 values at 1-5%.

289 ***Direction of influence for extrinsic parameters influencing key metrics for the microbiome***

290 Whilst PERMANOVA analyses show the extent of influence on microbiome structure in terms of
291 variability, to obtain directions as to whether an increase or decrease in these parameters cause

292 an increase or decrease in the properties of microbiome, we resorted to performing subset
293 regressions on one-dimensional realisation of microbiome (alpha, beta diversity measures etc).
294 These subset regressions permuted through all possible subsets of explanatory variables (extrinsic
295 parameters considered in this study) by ranking them in terms of quantitative fit after performing
296 cross-validation (Supplementary S4 to S10 and summarised in Fig. 3). Note that red and blue
297 backgrounds represent whether predictors have a positive or a negative influence along with the
298 significances, respectively in the regression model. In addition, all categorical variables were
299 "dummyfied" (a standard procedure) to represent as present/absent as a tag and were used in the
300 regression model to see whether their inclusion/exclusion has an effect on the final model. As
301 expected, measuring alpha diversity, inclusion of day 30 samples increases richness and Shannon
302 entropy. Although inclusion of day 7 samples led to an increased environmental pressure, it was
303 marginally significant and therefore deterministic nature of microbial communities at local and
304 terminal clade level should be taken with caution. In terms of how samples differ from each other,
305 LCBD was also considered in subset regression analysis with positive/red explanatory variables
306 causing community structure to become different from the average community structure as a mean
307 to identify groups that were markedly different. This was only observed for *Bray-Curtis* distance
308 metric (that considers abundances of taxa without their phylogenetic distances) at day 7, and
309 unweighted *UniFrac* (phylogenetic distance considering only presence/absence of taxa without
310 considering their abundance) for day 30.
311 An increase in *Protein_perc_ration* led to a positive effect on microbial diversity, whilst
312 simultaneously reducing the effect of environmental pressure on microbial community structure. In
313 terms of beta diversity measure (LCBD with different distances), overall there was a reduction in
314 beta diversity when considering *Protein_perc_ration*, although the trend was opposite for
315 unweighted UniFrac. *Energy_of_ration* also causes the microbial diversity to be more even as it
316 had a positive and significant influence on Shannon entropy. The *Feed_conversion_ratio* on the
317 other hand only caused shift in environmental pressure affecting the terminal clades by making
318 them more clustered though the NTI measure.

319 In terms of production systems, it was observed that samples belonging to production system N
320 had less influence by the environment and were possibly driven by competitive exclusion
321 principles. Of note, production system N also had the lowest (best) *Feed_conversion_ratio*. A
322 decrease in *Feed_conversion_ratio* was noted to lead to reduced environmental influence, which
323 aligns with production system N having a reduced environmental influence, and also that a higher
324 *Placement_birds/m*^{2} resulting in a reduced environmental influence. The same phenomena were
325 also observed when considering *Age_at_thin*, *Hockmark__percentage* and *Water_consumption*
326 *_per _bird* as explanatory variables. Interestingly, recorded *log_CFU_per_g_Campylobacter* led to
327 an increase in microbial diversity, with an increase in environmental pressure (at global scale: NRI,
328 and also at local terminal clustering: NTI) as well as causing a marked shift in terms of beta
329 diversity. Of all production systems, *Campylobacter* was only identified in production system N at
330 day 30 based on 16S rRNA abundance count (Supplementary S2), corroborated with independent
331 log CFU/g of *Campylobacter* measure.

332 **Discussion**

333 Our data clearly show that there is a difference in microbial community structure between
334 production systems with varying influence by extrinsic parameters considered within this study.
335 We observed diversity increase significantly for day 30 when compared to day 7. This is in line with
336 previous reports whereby the gastrointestinal (GI) tract of poultry comes into contact with
337 exogenous microorganisms immediately after hatch and as the host grows, this microbiome
338 becomes highly diverse until it reaches a relatively stable yet dynamic state [25]. An important
339 finding in this study is that amongst the different production systems, only N is where microbial
340 community assemblage seems to be random (less environmental pressure). Stocking density is
341 clearly a parameter which if varied, can alter microbial community structure significantly. This
342 demonstrates that production systems can be modified to alter the microbiome profile influencing
343 performance at farm level.

344 Dietary nutrient components are implicated in improving the performance of broiler chickens. An
345 increasing *Energy_of_ration* and decreasing *Protein_perc_ration* in feed over time, pertain to all
346 production systems within this study. These variables have a direct influence on gut microbial

347 composition, and in conjunction with differentiating parameters between production systems (i.e.
348 stocking density), lead to differences in microbial composition between production systems. The
349 role of diet is sufficiently important for microbial community structure assemblage as previously
350 described whereby digestion of non-starch polysaccharides (NSPs; found in the grain of chicken
351 feed) lead to production of SCFA, which are absorbed across mucosa and catabolised by the host
352 [15, 26]. SCFAs contribute to chicken nutrition and also lower pH which can inhibit acid-sensitive
353 pathogens and improve mineral absorption [17, 18]. Thus, an association exists between the
354 consistent and differentiating parmeters of production systems, that affect feed utilisation, leading to
355 competitive exclusion of genera based on competition for nutrients and other factors.
356 Genera that were differentially expressed between different production systems and days were
357 identified (some also part of the core microbiome). For day 30, HW vs O comparison,
358 *Phascolarctobacterium*, *Thalassospira* and *Bacteroides* were identified as increased for O.
359 *Phascolarctobacterium* is involved in SCFA production, including acetate and propionate and
360 described as an option for reduction of *Campylobacter* via competitive exclusion [27-29]. Here,
361 Omega-3 fed poultry systems harboured *Phascolarctobacterium* at a higher prevalence, although
362 we cannot state if this was directly associated with a reduction or absence of *Campylobacters* from
363 the cecal community. *Eisenbergiella* was increased for HW vs N comparison at day 30, and
364 *Alistipes* was increased for N when compared to HW at day 30. A reduction in *Eisenbergiella* has
365 been associated with gastrointestinal disorders linked to metabolic and microbiota changes (functional
366 dyspepsia) resulting in defective energy metabolism, amino acids, nucleotides and SCFA [30]. The HW
367 system may improve the metabolism-microbiome interaction and could result in a competitive exclusion
368 of bacterial pathogens via a fortified immune system. A dysfunctional microbiota can induce
369 metabolic, autoimmune and inflammatory diseases, and can seriously undermine gut function [31-
370 33]. *Barnesiella* was increased for HW when comparing to O. Presence of *Barnesiella* has been
371 associated with prevention and spread of highly antibiotic-resistant bacteria [34] where the gut
372 bacterial community may react to antibiotic inclusion in the diet and prophylactically encourage the
373 presence of *Barnesiella*, an effect demonstrated in mice where ampicillin treatment increased their

374 presence [35]. The competitive exclusion hypothesis is supported by the increase of *Alistipes* bacteria
375 in N group at day 30, and using the top-25 most abundant taxa identified at OTU level where clear
376 differences for N against HW and O were observed (also for genera *Bacteroides*). The presence
377 of *Subdoligranulum* bacteria in HW and O production systems, although not discriminatory, still
378 contributing to beta diversity, represents a sign of improved gut health as these bacteria are known
379 to be involved in production of SCFAs (e.g. butyrate) with an important role in gut physiology [36].
380 *Ruminiclostridium 5*, identified within HW and O production systems, also within core microbiome,
381 has been noted to impact SCFA concentration within the gut [37].
382 The European Union (EU) ban on antimicrobial growth promoters in 2006 has created an increased
383 need to devise alternative methods to improve performance and potentially reduce numbers of
384 pathogenic bacteria. Examples include use of natural plant derived products such as carvacrol
385 [38], addition of dietary prebiotics [39] and administration of live probiotic bacteria [40]. More
386 recently, prebiotic galacto-oligosaccharides (GOS) have been added to broiler feed and enhanced
387 the growth rate and feed conversion of chickens relative to those obtained with a calorie-matched
388 control diet [41]. Recent developments has observed chicken diets enriched with Omega-3 PUFA's
389 for benefits to human health [5]. Omega-3 fatty acids α-linolenic acid (ALA) (often found in plants),
390 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (found typically in marine oils), all
391 play a role in forming structural components of cell membranes, serve as precursors to bioactive
392 lipid mediators, and provide a source of energy [42]. PUFAs are also important as substrates for
393 inflammatory and anti-inflammatory acids, with EPA is believed to have anti-inflammatory
394 properties [43]. One of the proposed mechanisms as to how dietary antibiotics exert their growth
395 promoting benefits is via anti-inflammatory effects towards the intestinal epithelium, by inhibition
396 of production and excretion of catabolic mediators [44]. Increased levels of EPA following antibiotic
397 supplementation align with this non-antibiotic, anti-inflammatory theory of antibiotic growth
398 promotion [45]. In our study, we do not know if PUFAs were fully absorbed, and to what effect the
399 interaction is with host microbiome, however we do observe an increase in weight for production
400 system O which has Omega-3. Of note, production system N had the lowest (best) FCR.

401 Remarkably, *log_CFU_per_g_Campylobacter* led to an increase in microbial diversity, an increase
402 in environmental assemblage metrics NRI and NTI, and also increased divergence in community
403 structure from other samples. *Campylobacter* was identified in production system N at day 30
404 (Supplementary S2) along with measuring the log CFU/g of *Campylobacter* corroborating the 16S
405 data. It has been previously reported that *Campylobacter* typically appears within week two of the
406 life cycle [11-14, 46]. The lack of identification of *Campylobacter* at any of the day 7 samples is
407 anticipated. It is interesting that detection of *Campylobacter* was only observed at day 30 for
408 production system N. This may have been due to limitation of sampling points. Although we
409 recognise the diversity will increase naturally from day 7 to day 30, based on our data,
410 *Campylobacter* is associated with an increasing diversity. Broiler genetics is known to impact
411 microbial diversity [47, 48] and this may explain why in our study the environmental pressure was
412 significantly impacted positively by the presence of *Campylobacter*. Here the environmental
413 influence may be the chicken host genetics influencing microbial community structure.

414 **Conclusion**

415 Our findings demonstrate a relative role of different production system parameters in shaping the
416 bacterial communities' impact on the chicken microbiome, with stocking density playing a major
417 role influencing microbial dynamics. Specific genera with higher prevalence were identified within
418 production systems that have key roles in energy metabolism, amino acid, nucleotide and SCFA
419 utilisation. It is clear that parameters between production system (whether constant or variable) have an
420 impact on microbial diversity which subsequently influences feed breakdown and hence instigates
421 competitive exclusion of certain genera. Omega-3 had a positive impact on weight gain and
422 *Campylobacter* presence was linked with environmental pressure, which may be the external
423 environment or the host itself. Future studies that will direct the optimisation of extrinsic parameters
424 and optimisation of diets targeting microbes with the underlying benefits of improving performance
425 will aid in reducing pathogens such as *Campylobacter*. Our study is the first to investigate the relative
426 importance of production system parameters in an industrial farm environment without any intervention
427 strategy (studying cecal microbiome at its natural environment), to reveal the factors that link microbial

428 community structure to improved broiler performance, and reduced pathogenic bacteria such as
429 *Campylobacter*.
430
431 **Availability of supporting data**
432 The raw sequence files supporting the results of this article are available in the European
433 Nucleotide Archive under the project accession number PRJEB34742.
434
435 **Competing interests**
436 AM, AR and UL are employed by company Moy Park. AM is also a PhD student between Moy
437 Park and AFBI. All other authors declare no competing interests.
438
439 **Author contributions**
440 AM, AR, UL, UZI, NC and OG contributed to the study design. NC and OG managed the study.
441 CK, AM and ML performed the sample collection and DNA extraction. AM and OG performed the
442 library preparation and Illumina MiSeq sequencing at the LSHTM. UZI wrote the analysis scripts
443 to generate the figures and tables in this paper. UZI, AM and OG performed the bioinformatics and
444 statistical analysis. UZI, AM, NC and OG drafted the initial version of the manuscript with all authors
445 including ND, BWW and WTS contributing to redrafting.
446
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450 **List of Figures and Tables**
451 **Figure 1**: **Microbial diversity and community structure**. (a) represents alpha diversity (Richness
452 and Shannon entropy) and environmental filtering (NRI/NTI) measures respectively. Lines for
453 figure (**a)** connect two categories where the differences were significant (ANOVA) with * ($P < 0.05$),
454 ** ($P < 0.01$), or *** ($p < 0.001$). (b) beta diversity using Bray-Curtis distance measure along with
455 Top-25 genera observed in all samples grouped by categories. The tables represent taxa that were

456 found to be significant based on Subset analysis (Supplementary S1) i.e., those genera selected
457 in the subsets that explain roughly the same distance between samples as all the genera.
458 Additionally, if the taxa were found to be differentially expressed based on other analyses, such as
459 DESeq2 (Supplementary S11), MINT (Supplementary S2), Differential Heat Tree (Figure 2), the
460 categories they up and down-regulated are represented with corresponding up and down arrows.
461 For example, in HW30 vs O30 comparison, "(S), ↑O30 (D, H)" for *Phascolarctobacterium* should
462 be read as selected in subset analysis: (S) and upregulated in O30 according to both DESeq2 and
463 Differential Tree: ↑O30 (D, H).

464 **Figure 2**: **Taxa that persist and those that are differentially abundant.** (a) Core microbiome
465 analyses that persist in 85% of the samples for different production systems (day 7 and day 30).
466 In the heat maps the OTUs are sorted by their abundances with those on the top being low
467 abundant whereas those at the bottom are highly abundant. (b) Differential heat tree with taxonomy
468 key given in the middle, and the branches where they are upregulated are colored according to
469 their respective categories shown on top of each subpanel.

470 **Figure 3**: **Heatmap of key extrinsic parameters that influence different attributes of**
471 **microbiome**. The figure is based on subset regressions (Supplementary S4 to S10), where red
472 and blue represent the significant positive and negative beta coefficients that were consistently
473 selected in different regression models. The categorical variables are represented with a yellow
474 highlight (coded as 1 (present) or 0 (absent)) and if selected is interpreted as the samples
475 belonging to those categories having positive/negative influence on the respective microbiome
476 metrics.

477 **Table 1**: Statistics for PERMANOVA against performance parameters when using different
478 dissimilarity measures on microbiome data. R^2 represents the proportion of variability explained,
479 for example, using "Days" and "Bray-Curtis" dissimilarity, the days explain 21.2% variability in
480 microbial community structure.

481 **List of Supplementary Material**

482 **Supplementary S1-Subset Analysis.docx**: Subset analysis from BVSTEP routine listing top
483 subsets with highest correlation with the full genera table considering Bray-Curtis distance and
484 considering samples provided in the first column. For each subset, PERMANOVA was performed
485 against Groups considered in the first column.

486 **Supplementary S2-MINT Analysis.pdf**: **MINT study-wise discriminant analysis between**
487 **treatments (N, HW, and O)**. The algorithm is a two-step process where **(a)** two components were
488 found that reduce the classification error rates (using mahalanobis.dist in the function) in the
489 algorithm, showing the ordination of samples using all the genera in the first two components
490 (MINT PLS-DA) with ellipse representing 95% confidence interval and percentage variations
491 explained by these components in axes labels. In step two, **(b)** the number of discriminating genera
492 were found for each component, highlighted as diamonds, **(c)** is similar to **(a)** however the
493 ordination was considered using the discriminants from two components (MINT sPLS-DA); **(d)**
494 shows the heatmap of these discriminant genera, with both rows and columns ordered using
495 hierarchical (average linkage) clustering to identify blocks of genera of interest. Heatmap depicts
496 TSS+CLR normalised abundances: high abundance (pink) and low abundance (blue), along with
497 metadata drawn on top. Table on the right side shows the summary statistics for *Campylobacter*
498 reads obtained for each category.

499 **Supplementary S3-Top 25 Abundant Taxa.pdf**: Community structure based on relative
500 abundance of the top-25 most abundant taxa identified at different taxonomic groups, where
501 'others' refers to all taxa not included in the 'top-25'.

502 **Supplementary S4-Richness Subset Regression.docx**: Subset regression analysis using
503 Richness as the dependent variable.

504 **Supplementary S5-Shannon Entropy Subset Regression.docx**: Subset regression analysis
505 using Shannon Entropy as the dependent variable.

506 **Supplementary S6-NRI Subset Regression.docx**: Subset regression analysis using
507 phylogenetic alpha diversity (NRI) as the dependent variable.

508 **Supplementary S7-NTI Subset Regression.docx:** Subset regression analysis using
509 phylogenetic alpha diversity (NTI) as the dependent variable.

510 **Supplementary S8-LCBD Bray-Curtis Subset Regression.docx:** Subset regression analysis
511 using beta diversity measure (LCBD with Bray-Curtis distance) as the dependent variable.

512 **Supplementary S9-LCBD Unweighted UniFrac Subset Regression.docx:** Subset regression
513 analysis using beta diversity measure (LCBD with Unweighted UniFrac distance) as the dependent
514 variable.

515 **Supplementary S10-LCBD Weighted UniFrac Subset Regression.docx:** Subset regression
516 analysis using beta diversity measure (LCBD with Weighted UniFrac distance) as the dependent
517 variable.

518 **Supplementary S11-Pairwise Differential Analysis.xlsx:** Differential analysis of genera that are
519 up/down-regulated between different groups (Adjusted P values ≤ 0.05) with at least log2 fold
520 change from the base mean abundances for the samples considered in the first column.

521 **Supplementary S12-Bioinformatics_and_StatisticalAnalysis.docx:** Bioinformatics and
522 statistical analysis methods used within the study.

523 **Supplementary S13-Parameters Measured.docx:** A list of all of the parameters measured within
524 the study and their explanations.

525
526
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669

A) Alpha Diversity Measures

Richness ***

Shannon ***

Simpson ***

NRI **

NTI ***

B) Beta Diversity Measure and Taxa Differential Analysis

HW30 v's
Phascolarctobacterium
Thalassospira
Barnesiella
Subdoligranulum
Eisenbergiella
Alistipes
Ruminiclostridium 5
Ruminococcaceae UCG-014
Bacteroides

N30 v's H
Eisenbergiella
Alistipes

O30 v's N	
Thalassospira	(S), ↑
Bacteroides	

PERMANOVA

Treatment: $R^2 = 0.088$ ($p = 0.001$ ***)

Days: $R^2 = 0.211$ ($p = 0.001$ ***)

HW7 v's O7	
Lachnoclostridium	(S), ↑HW7 (H)
Eisenbergiella	(S)
Bacteroides	(S),
Escherichia-Shigella	(S), ↑O7 (D, H)

O7 v's N7	
Eisenbergiella	(S)
Bacteroides	(S), ↑O7 (D)
Escherichia-Shigella	(S)

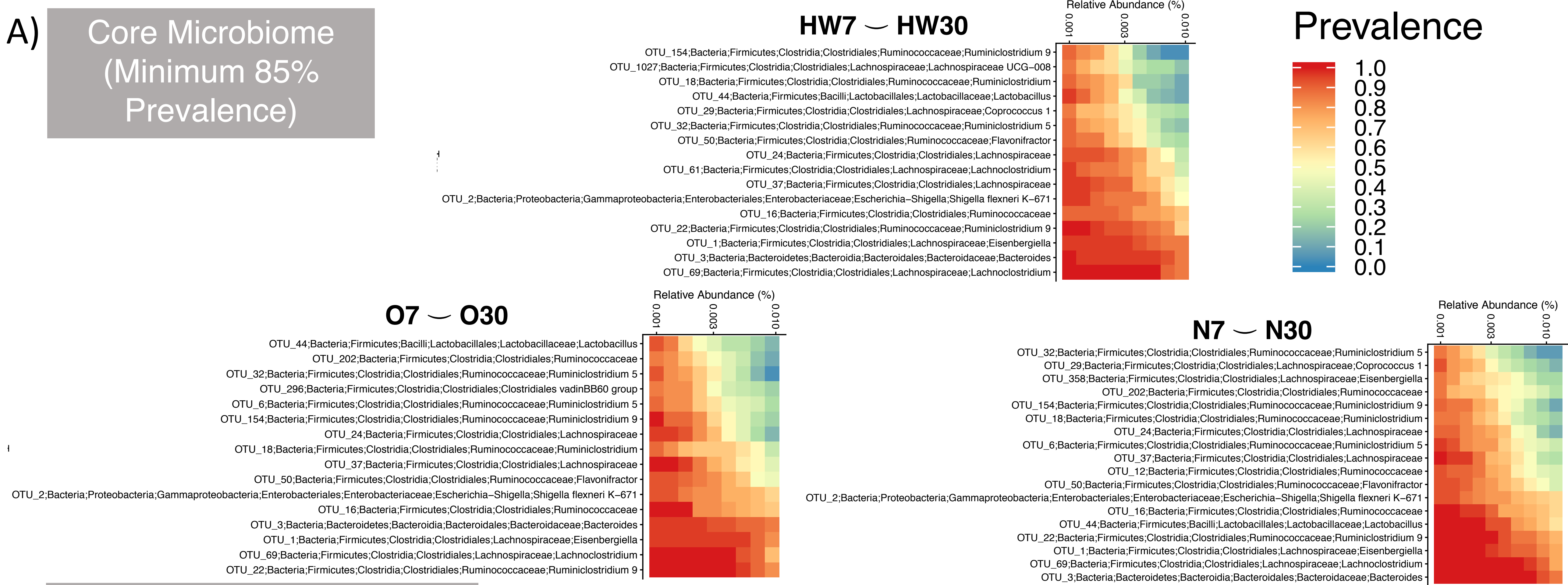
N7 v's HW7	
Lachnoclostridium	(S)
Eisenbergiella	(S)
Bacteroides	(S), ↑HW7 (H)

S = Subset Analysis
D = Deseq Analysis
H = Heat Tree Analysis
M = Mint Analysis

HW7 v's HW30	
Alistipes	(S), ↑HW30 (D, H) ↓HW30 (M)
Ruminococcaceae UCG-014	(S), ↑HW30 (H), ↓HW30 (M)
Bacteroides	(S), ↑HW7 (D, H), ↓HW7 (M)
Escherichia-Shigella	(S), ↑HW30 (H), ↓HW30 (M)

O7 v's O30	
Thalassospira	(S), ↑O30 (D, H)
Lachnoclostridium	(S), ↑O7 (D, H), ↓O7 (M)
Eisenbergiella	(S), ↑O7 (D, H), ↓O7 (M)
Alistipes	(S), ↑O30 (D, H), ↓O30 (M)
Bacteroides	(S), ↑O30 (M)
Escherichia-Shigella	(S), ↑O7 (D)

N7 v's N30	
Lachnoclostridium	(S), ↑N7 (H)
Eisenbergiella	(S), ↑N7 (D, H), ↓N7 (M)
Alistipes	(S), ↑N30 (D, H), ↓N30 (M)
Bacteroides	(S), ↓N7 (M)



B) Differential Heat Trees

N7 ⌒ HW7
O7 ⌒ HW7
HW7 ⌒ HW30
N7 ⌒ N30
O7 ⌒ O30
O30 ⌒ HW30
N30 ⌒ HW30
O30 ⌒ N30
O7 ⌒ N7

Root
Bacteria
Firmicutes
Clostridia
Clostridiales
Clostridiales vadinBB60 group
Lachnospiraceae
Ruminococcaceae
Bacteroidetes
Bacteroidia
Bacteroidaceae
Bacteroidales
Bacteroides
Proteobacteria
Tenericutes
Mollicutes
Cyanobacteria
Melainabacteria
Gastranaerophilales
Archaea
Eukaryota

Nodes
Log2 ratio median proportions
−3
−2
−1
0
1
2
3
Number of OTUs
1.0
48.8
192.0
431.0
765.0
1200.0
1720.0

	Richness	Shannon Entropy	NRI	NTI	LCBD (Bray-Curtis Distance)	LCBD (Unweighted UniFrac)	LCBD (Weighted UniFrac)
Day_7	-***	-***		+*	+**		
Day_30	+***	+***				+***	
Protein_perc_ration	+***	+***	-**	-**	-***	+***	-***
Feed_Change_Age_Grower_to_Finisher_22	-*						
Feed_Change_Age_Finisher_to_Withdrawal_34			-**				
Food_Conversion_Ratio				+***			
Production_System_N			-**				
Water_Consumption_per_Bird			-***				
Energy_of_ration		+***					
Placement Birds/m²				-*			
Age_At_Thin				-***			
Hockmark_percentage				-***			
Total_Mortality_percentage					-*		
Pododermatitis_percentage						+**	
log_CFU_per_g_Campylobacter	+*		+***	+***	+*		

Bray-Curtis Distance						
Predictors	*Df*	*SumsOfSqs*	*MeanSqs*	*F.Model*	R^2	*p*
Days	1	4.9513	4.9513	26.8712	0.21163 ***	**<0.001**
No. of Parent Flocks Used	1	1.065	1.065	5.7796	0.04552 ***	**<0.001**
Birds Placed	1	1.0209	1.0209	5.5407	0.04364 ***	**<0.001**
log CFU/g Campylobacter	1	0.9606	0.9606	5.2131	0.04106 ***	**<0.001**
Protein (% of Ration)	1	0.2729	0.2729	1.4813	0.01167 .	**<0.1**
Energy of Ration (Kcal/lb AME)	1	0.9375	0.9375	5.088	0.04007 ***	**<0.001**
Residuals	77	14.1881	0.1843	0.60642		
Total	83	23.3964	1			
Unweighted UniFrac Distance						
Predictors	*Df*	*SumsOfSqs*	*MeanSqs*	*F.Model*	R^2	*p*
Days	1	4.2369	4.2369	46.886	0.32727 ***	**<0.001**
No. of Parent Flocks Used	1	0.3242	0.3242	3.588	0.02504 **	**<0.01**
Birds Placed	1	0.6806	0.6806	7.532	0.05258 ***	**<0.001**
log CFU/g Campylobacter	1	0.386	0.386	4.272	0.02982 ***	**<0.001**
Protein (% of Ration)	1	0.1251	0.1251	1.385	0.00966	0.1744
Energy of Ration (Kcal/lb AME)	1	0.235	0.235	2.601	0.01816 *	0.0165
Residuals	77	6.9582	0.0904	0.53747		
Total	83	12.9461	1			
Weighted UniFrac Distance						
Predictors	*Df*	*SumsOfSqs*	*MeanSqs*	*F.Model*	R^2	*p*
Days	1	0.055783	0.055783	76.574	0.43079 ***	**<0.001**
No. of Parent Flocks Used	1	0.001894	0.001894	2.6	0.01463 .	0.0509

Birds Placed	1	0.005532	0.005532	7.594	0.04272 ***	**<0.001**
log CFU/g Campylobacter	1	0.00484	0.00484	6.644	0.03738 ***	**<0.001**
Protein (% of Ration)	1	0.00092	0.00092	1.262	0.0071	0.2534
Energy of Ration (Kcal/lb AME)	1	0.004429	0.004429	6.08	0.0342 **	**<0.01**
Residuals	77	0.056093	0.000728	0.43318		
Total	83	0.129491	1			

*. p<0.1 * p<0.05 ** p<0.01 *** p<0.001*