Impact of industrial production system parameters on chicken microbiomes: mechanisms to improve performance and reduce *Campylobacter*

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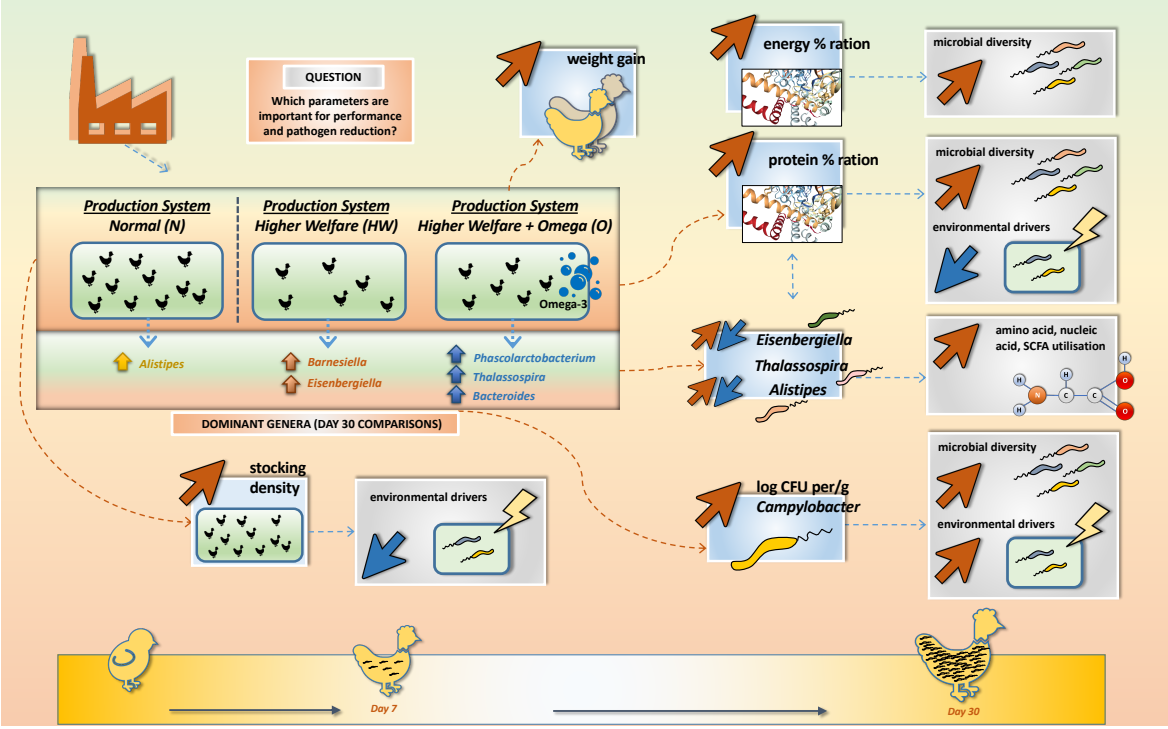
32

33 **Graphical Abstract:**



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Abstract

Background

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

Results

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

Conclusions

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

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

Background

Chickens are a key source of protein for humans where poultry production is predicted to produce approximately 130 million tons of chicken meat in 2020 [1, 2]. Sustainable poultry practices are needed to help maintain an adequate supply of poultry products for the increasing human population without compromising the chicken or human health [3]. Selective breeding programmes has resulted in chickens that efficiently convert food into body mass, as defined by feed conversion ratios (FCR) [4]. The fundamental component needed to ensure an efficient poultry production is highly dependent in having an optimised nutrition and production setup [1].

Production systems vary immensely between countries, businesses and at farm level. Certain parameters such as *Placement Birds/m*^{2} (stocking density), *Protein_perc_ration* (protein percentage within ration) and *Energy_of_ration* (energy content) in relation to the feed are key determinants that if varied, may directly influence chicken microbial community structure. Thus, impacting performance and potentially reduction of pathogenic bacteria. Chicken diets are typically formulated to enhance production efficiency. However, some diets are formulated to enhance human health, such as diets containing Omega-3 polyunsaturated fatty acids (PUFA's) [5]. There is anecdotal evidence at farm level to suggest that this enrichment has potential to improve chicken gut health and performance or reduce pathogen colonisation.

Many bacterial species found within the microbiome of farmed animals can potentially be considered pathogens and detrimental to human health. One of these pathogens is *Campylobacter* which is the leading cause of human foodborne bacterial gastroenteritis causing bloody diarrhoea, fever and abdominal pains in humans and can also cause post infectious sequelae such as Guillain-Barré syndrome which is a potentially fatal paralytic autoimmune illness [6]. *Campylobacter jejuni* (predominant species causing infection to humans) colonises chicken ceca with relatively high numbers (>10^9 CFU per gram) and can be pathogenic to the chicken, with this dependent on the genetics of host and strain of infection [7-10]. Although there exists many intervention studies, in an industrial farm environment, we currently do not understand why we

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

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

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

Materials and Methods

Ethics Statement

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

Experimental design, broilers and sample collection

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

Although the three production systems differ in receiving chicks from multiple flocks, our analysis suggested that they had no bearing on microbial community structure at finer level (Fig. 3 and discussion later) although some parameters were implicated as significant in PERMANOVA analysis. All chicks were Ross 308 as hatched (AH) and were supplied from the same Moy Park hatchery to all three farms on 11/10/2018. All birds were grown in typical industrial poultry houses and were raised on a four-stage diet made up of a starter, grower, finisher and withdrawal ration, however composition of these diets differed across the three growing regimes. Farm N – Birds were offered standard starter ration from days 0 to 11, standard grower ration from days 11 to 22 and standard finisher from day 22 to day 34 before moving to a standard withdrawal ration prior to slaughter. Farm HW - Birds were offered higher welfare starter ration from days 0 to 11, higher welfare grower ration from days 11 to 23 and higher welfare finisher from day 23 to day 31 before moving to a higher welfare withdrawal ration prior to slaughter. Farm O - Birds were offered higher welfare starter ration from days 0 to 11, higher welfare grower ration from days 11 to 20 and Omega-3 finisher ration from day 20 to day 30 before moving to an Omega-3 withdrawal ration prior to slaughter. Both Omega-3 finisher rations are identical to HW equivalent ration, but with addition of an Omega-3 premix produced by Devenish Nutrition Ltd (Belfast, UK) and added at the 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) (Oxoid, UK) buffer to make a 1/10 dilution and stomached
161 at 260 rpm for 1 minute. Further decimal dilutions were carried out in MRD to give a range of
162 dilutions suitable to achieve countable plates. 100 µl of the original suspension (10^{-1} dilution) was
163 inoculated onto duplicate plates of mCCDA (Oxoid, UK). The inoculum was spread uniformly over
164 the surface of the agar plate with a sterile spreader until fully absorbed. This spread plating was
165 repeated for all the other decimal dilutions. When low numbers of *Campylobacter* were expected,
166 the limit of detection was increased by also spread plating 1 ml of the original suspension. The 1
167 ml inoculum was inoculated over three plates. The plates were incubated in a microaerophilic
168 atmosphere (5% oxygen, 10% carbon dioxide and 85% nitrogen) at 41.5ºC and examined after 44
169 ± 4 hours for typical colonies of *Campylobacter* spp. Plates containing less than 300 colonies were
170 counted. Colonies were considered typical if they were greyish (often with a metallic sheen), flat
171 and moist, with a tendency to spread. *Campylobacter* numbers were expressed as CFU/g cecal
172 content. Genomic DNA (gDNA) was also extracted from chicken ceca for 16S metagenomics
173 experiments.

174 **Poultry Growth and Performance Measurements**

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

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

182 Cecal gDNA was extracted using QIAamp DNA Stool Mini Kit according to the manufacturer's
183 instructions and stored at -20°C. 16S metagenomic sequencing library construction was performed

184 using Illumina guidelines (Illumina, U.S.A). The 16S ribosomal primers used were V3
185 (tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag) and V4
186 (gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaatcc) [22, 23]. A second PCR step was
187 performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit.
188 Sequencing was performed on Illumina MiSeq at LSHTM using a v3 300 bp paired-end kit.

189 **Bioinformatics and Statistical Analysis**

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

191 **Results**

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

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

210 Ecological drivers of microbial community were explored observing the clustering in the
211 phylogenetic tree of the OTUs and utilising phylogenetic alpha diversity measures such as

212 NRI/NTI. Positive NRI/NTI values indicate strong phylogenetic clustering (driven by environmental
213 filtering), whereas reduced values represent phylogenetic overdispersion (environment has little or
214 no role to play). Here, using NRI and NTI we can observe an increase in N, HW and O samples
215 respectively from day 7 to day 30. Since chicken ceca are already a constrained environment to
216 begin with (as opposed to real environmental datasets), the values <0[a] (traditionally this implies
217 stochasticity) may not be feasible to ascertain randomness/stochasticity/competitive exclusion
218 principle, and hence values should be taken relatively with an increasing value implying increasing
219 host environmental pressure (in the immediate case it is the environment within a chicken, whilst
220 extrinsic environmental factors such as surroundings where chickens are confined and their diet
221 may also have a role to play). Fig. 1a corroborates our findings from our previous longitudinal study
222 [14].

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

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

[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 were differentially expressed (Fig. 2), and iii) MINT analysis to consolidate the genera that have
237 simultaneous discriminatory power at both spatial (production system N, HW and O) and temporal
238 scales (day 7 and day 30)(Supplementary S2). To aid interpretation, all up/downregulated taxa are
239 annotated with up and down arrows in Figure 1 according to which analysis they were selected in
240 and with taxa from subset analysis as the seeding point. Redundancy in these analyses was to
241 ensure there was no biases associated with the mathematical algorithms to find discriminating
242 terms. In some cases, direction of the discrimination was contradictory, for example, there were a
243 few cases where DESeq2 and Heat Tree agreed, whilst MINT analysis showed a reciprocal trend.
244 This latter disagreement may possibly be because MINT consolidates all possible sources of
245 variation. Nonetheless, we wanted to be comprehensive and therefore discuss only those taxa
246 which achieve a majority consensus on multiple analyses albeit MINT underperforming in most
247 cases.
248 For production system HW, *Alistipes*, *Ruminococcaceae UCG-014* and *Escherichia-Shigella* were
249 significant genera increasing from day 7 to day 30. *Bacteroides* displayed significant increase at
250 day 7 (decreasing at day 30) using DESeq2 and Heat Tree. For production system O,
251 *Thalassospira*, *Alistipes* and *Bacteroides* increased at day 30 (*Bacteroides* directionality was only
252 observed with MINT analysis). *Lachnoclostridium*, *Eisenbergiella*, *Escherichia-Shigella* all
253 increased at day 7 (decreasing at day 30). For production system N, *Lachnoclostridium*,
254 *Eisenbergiella* increased at day 7 (decreasing at day 30). *Bacteroides* was identified as decreasing
255 at day 7 (increasing at day 30; directionality was only observed with MINT analysis). In general,
256 *Alistipes* was observed to increase consistently at day 30 for all production systems, whilst all other
257 genera showed mixed trends. Although it should be noted that *Alistipes* was present at higher
258 abundance at production system N as compared to others.
259 Key genera were identified when comparing production systems at day 7. For HW vs O
260 comparison, *Lachnoclostridium* was identified as increased for HW. *Escherichia-Shigella* was
261 identified increased for production system O. For O vs N comparison, *Bacteroides* was increased
262 for O. This was also replicated for HW when comparing to N. Key genera were identified when
263 comparing production systems at day 30. For HW vs O comparison, *Phascolarctobacterium*,

264 *Thalassospira* and *Bacteroides* were identified as increased for O, whereas *Barnesiella* was
265 increased for HW when comparing to O. *Subdoligranulum*, *Eisenbergiella*, *Alistipes*,
266 *Ruminiclostridium* 5 and *Ruminococcaceae UCG-014* were all identified as part of the subsets that
267 explain beta diversity, although they were not implicated as discriminating in differential analyses.
268 For N vs HW comparison at day 30, *Eisenbergiella* was increased for HW, and *Alistipes* was
269 increased for N. For O vs N comparison at day 30, *Bacteroides* was observed with subset analysis
270 alone. *Thalassospira* was observed, yet its discriminatory power was inconclusive.
271 Core microbiome where genera persist in 85% of the samples (something that is traditionally used
272 to define the prevalence of core microbiome) for different production systems (day 7 and day 30)
273 was assessed (Fig. 2). Genera identified include *Bacteroides*, *Lachnoclostridium*, *Eisenbergiella*,
274 *Ruminoclostridium 9*, *Lactobacillus*, *Ruminococcaceae*, *Shigella flexneri K−671*, *Flavonifractor*,
275 *Ruminococcaceae*, *Lachnospiraceae*, *Ruminiclostridium 5*, *Ruminiclostridium*, *Coprococcus 1*,
276 *Ruminiclostridium 5* at varying level of abundance. In Fig. 2, OTUs are sorted by their abundances
277 with those on the top being low abundant prevalent OTUs, whereas those at the bottom are highly
278 abundant prevalent OTUs.
279 ***Parameters deriving microbial community structure***
280 Analysis of parameters that had a significant effect on microbial diversity were assessed using
281 PERMANOVA against performance parameters when using different dissimilarity measures on
282 microbiome data (Table 1). Using R^2, if significant, to represent the variability explained in the
283 community structure for Bray-Curtis distance, the parameter with the greatest impact was days,
284 explaining 21.2% of the variation. Next, key parameters of interest were:
285 *log_CFU_per_g_Campylobacter* (4.1% variability), *Energy_of_Ration* (4.0% variability) and
286 *Protein_perc_ration* (1.2% variability). Using both unweighted and weighted *UniFrac* as a beta
287 diversity measure, the pattern was more or less similar with the same trends with days having the
288 greatest impact on microbial diversity (32.7% and 43.0% respectively). All other parameters had
289 R^2 values at 1-5%.
290 ***Direction of influence for extrinsic parameters influencing key metrics for the microbiome***

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

other hand only caused shift in environmental pressure affecting the terminal clades by making them more clustered though the NTI measure.

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

Discussion

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

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

372 associated with prevention and spread of highly antibiotic-resistant bacteria [34] where the gut
373 bacterial community may react to antibiotic inclusion in the diet and prophylactically encourage the
374 presence of *Barnesiella*, an effect demonstrated in mice where ampicillin treatment increased their
375 presence [35]. The competitive exclusion hypothesis is supported by the increase of *Alistipes* bacteria
376 in N group at day 30, and using the top-25 most abundant taxa identified at OTU level where clear
377 differences for N against HW and O were observed (also for genera *Bacteroides*). The presence
378 of *Subdoligranulum* bacteria in HW and O production systems, although not discriminatory, still
379 contributing to beta diversity, represents a sign of improved gut health as these bacteria are known
380 to be involved in production of SCFAs (e.g. butyrate) with an important role in gut physiology [36].
381 *Ruminiclostridium 5*, identified within HW and O production systems, also within core microbiome,
382 has been noted to impact SCFA concentration within the gut [37].
383 The European Union (EU) ban on antimicrobial growth promoters in 2006 has created an increased
384 need to devise alternative methods to improve performance and potentially reduce numbers of
385 pathogenic bacteria. Examples include use of natural plant-derived products such as carvacrol
386 [38], addition of dietary prebiotics [39] and administration of live probiotic bacteria [40]. More
387 recently, prebiotic galacto-oligosaccharides (GOS) have been added to broiler feed and enhanced
388 the growth rate and feed conversion of chickens relative to those obtained with a calorie-matched
389 control diet [41]. Recent developments has observed chicken diets enriched with Omega-3 PUFA's
390 for benefits to human health [5]. Omega-3 fatty acids α-linolenic acid (ALA) (often found in plants),
391 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (found typically in marine oils), all
392 play a role in forming structural components of cell membranes, serve as precursors to bioactive
393 lipid mediators, and provide a source of energy [42]. PUFAs are also important as substrates for
394 inflammatory and anti-inflammatory acids, with EPA is believed to have anti-inflammatory
395 properties [43]. One of the proposed mechanisms as to how dietary antibiotics exert their growth
396 promoting benefits is via anti-inflammatory effects towards the intestinal epithelium, by inhibition
397 of production and excretion of catabolic mediators [44]. Increased levels of EPA following antibiotic
398 supplementation align with this non-antibiotic, anti-inflammatory theory of antibiotic growth
399 promotion [45]. In our study, we do not know if PUFAs were fully absorbed, or to what effect the

interaction is with host microbiome, however we do observe an increase in weight for production system O which has Omega-3. Of note, production system N had the lowest (best) FCR.

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

Conclusion

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

428 strategy (studying cecal microbiome at its natural environment), to reveal the factors that link microbial
429 community structure to improved broiler performance, and reduced pathogenic bacteria such as
430 *Campylobacter*.
431
432 **Availability of supporting data**
433 The raw sequence files supporting the results of this article are available in the European
434 Nucleotide Archive under the project accession number PRJEB34742.
435
436 **Competing interests**
437 AM, AR and UL are employed by company Moy Park. AM is enrolled on a PhD programme at
438 QUB, and undertook research work at AFBI and Moy Park. All other authors declare no competing
439 interests.
440
441 **Author contributions**
442 AM, AR, UL, UZI, NC and OG contributed to the study design. NC and OG managed the study.
443 CK, AM and ML performed the sample collection and DNA extraction. AM and OG performed the
444 library preparation and Illumina MiSeq sequencing at the LSHTM. UZI wrote the analysis scripts
445 to generate the figures and tables in this paper. UZI, AM and OG performed the bioinformatics and
446 statistical analysis. UZI, AM, NC and OG drafted the initial version of the manuscript with all authors
447 including BDG, ND, BWW and WTS contributing to redrafting.
448
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451 Research Fellowship (NE/L011956/1).
452 **<u>List of Figures and Tables</u>**
453 **Figure 1**: **Microbial diversity and community structure**. (a) represents alpha diversity (Richness
454 and Shannon entropy) and environmental filtering (NRI/NTI) measures respectively. Lines for
455 figure (**a)** connect two categories where the differences were significant (ANOVA) with * ($P < 0.05$),

456 ** (P < 0.01), or *** (p < 0.001). (b) beta diversity using Bray-Curtis distance measure along with
457 Top-25 genera observed in all samples grouped by categories. The tables represent taxa that were
458 found to be significant based on Subset analysis (Supplementary S1) i.e., those genera selected
459 in the subsets that explain roughly the same distance between samples as all the genera.
460 Additionally, if the taxa were found to be differentially expressed based on other analyses, such as
461 DESeq2 (Supplementary S11), MINT (Supplementary S2), Differential Heat Tree (Figure 2), the
462 categories they up and down-regulated are represented with corresponding up and down arrows.
463 For example, in HW30 vs O30 comparison, "(S), ↑O30 (D, H)" for *Phascolarctobacterium* should
464 be read as selected in subset analysis: (S) and upregulated in O30 according to both DESeq2 and
465 Differential Tree: ↑O30 (D, H).

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

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

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

483 **<u>List of Supplementary Material</u>**

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

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

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

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

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

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

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

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

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

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

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

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

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

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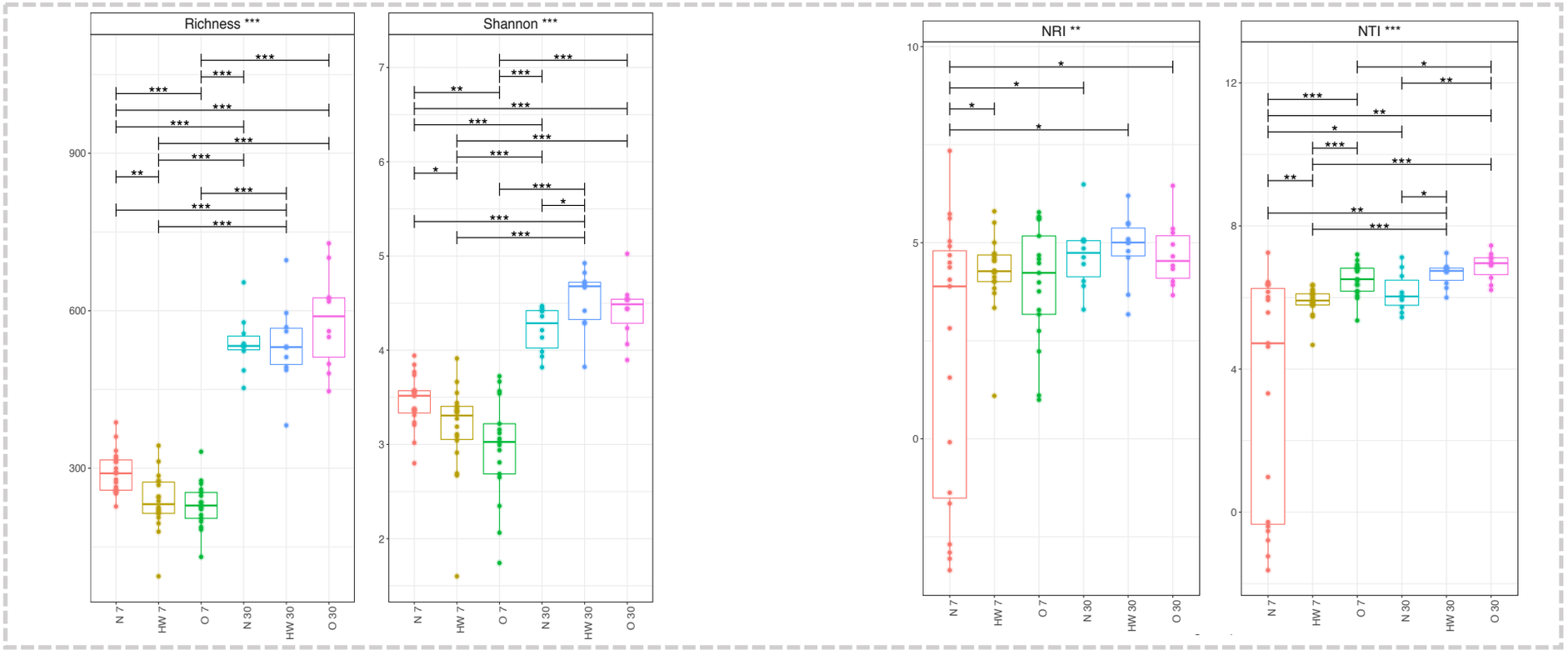
671

A) Alpha Diversity Measures

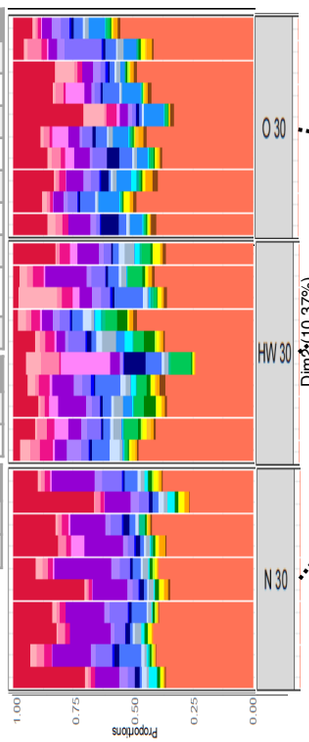


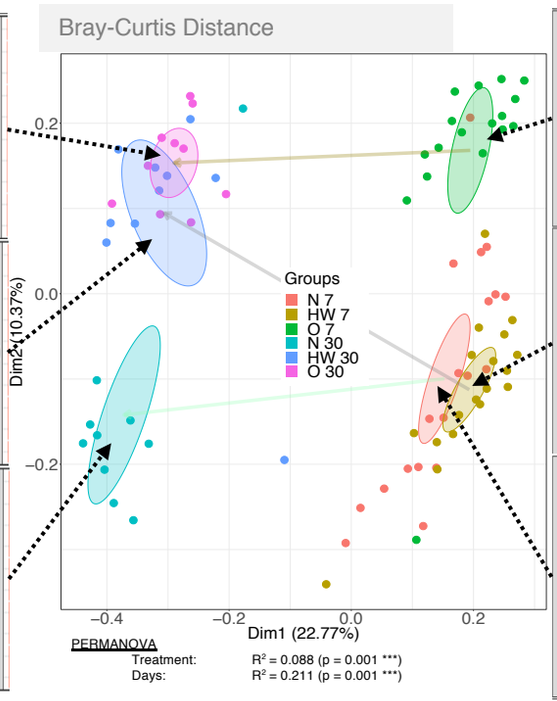
B) Beta Diversity Measure and Taxa Differential Analysis

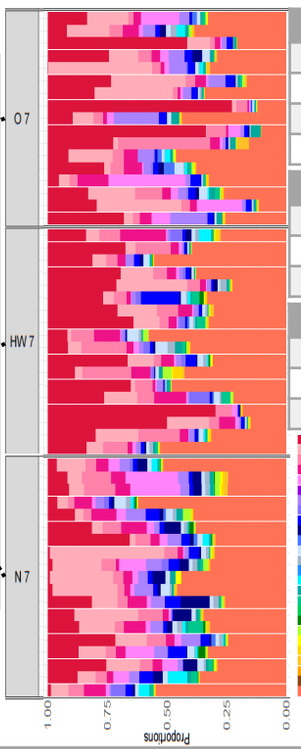
HW30 v's O30	
Phascolarctobacterium	(S), ↑O30 (D, H)
Thalassospira	(S), ↑O30 (D,H)
Barnesiella	(S), ↑HW30 (D, H)
Subdoligranulum	(S)
Eisenbergiella	(S)
Alistipes	(S)
Ruminiclostridium 5	(S)
Ruminococcaceae UCG-014	(S)
Bacteroides	(S), ↑O30 (H)

N30 v's HW30	
Eisenbergiella	(S), ↑HW30 (D)
Alistipes	(S), ↑N30 (H)

O30 v's N30	
Thalassospira	(S), ↑O30 (D), ↑N30 (H)
Bacteroides	(S)



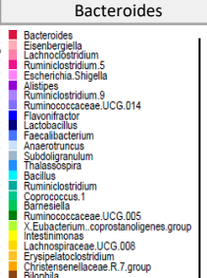




HW7 v's O7	
Lachnoclostridium	(S), ↑HW7 (H)
Eisenbergiella	(S)
Bacteroides	(S),
Escherichia-Shigella	(S), ↑O7 (D, H)

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)



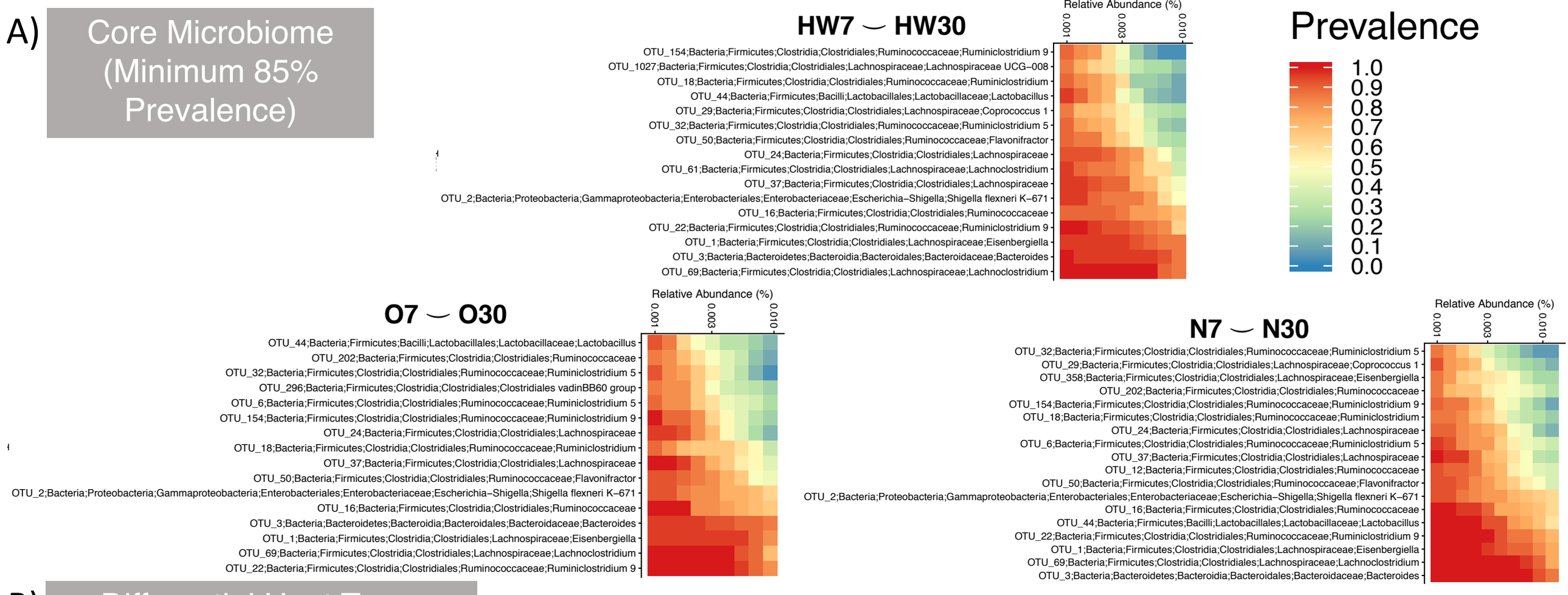
S = Subset Analysis
D = Deseq Analysis
H = Heat Tree Analysis
M = Mint Analysis

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)

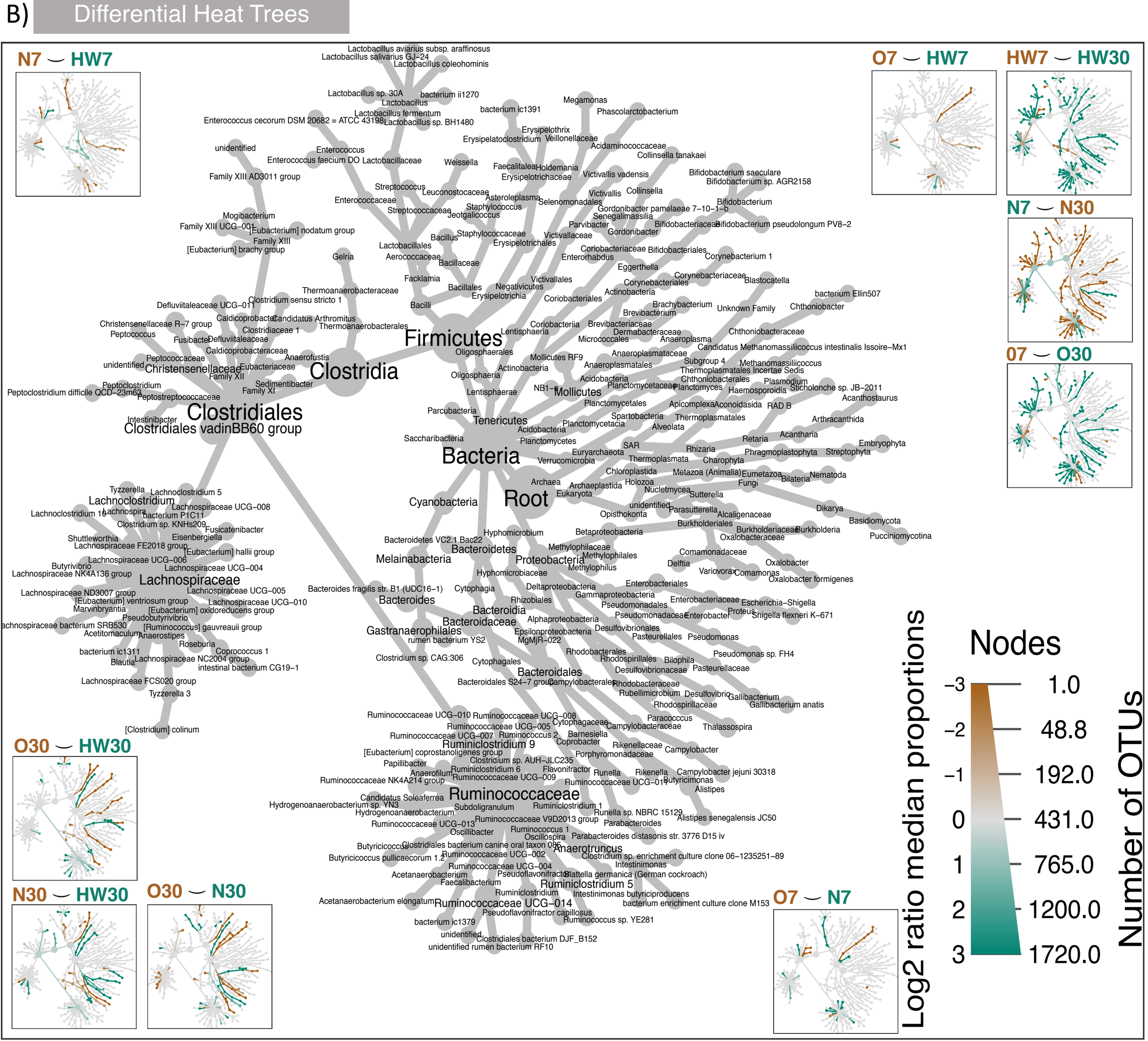
07 v's O30	
Thalassospira	(S), ↑O30 (D, H)
Lachnoclostridium	(S), ↑O7 (D, H), ↓O7 (M)
Eisenbergiella	(S), ↑O7 (D, H), ↓O7 (M)
Alistipes	(S), ↑O30 (D, H), ↓O30 (M)
Bacteroides	(S), ↑O30 (M)
Escherichia-Shigella	(S), ↑O7 (D)

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)

A) Core Microbiome (Minimum 85% Prevalence)



B) Differential Heat Trees



	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/m^2				-*			
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*