Role of caecal microbiota in the differential resistance of inbred chicken lines to colonization by *Campylobacter jejuni.*

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Running title: Role of gut microbiota in avian resistance to *Campylobacter*.

Abstract

Campylobacter is the leading foodborne bacterial diarrhoeal illness in many countries, with up to 80 % of human cases attributed to the avian reservoir. The only control strategies currently available are stringent on-farm biosecurity and carcass treatments. Heritable differences in the resistance of chicken lines to *Campylobacter* colonisation have been reported and resistance-associated quantitative trait loci are emerging, albeit their impact on colonization appears modest. Recent studies indicated a protective role of the microbiota against colonization by *Campylobacter* in chickens. Furthermore, in murine models, differences in resistance to bacterial infections can be partially transferred between lines by transplantation of gut microbiota. In this study, we investigated whether heritable differences in colonization of inbred chicken lines by *Campylobacter jejuni* are associated with differences in caecal microbiota. We performed homologous and heterologous caecal microbiota transplants between line 6_1 (resistant) and line N (susceptible), by orally administering caecal contents collected from 3-week-old donors to day-of-hatch chicks. Recipient birds were challenged (day 21) with *C. jejuni* 11168H. In birds given homologous microbiota, the differential resistance of lines to *C. jejuni* colonization was reproduced. Contrary to our hypothesis, transfer of caecal microbiota from line 6_1 to line N significantly increased *C. jejuni* colonization. No significant difference in the overall composition of the caecal microbial communities of the two lines was identified, albeit line-specific differences for specific operational taxonomic units were identified. Our data suggest that while heritable differences in avian resistance to *Campylobacter* colonization exist, these are not explained by significant variation in the caecal microbiota.

Keywords: *Campylobacter*, chickens, microbiota, colonization, resistance.

Importance

Campylobacter is a leading cause of foodborne diarrhoeal disease worldwide. Poultry are a key source of human infections but there are currently few effective measures to *Campylobacter* in poultry during production. One option to control *Campylobacter* may be to alter the composition of microbial communities in the avian intestines by introducing beneficial bacteria which exclude the harmful ones. We previously described two inbred chicken lines which differ in resistance to intestinal colonization by *Campylobacter*. Here, we investigated the composition of the microbial communities in the gut of these lines, and whether transferring gut bacteria between the resistant and susceptible lines alters their resistance to *Campylobacter*. No major differences in microbial populations were found and, resistance or susceptibility to colonization was not conferred by transferring gut bacteria between lines. The data suggest that gut microbiota did not play a role in resistance to *Campylobacter* colonization, at least in the lines used.

Introduction

Campylobacter is the main bacterial cause of zoonotic foodborne infections in many countries. In the United Kingdom, approximately 90 % of human cases are caused by *Campylobacter jejuni*, with *C. coli* and other species playing a relatively minor role (1). Symptoms can range from mild gastroenteritis to severe haemorrhagic diarrhoea that can last as long as two weeks and occasionally relapse. In addition, campylobacteriosis can involve severe sequelae, including inflammatory bowel disease and debilitating inflammatory neuropathies such as the Guillain-Barré syndrome. Recent estimates place its economic cost at £50 million per year in the United Kingdom (2), where 63,946 laboratory-

68 confirmed cases of human infection were recorded in 2017 (1) and 9.3 cases are predicted
69 to be unreported for every one captured by national surveillance (3).
70 Poultry are an important reservoir of human campylobacteriosis, with some estimates
71 attributing up to 80 % of human infections to this source (4). The caeca are a key site of
72 persistence of *Campylobacter* in chickens, where numbers of *C. jejuni* can reach as high as
73 10^{10} colony forming unit (CFU)/g of contents. Given such levels, contamination of carcasses
74 with numbers of *C. jejuni* predicted to be adequate for human infection is challenging to
75 prevent during the slaughter process. Control of *Campylobacter* relies mainly on stringent
76 on-farm biosecurity measures and carcass treatments, including freezing, rapid surface
77 chilling or the application of organic acid solutions or chlorinated water, where permitted by
78 national regulations. There are currently no effective commercial vaccines for
79 *Campylobacter* in poultry and, even though some protective candidates have been
80 described in the literature, these often confer modest protection that has proven
81 challenging to reproduce across repeated studies and laboratories (5).
82 Inbred chicken lines 6_1 and N are known to exhibit heritable differences in resistance to
83 colonization by several *C. jejuni* strains (6) and recent work using backcross and advanced
84 intercross populations of these lines has identified quantitative trait loci (QTL) associated
85 with this phenotype (7). Differential resistance to *C. jejuni* colonization has also been
86 detected between other chicken lines and was associated with variation in caecal and
87 systemic transcriptional responses (8, 9, 10). Heritable differences in resistance to
88 *Campylobacter* also appear to exist in commercial broilers, although only 10 % of the
89 variation in *Campylobacter* colonization phenotype was explained by host genetics in a
90 population studied recently (11).

In murine models, *Campylobacter*-induced enteritis and colonization requires the prior depletion of the indigenous microbiota with antibiotics (12). This study also indicated that *Enterococcus faecalis* may be a protective constituent of the microbiota. More recently, *Clostridium* cluster XI, *Bifidobacterium* and *Lactobacillus* spp. were reported to be significantly enriched in mice that were protected against *Campylobacter*-induced colitis (13). These authors also demonstrated that oral administration of sodium deoxycholate, a secondary bile acid that is produced via the metabolism of the aforementioned bacteria, reduced enteritis. Moreover, removal of these bacterial taxa through antibiotic treatment enhanced the severity of *Campylobacter*-induced colitis (13).

In chickens, Han *et al* demonstrated that the presence of intestinal microbiota is protective against *Campylobacter* by comparing colonization levels in *C. jejuni*-challenged birds that had naturally-acquired microbiota or which had been reared under germ-free conditions or treated with antibiotics (14). However, the individual components of the microbiota that were associated with the protective effect and relative role(s) of direct competition versus immune priming by the microbiota were not investigated. More recently, Connerton *et al* studied the effects of *Campylobacter* colonization on the caecal microbiota of chickens and found that colonization by *Campylobacter* significantly alters the composition of the gut microbiota, with decreases in the abundance of operational taxonomic units (OTUs) in the families of *Lactobacillaceae* and *Clostridium* cluster XIVa (15). However, they also found that the age of bird had an effect on the composition of the microbiota and that the effect of age exceeded that of *Campylobacter* infection as time progressed (15).

In this study, we sought to investigate whether intestinal microbiota plays a role in the differential resistance of chicken inbred lines 6_1 (resistant) and N (susceptible) to

115 colonization by *C. jejuni.* This involved analysing the caecal microbiome of birds of each line
116 at three weeks of age, when they are known to differ in resistance to *C. jejuni* challenge (6;
117 7), and performing homologous and heterologous microbiota transplants between the two
118 lines. A precedent exists in the literature for transferring resistance against bacterial
119 colonization in this way. For example, when using inbred mouse lines that differ in
120 resistance to colonization by the murine attaching & effacing pathogen *Citrobacter*
121 *rodentium*, reciprocal transfer of the microbiota to the heterologous line altered
122 susceptibility to colonization (16). Furthermore, faecal microbiota transplants are now
123 accepted treatments for acute and recurrent *Clostridium difficile* infections in humans, even
124 in cases where antibiotic treatment failed (17; 18). The rationale for our study is given
125 further impetus by the recent observation that introduction of adult microbiota into flocks
126 of neonatal chicks has a mild protective effect against *Campylobacter* colonization and
127 altered the gut microbiome (19).
128
129 **Materials and Methods**
130
131 **Bacterial strains and culture conditions**
132 *C. jejuni* 11168H was obtained from the National Collection of Typed Cultures and has
133 been fully sequenced (20) and confirmed to be proficient in colonization of chickens (7). It
134 was cultured on modified charcoal-cephoperazone-deoxycholate agar (mCCDA) (Oxoid, UK)
135 or in Mueller-Hinton Broth (MH; Oxoid), at 37 °C in a microaerophilic workstation (Don
136 Whitley Scientific, UK) in a low oxygen atmosphere (5 % O_2, 5 % CO_2 and 90 % N_2). Broth
137 cultures of *Campylobacter* were grown with shaking at 400 rpm.
138

Experimental animals

All procedures were conducted under Home Office project license PCD70CB48, according to the requirements of the Animal (Scientific Procedures) Act 1986, with the approval of local ethical review committees. A total of 88 chickens were used in licensed procedures. Forty four chickens of each of the inbred lines 6_1 (http://www.narf.ac.uk/chickens/line-6/) and N (http://www.narf.ac.uk/chickens/line-n/) were obtained on the day of hatch from the National Avian Research Facility at The Roslin Institute, a Home Office licensed breeding establishment. Eggs were incubated and hatched under specified pathogen-free (SPF) conditions. Animals were housed in four groups of 22 in colony cages in a single study. Groups were of mixed sex and were individually wing-tagged for identification. Water and sterile irradiated feed based on vegetable protein (DBM Ltd., UK) were provided *ad libitum*. A further five birds of each line, reared under SPF conditions, were culled at 3 weeks of age by cervical dislocation to act as donors of caecal microbiota.

As previously described (6), chicken lines 6_1 and N were derived originally from White Leghorn flocks at the Avian Disease and Oncology Laboratory of the US Department of Agriculture, Agricultural Research Service, the former Regional Poultry Laboratory in East Lansing, MI, USA. The lines were maintained by random mating within the flock at the Institute for Animal Health (IAH) since 1972 (line 6_1) or 1982 (line N), before being transferred to the SPF unit of the National Avian Research Facility in 2013, where they have been maintained since. Inbred chicken line 6_1 was obtained from a White Leghorn background in 1939 to be resistant to avian leukosis virus (21). These two inbred lines have previously been reported to differ in resistance to intestinal colonization by *C. jejuni* when challenged on the day of hatch (6) or at three weeks old (7), as well as to enteric colonization by *Salmonella enterica* serovar Typhimurium (22).

Microbiota transplant experiment

The donor birds were housed in separate floor pens in the SPF unit of the NARF until three weeks of age. At this age, they were culled by cervical dislocation and caecal contents from the donor birds of each line were collected for separate DNA extractions to assess variability in their microbiota and for transplantation. For DNA extraction, the samples were promptly processed without freeze-thawing as described below. For transplants, the caecal contents of five birds were mixed within line in equal weight and diluted 1:6 (v/v) in sterile phosphate-buffered saline (PBS) to provide a mixture of sufficiently low viscosity that it could be reliably administered by oral gavage using a syringe and blunt-ended needle. Homologous transplants (6_1 microbiota into line 6_1 or N microbiota into line N) and heterologous transplants (6_1 microbiota into line N or N microbiota into line 6_1) were performed by administering 100 µl of a suspension of caecal contents by oral gavage, within 30 minutes of collection from donors, under aerobic conditions. Four birds from each group were sampled at 1, 7 and 21 days after the microbiota transplant. At 21 days post-transplant, all remaining birds (10 per group) were inoculated with 10^4 CFU of *C. jejuni* 11168H administered by oral gavage in a volume of 100 µl, diluted in sterile PBS. All infected birds were culled by cervical dislocation at 9 days post-challenge to enumerate caecal *Campylobacter* by plating 100 µl of serial 10-fold dilutions of caecal contents in PBS on mCCDA plates. At the same time, samples of caecal contents were promptly transported on ice to the laboratory for DNA isolation and analysis of the microbiota. Differences in caecal colonization by *Campylobacter* were investigated using a one-way, two-sided ANOVA test in Minitab (Minitab LLC, USA). *P* values ≤ 0.05 were taken to be significant. Power analysis using measures of inter-animal variance from our past research on *Campylobacter* vaccines,

187 mutants and heritable resistance indicated that 10 birds per group can detect a 2 $\log_{10}$
188 CFU/g difference with 80 % power at a significance level of $\alpha = 0.05$.
189
190 **DNA extraction**
191 DNA extractions were performed using pooled contents from both caeca of each bird,
192 with a separate extraction for each individual. Extraction was performed using a DNeasy
193 Powersoil kit (Qiagen, Valencia, CA, USA) with minimal delay from the time of collection and
194 without freezing. Samples were extracted in a single batch at the earlier time-points and in
195 two batches at the last time-point, with samples collected from birds which received the
196 transplant from the same donor birds extracted in the same batch. Due to the low volume
197 of caecal contents at 1 day post-hatch, the entire caeca (tissues and contents) were used for
198 DNA extraction. For birds of all other ages, only caecal contents were used. Caecal contents
199 or tissues were transferred to bead containing tubes with Powersoil solution c1 and were
200 heated at 65 °C for 10 minutes. A bead beating step was performed using a Precellys 24
201 Homogenizer (Bertin Technologies, France) at 5000 rpm for 45 seconds. After this step, DNA
202 extraction was carried out following the manufacturer's protocol. A reagent-only control
203 was produced for every DNA extraction batch. All negative control samples returned
204 between 22 and 810 reads per sample, whereas the cutoff of the caecal samples was 43,808
205 reads per sample. As such, any low level contamination is unlikely to have impact our
206 analysis given the high biomass of the caecal samples. DNA was also extracted from a
207 ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvine, CA, USA) in the same
208 manner as for caecal samples and this was used as a mock community positive control.
209 After DNA extraction, DNA samples were stored at -80 °C until sequence analysis, for up to
210 three months.

211

212 **Amplicon Library Construction and Sequencing**

213 Barcoded primers specific to the variable 4 (V4) region of 16S bacterial ribosomal DNA
214 were used for amplification by polymerase chain reaction (23). PCR was performed with Q5
215 High-Fidelity 2x Master Mix (New England BioLabs, Beverly, MA, USA) with denaturation at
216 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 20 seconds, 55 °C for 15 seconds and
217 72 °C for 5 minutes, with a final extension at 72 °C for 10 minutes. PCR amplicons were
218 purified using the Ampure XP PCR purification system (Beckman Coulter, La Brea, CA, USA).
219 The concentration of purified amplicons was measured using the Qubit dsDNA HS Assay kit
220 (Thermo Fisher Scientific, Hemel Hempstead, UK). Amplicons were then pooled at
221 equimolar concentrations into a single library, whereby samples from each individual bird
222 could be identified via unique barcodes. A mock DNA control sample from Zymobiotics was
223 included as a control for the PCR step, containing bacterial DNA comprised of 25 %
224 *Enterobacetriaceae* and 12.5 % of each of another 6 bacterial species. The pooled library
225 was sequenced by paired-end 250 base pair reads on the Illumina Miseq platform (Illumina,
226 San Diego, CA, USA) using v2 chemistry. Sequencing was carried out by Edinburgh
227 Genomics, The University of Edinburgh.

228

229 **Bioinformatic analysis**

230 The microbiome helper pipeline (24) was used in this study following version 1 of the 16S
231 Bacteria and Archaea standard operating procedure from the developer. In brief, paired-end
232 reads were stitched with PEAR v0.9.6 (25). Stitched reads were filtered by quality score
233 (q=30) and length (250 bp) with the *read_filter.pl* command. Chimeric sequences were
234 removed from the samples with VSEARCH v2.7.0 (26) using the RDP trainset database (27).

235 QIIME wrapper scripts version 1.9.1 was used for OTU classification (28). SortMeRNA v2.1b
236 (29) was used as the reference-based OTU picking method while SUMACLUST v1
237 https://git.metabarcoding.org/obitools/sumaclust/wikis/home/) was used for the *de novo*
238 OTU picking method. Samples were rarefied by using the lowest number of reads from any
239 sample in the analysis excluding negative control samples. Finally, OTU tables were
240 generated in BIOM format for diversity analysis and abundance comparison with R version
241 3.4.2. Non-Metric Multidimensional Scaling (NMDS) plots were constructed using Bray-
242 Curtis dissimilarity values and statistical analyses comparing the distance of bacterial
243 community compositions between groups were performed using the adonis function in R,
244 which is part of the *vegan* package (31). Comparisons of bacterial abundance were
245 calculated with analysis of composition of microbiomes (ANCOM) (32). Data visualization
246 was performed with the *ggplot2* package (33). *P* values ≤ 0.05 were taken to be significant.
247
248 **Accession number(s).**
249 Sequencing reads can be accessed in the European Nucleotide Archive under accession
250 number PRJEB35577.
251
252 **Results**
253
254 **Reciprocal transfer of caecal microbiota from resistant or susceptible inbred lines does not**
255 **confer the phenotype against *C. jejuni* colonization.**
256 We hypothesised that differential resistance of inbred lines 6_1 and N to *C. jejuni*
257 colonization will be associated with variation in indigenous microbiota at a key site of
258 persistence, and that heterologous transplants of caecal microbiota would transfer

susceptibility (microbiota from line N into line 6_1) or resistance (microbiota from line 6_1 into line N) to *Campylobacter* challenge. Caecal microbiota was pooled from five donor birds of each line at 21 days of age. Following homologous or heterologous administration of caecal contents from the donor birds to day of hatch recipient chicks, 4 recipient birds were sampled from each group at 1, 7 and 21 days post-hatch and on day 21, the remaining 10 birds from each group were challenged with 10^4 CFU of *C. jejuni* 11168H as described in Materials and Methods. The data presented derive from a single study of this design.

In birds of each line given homologous caecal microbiota, the previously described differences in *C. jejuni* colonization were reproduced (Figure 1). A mean of 1.3 x 10^7 CFU/g caecal contents was detected in susceptible line N birds given line N microbiota, whereas for resistant line 6_1 birds given line 6_1 microbiota, no *Campylobacter* was isolated at the limit of detection by direct plating (2 $\log_{10}$ CFU/g). In the groups that received heterologous microbiota, none of line 6_1 birds given microbiota from susceptible line N were colonized at the limit of detection (Figure 1). In birds of line N given microbiota from the resistant line 6_1, a mean of 2.1 x 10^8 CFU/g caecal contents was detected, which represents a statistically significant increase compared to the birds of the same line which received homologous microbiota (Figure 1; $P = 0.002$).

The global composition of the caecal microbiota of donor birds varying in *Campylobacter* resistance is not significantly different but line-specific OTUs exist

From a total of 106 samples (including positive and negative controls) in this experiment, a total of 23,065,560 reads were sequenced on the Illumina Miseq platform. After quality filtering and the chimeric reads removal step, there were 9,911,881 reads from all caecal content samples that passed through the OTUs classification step. The average number of

283 reads per caecal sample was 101,620 ± 46,029. All samples were rarefied at 43,808 reads. In
284 the mock bacterial population control sample used as the DNA extraction control, we
285 obtained on average 11.15 % (± 7.97 %) abundance of the six bacterial species and
286 *Enterobacteriaceae* were present at 31 %. In the mock DNA control sample used as the
287 control for the PCR step, we obtained on average 13.24 % (± 5.28 %) abundance of the six
288 bacterial species and *Enterobacteriaceae* were present at 19 %. The relative abundance of
289 the individual bacterial species in the mock controls is given in Supplementary Table 1.
290 While we observed some differences between the observed and the expected mock
291 community compositions, any biases are likely to be consistent across samples.
292 Furthermore, while *Listeria* was under-represented in the PCR control sample, it is not
293 anticipated to be a major genus in the intestinal microbiota of chickens.
294 The total number of OTUs generated from the classification step was 1,297 OTUs (from
295 all caecal and positive control samples). The average number of OTUs from caecal samples
296 was 662 OTUs per sample. This compares favourably to independent analysis of microbial
297 diversity in broiler chicken caeca (34; 35) Using a non-metric multidimensional scaling
298 (NMDS) plot to compare the caecal microbiota obtained from donor lines 6_1 and N at 21
299 days of age, no significant difference was observed between them (Figure 2; $P = 0.061$ by
300 the adonis test). The caecal bacterial communities of the donor birds were dominated by
301 the phylum *Firmicutes* (Figure 3). At family level, unknown families in the orders
302 *Clostridiales* and *Ruminococcaceae* dominated in both chicken lines (Figure 3). However, a
303 comparison of bacterial abundance between lines at the level of individual OTUs using the
304 analysis of composition of microbiomes method (ANCOM) revealed three significantly
305 different OTUs between the lines from the *Ruminococcaceae* family (*P* values < 0.05). One
306 OTU in the genus *Oscillospira* was present at a mean of 146 ± 64 reads in caecal microbiota

307 from resistant line 6_1 donor birds but was completely absent from susceptible donor birds of
308 line N. An OTU of an unclassified genus was found to be significantly more abundant in line
309 N while another OTU of an unclassified genus was found to be significantly more abundant
310 in line 6_1.
311
312 **Age rather than the origin of transplanted microbiota had a dominant effect on the caecal**
313 **microbial communities studied**
314 We next investigated whether microbiota transplants influenced the composition of the
315 caecal microbiota over time. The NMDS plot in Figure 4 indicates that age rather than the
316 treatment received was the major factor that influenced the caecal microbiome. The
317 microbiota of day-old birds clustered separately from 21- and 30-day-old birds, with the 7-
318 day-old birds showing an intermediate clustering (*P* values ≤0.001 at all time intervals
319 studied). At family and phylum levels, no significant differences were detected between the
320 microbial communities found in the caeca of line 6_1 or N birds given homologous or
321 heterologous microbiota over time (Figure 5). The microbiota of chickens at 1 day post-
322 transplant had a lower diversity of bacteria and was mainly dominated by the phylum
323 *Proteobacteria* (Figure 5). At day 1 post-transplant the microbiota clustered separately from
324 that of donor chickens (Figure 4), which was dominated by the *Firmicutes* phylum (Figure 5).
325 Within this phylum, the *Enterobacteriaceae* family dominated at 1 day post-transplant
326 (Figure 5). The analysis pipeline used in this study was not able to identify the bacteria to
327 the species level; however, nucleotide sequence alignments using BLAST searches with
328 representative sequences of this OTU indicated that the dominant bacterium at 1 day post-
329 transplant was *Escherichia coli*. The intermediate phenotype of 7-day-old chickens was
330 largely a consequence of the presence of bacteria in the *Bacillaceae* family (Figure 5).

331 To further investigate the stability of the transplanted microbiota we used a multivariate
332 comparison of all the treatment groups at each time-point separately to determine if the
333 origin of the transplant or recipient line contributed significantly to the clustering of the
334 microbiota. This analysis revealed that the donor transplant had some effect on the
335 composition of the microbiota, but not at all time-points studied (Figure 6). Using the adonis
336 test, the origin of transplant influenced the caecal microbiota of 1 day-old recipient chickens
337 but the genotype of the recipient did not (P = 0.007 and 0.071, respectively). At 7 days of
338 age, neither transplanted bacteria nor the genotype of the recipient birds had a significant
339 effect on the caecal microbiota of the recipient (P = 0.068 and 0.232, respectively), possibly
340 owing to the low number of birds sampled at each of the first three time-points. After 21
341 days of age, the genotype of the recipient significantly affected the caecal microbiota of the
342 recipient (P = 0.002 and 0.001 at 21- and 30-days-old, respectively). The transplanted
343 microbiota did not affect the caecal microbiota at 21-days of age (P = 0.261), but had a
344 significant effect in the 30-day-old recipients (P = 0.012).
345 Lastly, because we observed significant effects of the transplant at some time intervals
346 (Figure 6), but the average bacterial abundance at phylum and family levels was not
347 statistically different (Figure 5), we investigated whether the microbiota transplants
348 changed the relative abundance in the recipient birds of the same OTUs which we identified
349 to be significantly different in donor birds. An OTU in an unknown genus of
350 *Ruminococcaceae* was more abundant in the donor birds of line N (Figure 7A); an OTU from
351 unknown genus of *Ruminococcaceae* was significantly more abundant in the donor birds of
352 line 6_1 (Figure 7B); and an OTU in the genus *Oscillospira* was significantly more abundant in
353 the donor birds of line 6_1 (Figure 7C). The abundance of these OTUs per line following
354 homologous or heterologous microbiota transplants was determined using ANCOM analysis.

355 We found that these OTUs did not show significant differences when compared between
356 recipient lines or donor bacteria at 1 or 7 days post-transplant (Figure 7). The only
357 significant effect was that of the genotype of the recipient line at both 21 and 30 days of age
358 (Figure 7). This suggest that the transplanted bacteria may have only been able to persist in
359 the recipient birds for a limited period of time.

360 At the level of individual OTUs we also examined the relative abundance of
361 *Campylobacter* (Figure 8). Sequence reads corresponding to *Campylobacter* OTUs were only
362 detected in the susceptible line N birds and, within this line, the birds which received the
363 heterologous microbiota transplant had significantly higher abundance of *Campylobacter*
364 compared to the birds which received homologous microbiota (Figure 8; $P < 0.05$),
365 consistent with the bacterial counts detected (Figure 1).

366

367 **Discussion**

368 Control of *Campylobacter* infections in poultry remain challenging and, to date, no
369 methods for effective control at the farm level have been developed, other than the
370 application of stringent biosecurity. Previous literature has demonstrated that the intestinal
371 microbiota can play a role in resistance to enteric pathogens in mice (16), chickens (14; 19)
372 and pigs (36). Consequently, we investigated the contribution of caecal microbiota to the
373 differential resistance of inbred chicken lines 6_1 and N to colonization by *C. jejuni*, which
374 have been demonstrated by experimental inoculation with several *C. jejuni* strains (6; 7).
375 The same lines also differ in resistance to enteric carriage of *S*. Typhimurium in the same
376 direction (22) and we reasoned that differences in their microbiota may contribute to this.
377 To this end, we performed homologous and heterologous microbiota transplants between
378 these two lines of chickens, followed by inoculation with a dose per bird of 10^4 CFU of *C.*

379 *jejuni* 11168H. Contrary to a precedent in the literature which described resistance to
380 *Citrobacter* being transferable between strains of inbred mice following transfer of faecal
381 microbiota (16), we observed a significant increase in susceptibility of line N to *C. jejuni*
382 following the transfer of caecal microbiota from resistant line 6_1 birds (Figures 1 and 8). The
383 underlying basis of this effect will require repetition and further investigation.

384 The colonization phenotypes observed following heterologous transfer of microbiota are
385 to be interpreted in the context of 16S rDNA amplicon analysis. This revealed no statistically
386 significant difference in the clustering of the microbiota of the donor birds, albeit visually
387 there appeared to be separation of the microbiota of the two lines by principal component
388 analysis plots (Figure 2). It is possible that if we had sampled more birds of each line
389 differences at the level of the global community, phyla or families may have become
390 significant. A similar separation of caecal microbial communities by the recipient line was
391 detected (Figure 6), which was significantly different at the latter two time-points, possibly
392 owing to the higher number of birds analysed. As we only examined *C. jejuni* colonization of
393 the caeca of lines 6_1 and N for parity with preceding studies (6, 7), we cannot preclude the
394 possibility that microbial transplants may have affected faecal excretion of *Campylobacter*
395 and bird-to-bird transmission, as was reported to be significantly impaired following faecal
396 microbiota transplantation in a seeder-bird challenge model up to a typical slaughter age of
397 broiler chickens (19).

398 It could be argued that the microbiota transplant did not successfully establish in the
399 recipient birds as no significant differences were observed at the level of the entire
400 microbiome after the transplant (Figure 5). However, when dissected across the time-
401 course, the microbiota transplant did exert a significant effect on the microbiota of the
402 recipient birds (Figure 6), albeit later in the experiment the line of recipient birds had a

larger influence. RNA sequencing analysis using caecal mucosa from these two chicken lines supports the notion that bird genetics have the greatest influence on *C. jejuni* colonization as we observed the largest number of differences in gene expression between uninfected birds of the two lines, with the expression of relatively few additional genes affected by *Campylobacter* infection (Russell KM, Smith J, Bremner A, Chintoan-Uta C, Vervelde L, Psifidi A and Stevens MP submitted for publication). Previous experiments have made similar observations, with the line of chickens being described as one of the main factors which influences the intestinal microbiota (35). While we detected some significant differences in the prevalence of specific OTUs between donor birds, we could not conclusively demonstrate the early transfer of these OTUs in reciprocal transplants, albeit we did observe these OTUs to be present in similar proportions to donor birds in recipient birds of the same line later following inoculation (Figure 7). Alternatively, given the delay in observing this phenotype, it is possible that these OTUs were differentially selected from the environment by each recipient line, as with increasing age bird line exerted a stronger effect on the microbiota composition.

We observed that the age of birds has a large effect on the composition of the microbiota. At one day post-hatch, irrespective of the origin and composition of donor microbiota, we observed a large population of *Proteobacteria* (and more specifically *E. coli*) in the caeca. By one week following inoculation with microbiota *Firmicutes* dominate the caeca. Similar observations were reported in other microbiota studies in chickens (37; 38). It is not known what causes this proliferation of *E. coli* in neonatal chicks but it may plausibly be linked to the susceptibility of neonatal chickens to colibacillosis, which is widely recognised as a key cause of mortality of chicks in hatcheries and soon after placement (39). A large influence of the age of the chickens on the composition of their microbiota was also

427 reported in relation to colonisation by *C. jejuni* (15) and was identified via meta-analysis of
428 available datasets (40).

429 Our study determined that, at least in the case of these two particular inbred chicken
430 lines under our experimental conditions, the microbiota does not play a major role in their
431 differential resistance to *Campylobacter* colonization and that the transplantation of the
432 microbiota from resistant to susceptible birds may not be a viable control strategy. Recent
433 evidence in mice (41) highlights variability in the effect of the transplant when using
434 recipient mice of different ages. Indeed, it has been reported that while faecal microbiota
435 transfer reduced *C. jejuni* colonization and transmission when given to neonatal chicks, it
436 had little impact when administration was delayed to day 7 of age (19). The observations of
437 these authors indicates that the concept of microbiota transplantation has merit, however,
438 while they found the microbiota of recipients to be affected by the transplant, they too
439 observed expansion of OTUs that were not a major component of the transplanted material
440 (e.g. *Lactobacilli*; 19). This may indicate that the transplant changes the gut environment to
441 favour other microbes, as much as transfer them directly. Such changes may account for the
442 significant increase in *C. jejuni* colonisation in the susceptible line following transplant of
443 caecal contents from the resistant line. Where future studies reliably detect protective
444 effects, they may therefore need to consider impacts on metabolites and the mucosal
445 immune system, not just the microbes present *per se*.
446

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625

626 **Figure Legends**

627

628 **Figure 1. Transfer of caecal microbiota between inbred lines 6_1 and N is not protective**
629 **against *C. jejuni* colonization.** Chickens were given homologous or heterologous caecal
630 microbiota from 3-week-old donor birds on the day of hatch and infected with 10^4 CFU of *C.*
631 *jejuni* 11168H at 21 days post-transplant. Ten chickens were sampled in each group at 9
632 days post-infection and significant differences were identified using a one-way two-sided
633 ANOVA (Minitab, UK). Birds from line N which received microbiota from line 6_1 had a
634 significantly higher number of caecal *C. jejuni* compared to the line N birds that received line
635 N microbiota (P <0.05; asterisk). For groups noted on the X axis, the first letter denotes the
636 recipient line and the second letter denotes the donor line.

637

638 **Figure 2. Caecal microbial communities of donor birds of inbred lines that exhibit heritable**
639 **differences in resistance to *C. jejuni* colonisation are not significantly different.** The figure
640 shows a non-metric multidimensional scaling (NMDS) plot of the caecal microbiota from the
641 5 donor chickens of each line 6_1 and N at 21 days of age. While spatially the two lines
642 clustered separately, there was no statistically significant difference between the microbiota
643 of the two lines of chickens when investigated using the adonis test (P = 0.061).

644

645 **Figure 3. The bacterial composition of the caecal microbiota of 21-day-old donor chickens**
646 **of lines 6_1 and N is dominated by *Firmicutes* at phylum level and *Ruminococacceae* and an**
647 **unknown family in the order *Clostridiales* at family level.** Five birds were sampled in each
648 of the donor lines. The data represents the composition of the individual samples averaged

post-sequencing. The overall composition of the microbiota was not significantly different between the two lines.

Figure 4. Composition of the caecal microbiota of the recipients of transplants was primarily determined by the age of birds rather than the treatment received. The NMDS plot shows the clustering of caecal samples by bacterial community composition for all recipient chickens, grouped by age (12-16 birds were sampled at days 1, 7 and 21 and 40 birds at day 30), and for donor chickens (10 birds sampled at 21 days of age). Samples from all ages were found to cluster separately by the adonis test ($P \leq 0.001$). The Bray-Curtis dissimilarity values were used to calculate the dissimilarity between samples.

Figure 5. Caecal microbiota of lines 6_1 and N that received homologous or heterologous microbiota transplants are not significantly different. No significant differences were detected in the average bacterial abundance at phylum (A) or family (B) level in the caecal microbiota of inbred lines 6_1 and N given a homologous or heterologous microbiota transplant. Five birds were sampled per group for the donor birds, 2-4 birds per group at days 1, 7 and 21 and 10 birds per group at day 30. For groups noted on the X axis, the first letter denotes the recipient line and the second letter denotes the donor line.

Figure 6. Caecal microbiota transplants influenced the composition of the microbiota early in the experiment, but bird line had a dominant effect with increasing age. NMDS plot of gut microbiota at each time point: day 1 - top left, day 7 - top right, day 21 - bottom left, day 30 - bottom right. *P* values for the effect of the transplant or the bird line were obtained using the adonis test and are presented within boxes on each plot.

673

674 **Figure 7. The abundance of specific OTUs in donor microbiota and in caeca following**
675 **homologous or heterologous transplants.** (A) Unknown genus in the *Ruminococcaceae*
676 family. (B) A different unknown genus in the *Ruminococcaceae* family. (C) The genus
677 *Oscillospira*. Differences were investigated using ANCOM, *P* value for all comparisons is
678 <0.05 (asterisks). For groups noted on the X axis, the first letter denotes the recipient line
679 and the second letter denotes the donor line.

680

681 **Figure 8. The abundance of *Campylobacter* detected by direct plating on mCCDA was**
682 **validated by the abundance of OTUs detecting by sequencing.** The graphs shows the
683 abundance of sequence reads corresponding to the OTU for the genus *Campylobacter*
684 across all groups. Ten birds were sampled per group at day 30 of age. Significant differences
685 were identified using a one-way two-sided ANOVA (Minitab, UK). Birds from line N which
686 received microflora from line 6_1 had a significantly higher number of reads compared to the
687 other three group (*P* <0.05). Only the difference between the N/6 and N/N groups is shown
688 on the graph (*). For groups noted on the X axis, the first letter denotes the recipient line
689 and the second letter denotes the donor line.

Figure 1

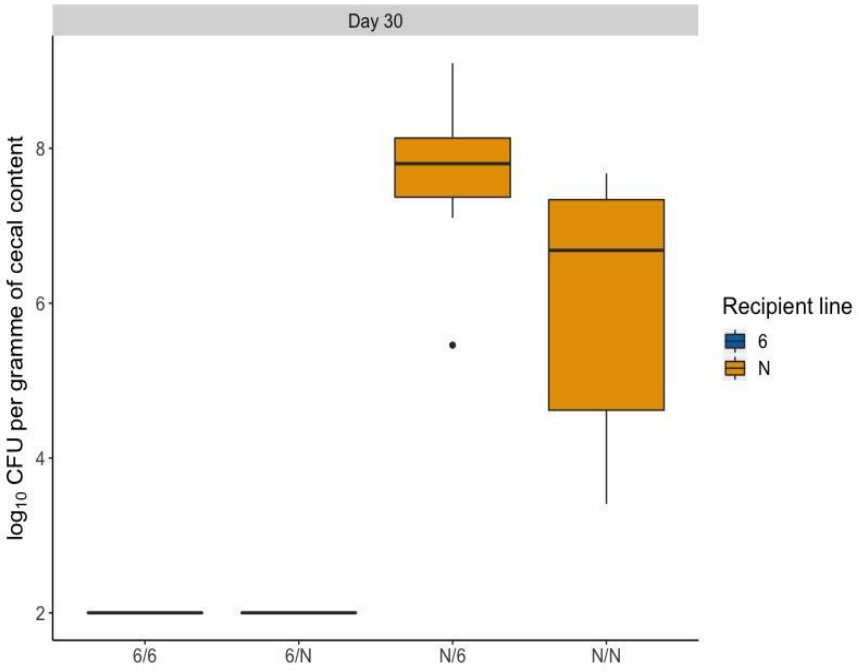
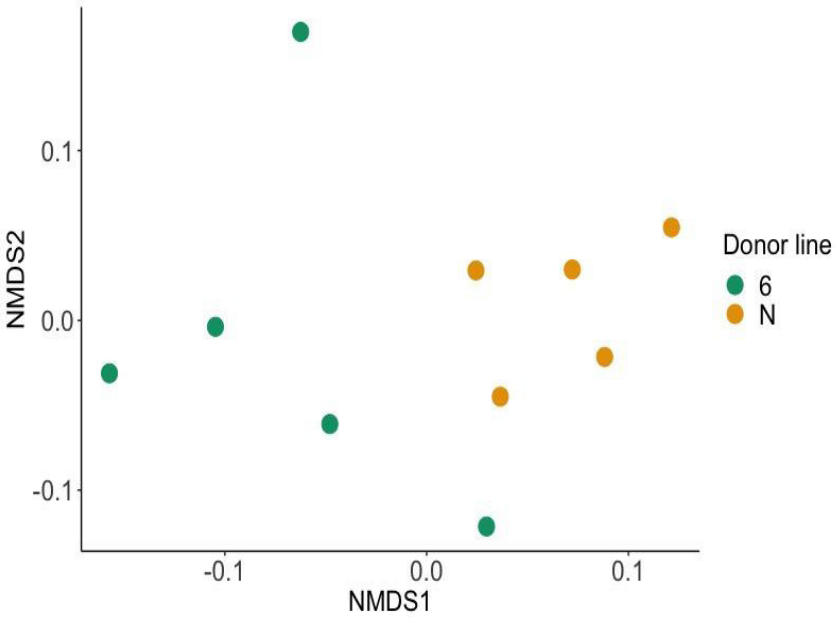


Figure 2



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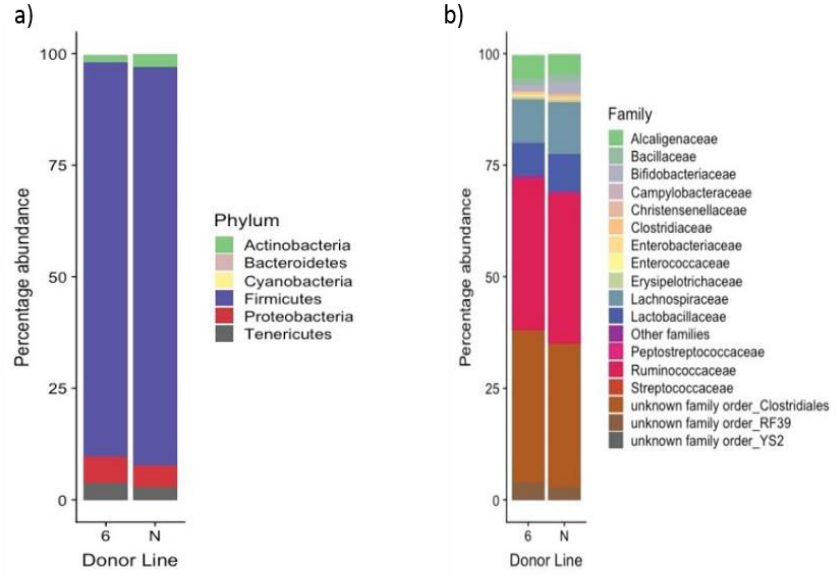

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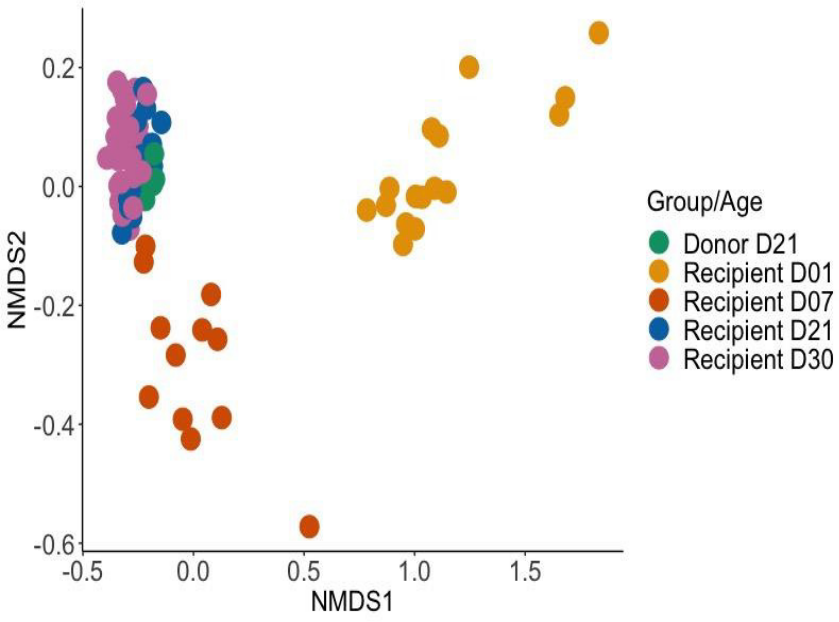

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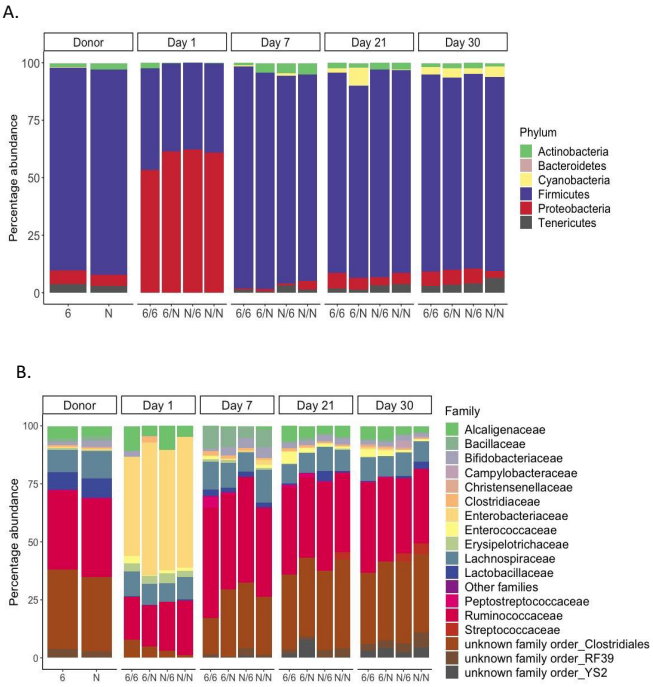

Figure 6

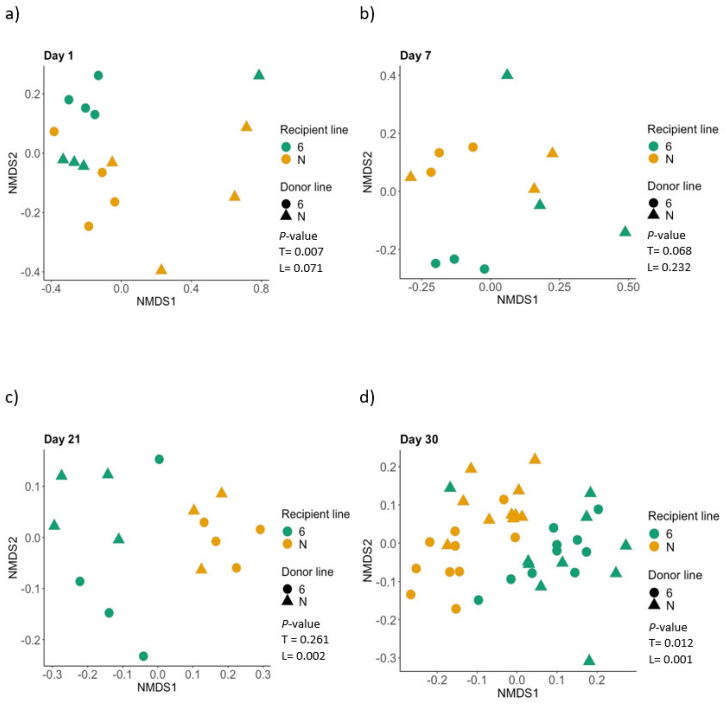

Figure 7a

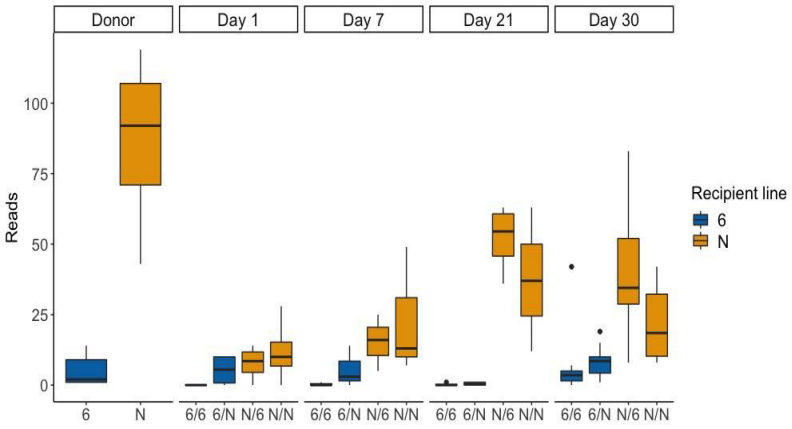

Figure 7b

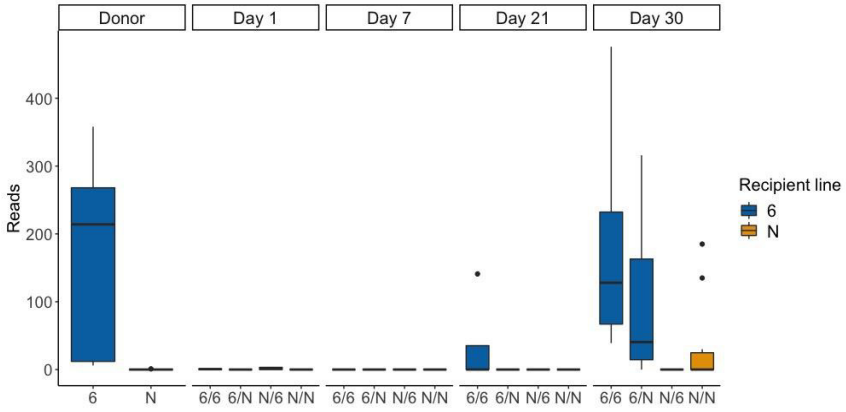

Figure 7c

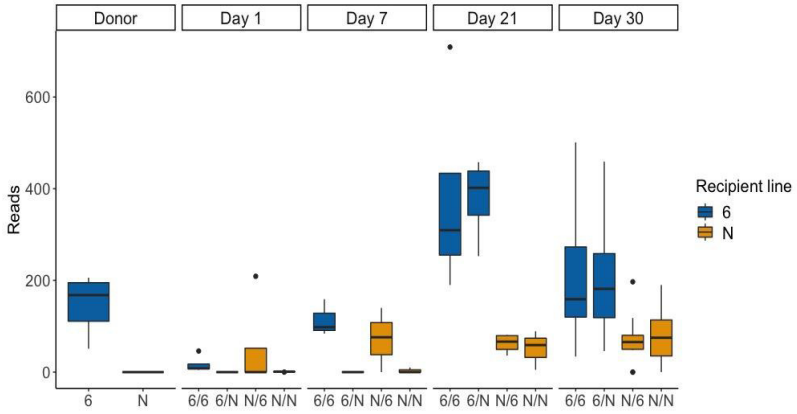

Figure 8