INFLUENCE OF DIETARY OLIGOSACCHARIDES ON THE GUT MICROBIOME

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ABSTRACT

Chicken meat is an important source of dietary protein in the global food chain. Although broiler chicken production is efficient compare to other sources of animal protein productivity will need to be increased through sustainable and ethical approaches to provide hygienically safe meat to meet the needs of a growing human population. Traditional methods of improving productivity have plateaued and new approaches are required to improve returns. Manipulation of the gut microbiota with antibiotic growth promotors has been a mainstay to improve growth performance, however this approach has fallen out of favour with regulators and consumers due to concerns about the spread of antimicrobial resistance. A potential alternative is the prebiotic galacto-oligosaccharide (GOS) - a by-product of the dairy industry that has been shown to affect human physiology. The articles presented here are the outputs of a systems biology approach to describing the effects of GOS on broiler chickens and upon colonization by the zoonotic pathogen *Campylobacter jejuni*. The focus of this thesis are findings generated by analysis of the gut microbiota using 16S rDNA cataloguing. The results show that the growth performance of fast-growing broiler chickens is improved by dietary GOS compared to birds sustained on an isocaloric control diet. Additionally, results presented here show how the dynamics of *C. jejuni* colonization are affected by the age of the bird at challenge alongside the accompanying changes to the gut microbiota and innate immunity. Finally, results demonstrate that phage therapy can reduce *C. jejuni* colonization of the gastrointestinal tract by >2 $\log_{10}$ CFU ml^{-1} without provoking dysbiosis of the gut microbiota. The novel findings presented here are of interest to the poultry industry and are the first step in determining a mode of action for GOS and potentially other prebiotic oligosaccharides. This work provides a foundation for future research with the goal of designing functional feed compositions to sustainably enhance chicken production whilst making the chicken gut less hospitable to pathogenic bacteria.

ACKNOWLEDGEMENTS

I would like to thank my academic supervisor Prof Ian Connerton. Thank you to Neville Fish and Dairy Crest for funding this work. Thank you so much to Geri LaFontaine, Pippa Connerton and all the members of the Connerton lab.

Dedicated to the memory of Jerry Garcia.

STATEMENT ABOUT JOINT AUTHORSHIP

Statement from joint principal author Phillippa L Connerton with regard to Connerton et al. (2018)

I agree with the description of the extent of the contribution of Philip Richards to the paper entitled: The effect of the timing of exposure to Campylobacter jejuni on the gut microbiome and inflammatory responses of broiler chickens.

Statement from joint principal author Geraldine M. Flaujac Lafontaine with regard to Lafontaine et al. (2020) and Connerton et al. (2018)

I fully endorse and agree with your statement describing the extent of your contribution to the joint papers Connerton et al. (2018) and Lafontaine et al. (2020).

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To whom it may concern

Dear Sir/Madam

Ref: Philip Richards

I am the primary PhD supervisor of Philip Richards and this letter is to confirm his contribution to the open access manuscripts presented in his thesis submitted 28th February 2020.

Philip Richards has contributed to the research team working on prebiotics as both primary researcher and bioinformatics researcher. These skills are evident in the manuscripts and are entirely consistent with research towards a PhD in the School of Biosciences.

I have no reservations in stating that the work specified in his thesis was developed by him in the course of his research and represents an original research portfolio as defined by the University of Nottingham.

Yours sincerely,



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CHAPTER 1. INTRODUCTION

1.1 Summary

The research submitted in this thesis improves our understanding of the effect that prebiotic oligosaccharides have on the gut microbiome; with the specific aim of describing the host:microbe:diet interactions that take place in broiler (meat) chickens. My research has focused on observing and describing what effect the introduction of dietary, host-inaccessible galacto-oligosaccharides (GOS) has on the bacterial ecology of the chicken caecal pouch and how this might influence growth performance and colonization resistance to zoonotic pathogens. In collaboration with colleagues the host response that accompanies the addition of in-feed GOS has been characterized through the profiling of gastrointestinal innate immunity. The results generated by this multi-disciplinary approach provide a foundation for an understanding of how host:microbiome:diet interactions might be deliberately managed to improve zootechnical performance. These findings can also inform future work on formulating feed with specific prebiotics to make the gastrointestinal tract of chickens less hospitable to zoonotic pathogens, including *Salmonella*, *Campylobacter* and other undesirable agents. The goal of these studies is the deliberate application of host-inaccessible dietary carbohydrates as an intervention against specific causes of sub-optimal performance and/or disease.

Please note that in this work *microbiota* will refer to the population of microorganisms within an ecosystem and *microbiome* will refer to the total gene repertoire of these microorganisms, as described by Ursell et al. (2012). A prebiotic is a substrate that is selectively utilized by host microorganisms to confer a health benefit (Gibson et al., 2017). *Microbiota-accessible carbohydrates* (*MACs*) are a broader category of carbohydrates that are metabolically available to the gut microbiota and include dietary prebiotics as well as those carbohydrates secreted by the host or produced by microbes within the gut (Sonnenburg and Sonnenburg, 2014).

1.2 Project motivation

This project was undertaken as part of a wider initiative from Dairy Crest Ltd, who wished to develop methods to assess the efficacy of prebiotic galacto-oligosaccharides (Nutrabiotic^{TM}) that have potential applications in infant formulae and farm animal (broiler chickens and piglets) feed. The sponsors objectives were to: (1) examine the ability of the different "prebiotic" materials, purified of any digestible components, to support the growth of a range of different micro-organisms selected from "probiotic" micro-organisms

and micro-organisms that characterise a healthy microbiota in each of the target species (human infants, broilers and piglets); (2) assess the metabolism of the different components in each of the "prebiotic" materials. The outcomes to these systematic studies were to form the basis of patentable IP before publication of the research to provide peer-reviewed research reports in support of commercial activities. To meet these objectives required a dedicated research team with challenging deadlines. This thesis represents a component of the research progress focussed on broiler chickens.

1.3 The chicken gut microbiota

Population trends indicate that demand for dietary protein will increase by 50% by 2050 (Westhoek et al., 2011). Increased broiler chicken production can augment the global response to this problem as it is among the most ecologically sound origins of meat protein (Vries and Boer, 2010). A uniquely comprehensive knowledge of how host factors affect growth performance has promoted huge gains in the metabolic efficiency and productivity of broiler chickens. However, the gains once made through selective breeding (Tallentire et al., 2018) and feed optimisation are declining as a sizeable proportion of birds fail to attain potential in terms of health, growth rate and feed efficiency. This may be due to host-microbe interactions and recent research efforts have focused on improving flock performance through deliberate promotion of gut bacteria beneficial to health and nutrition (Immerseel et al., 2017). It has been recognised for over four decades that commensal bacteria colonizing the gastrointestinal tract of the domestic chicken (*Gallus gallus*) affect growth performance and susceptibility to infectious disease (Barnes et al., 1972, 1979). Historically the productivity of farmed chickens was improved through administration of antibiotic growth promoters (AGP). The mechanisms by which AGPs improve bird performance are poorly understood but are believed to be linked to suppression of pathogenic bacteria and down-regulation of host pro-inflammatory innate immunity (Kumar et al., 2018; Niewold, 2007). Concerns over the spread of antimicrobial resistance throughout bacterial communities has led to a ban in the EU (Commission Regulation (EC) 1831/2003) of the prophylactic use of AGPs (such as monensin sodium, salinomycin sodium, avilamycin, flavophospholipol) and has increased demand for antibiotic-free chicken meat and chicken products in other markets.

In common with many published descriptions of the chicken gut microbiota, the main focus of the microbiological analysis presented here is on the bacterial population of the caecal lumen. In birds the caeca (*singular* caecum) are two blind pouches attached to the gastrointestinal tract where the large and small intestines join (the ileal-caecal junction). In

birds the caeca are thought to be involved in fibre metabolism as they have a relatively larger size in herbivorous birds (Grond et al., 2018) and grow in broiler chickens in response to increased levels of dietary fibre (Jørgensen et al., 1996). Correspondingly, the caecal microbiome of broiler chickens encodes a diverse array of biochemical pathways involved in the breakdown of polysaccharides (Sergeant et al., 2014). The caeca are also of interest as this is where the greatest numbers of zoonotic pathogen *C. jejuni* are to be found in colonized chickens (Connerton et al., 2018; Hendrixson and DiRita, 2004). Exposure to poultry meat contaminated with digesta from *C. jejuni*-colonized birds is a key source of foodborne illness (Osimani et al., 2017) where the risk of exposure is reduced by lowering the levels of *Campylobacter* in the gastrointestinal tract (Rosenquist et al., 2003). In order to seek pathogen control at the level of the chicken carcass, the EU have adopted a microbiological sampling plan for broiler chicken carcasses with a limit of 1,000 CFU g^{-1} (Commission Regulation (EU) 2017/1495). The studies presented here therefore focus on the caeca, based on the supporting evidence for the role of these organs in the metabolism of host-inaccessible carbohydrate and as reservoirs of zoonotic pathogens. This does not exclude the role of bacterial colonizing other sites in the gastrointestinal tract on performance and health (see section 3.4).

1.4 Prebiotic carbohydrates

The current consensus definition of prebiotics encompasses a range of substrates that includes both oligosaccharides and polyphenols (see Section 1.1; Gibson et al., 2017). Commercial sources of non-digestible oligosaccharides, including GOS, fructooligosaccharides (FOS), mannoligosaccharides (MOS), isomaltooligosaccharides (IMOS), xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS) are marketed for use in human and/or animal nutrition to promote gut health (Macfarlane et al., 2006; Saville and Saville, 2019). GOS and FOS have been the subjects of research efforts devoted to dietary prebiotics due to their inclusion in infant formula as a substitute for human milk oligosaccharides (Verkhnyatskaya et al., 2019). GOS are typically made up of a terminal reducing lactose linked to between 1 and 6 galactose moieties (Goh and Klaenhammer, 2015) and are manufactured using β-galactosidase-catalysed transglycosylation of lactose-whey, a by-product of dairy cheese production. This approach generates oligosaccharides with between 2 and 8 degrees of polymerization (DP) that in combination with the variety of possible linkage types gives rise to a huge number of different GOS structures (Bueren et al., 2017; Leeuwen et al., 2016, Logtenberg et al., 2020). These linkages are typically β(1→4), β(1→6) and β(1→3) with the proportions of each determined by the properties of

β-galactosidase(s) from different producing organisms. FOS are linear fructose oligomers produced by enzymatic modification of plant material, giving oligosaccharides with restricted structural heterogeneity, or from the β-fructofuranosidase-catalysed transfructosylation of sucrose (Niness, 1999; Goh and Klaenhammer, 2015). *In vitro* studies have determined that DP and structure affects GOS availability to probiotic bacteria at both species and strain level (Thongaram et al., 2017; Böger et al., 2019). By independently cultivating a number of probiotic bacteria with purified GOS (Vivinal® GOS, FrieslandCampina Domon, NL) and analysing the residual GOS components in stationary phase cultures, using advanced chromatography techniques, Böger et al. (2019) showed that lactic acid bacteria were often limited to DP2-3 GOS molecules, with bifidobacteria having a greater range (Böger et al., 2019). Correspondingly, the structure and DP of FOS have also been shown to influence the growth of *Bifidobacterium lactis* and *Lactobacillus acidophilus* (Wang et al., 2020). Although these studies of GOS and FOS provide some of the first empirical evidence for the preference of strains of probiotic bacteria for specific isomers of prebiotic carbohydrates, the fate of these carbohydrates *in vivo* when metabolised by the diverse bacterial population colonizing the broiler gut has yet to be determined.

1.5 Galacto-oligosaccharide has prebiotic qualities

GOS are generated from lactose, a by-product of the dairy industry, by using β-galactosidase-catalysed transglycosylation to create molecules of differing lengths and linkage types (Bueren et al., 2017; Leeuwen et al., 2016). Studies of prebiotic GOS consumption in humans have reported that it alleviates the symptoms of lactose-intolerant subjects (Azcarate-Peril et al., 2017), but may also increase fasting blood glucose levels (Liu et al., 2017). These studies, and that of other groups specifically focussing on the faecal microbiota, have reported that prebiotic GOS in humans is associated with increased proportions of *Bifidobacterium* spp. (Azcarate-Peril et al., 2017; Davis et al., 2011; Liu et al., 2017). Although Liu et al. (2017) and Davis et al. (2011) did not associate increased *Lactobacillus* levels with GOS, Azcarate-Peril et al. (2017) did. Gastrointestinal *Lactobacillus* spp. have frequently been linked with changes in the gut microbiota and potentially therapeutic immune modulation, primarily in mice (Azad et al., 2018). Supplementation of broiler chicken feed with GOS did not affect growth performance of broiler chickens or the levels of *Lactobacillus* recovered from faeces (Jung et al., 2008), but the authors did report that prebiotic GOS increased faecal levels of *Bifidobacterium* spp.

1.6 Approaches to studying the gut microbiota

Understanding of the role of the gut microbiota, and correspondingly the mode of action of AGPs, in bird performance has not kept pace with other aspects of poultry production. The laboriousness and complexity of isolating and culturing the physiologically diverse populations of bacteria resident in the gastrointestinal tract has historically restricted culture analysis to a few key organisms of pathogenic or biotechnological interest. Although surveys of the chicken microbiota did continue at a steady pace throughout the 1970s, 1980s and 1990s, research has flourished in the period since 16S rDNA sequencing approaches have been widely adopted (Rychlik, 2020). Developments in NGS high-throughput sequencing technologies and the steep fall in cost per base expense have made both 16S and WGS sequencing affordable to more laboratories. Similarly, parallel development and release of well supported, open-source curation/analysis pipelines such as QIIME and mothur (Caporaso et al., 2010; Schloss et al., 2009), has allowed researchers easy entry into bioinformatic analysis. Built into these analytical pipelines are pre-existing concepts in ecology such as the Simpson index (Simpson, 1949) and Yue and Clayton θ similarity coefficient metrics (Yue and Clayton, 2005), plus new ones developed for metagenomic data, eg. LEfSE for determining biomarkers (Segata et al., 2011). This is a relatively new discipline and it must be cautioned that there are critiques of many of the routinely employed statistical tests and their applicability to compositional sequencing data (Gloor et al., 2017).

1.7 The chicken caecal microbiota

The caecal microbiota of young chickens is dominated by the phylum Proteobacteria (Chintoan-Uta et al., 2020; Litvak et al., 2019; Ocejo et al., 2019), whereas the phyla distribution within the caecal microbiota of mature chickens is broadly similar to that observed in mammals (Rychlik, 2020). The phyla are dominated by Firmicutes (50-85%) and Bacteroidetes (0-20%) with much lower levels of Proteobacteria, as determined by 16S rDNA amplicon profiling (Chintoan-Uta et al., 2020; Kumar et al., 2018; Sergeant et al., 2014). These observations are supported by the work of Medvecky et al. (2018) who cultured 133 anaerobic bacteria from caecal content collected from chickens and reported that 63% were *Firmicutes*, 22% were *Bacteroidetes* and 11% Actinobacteria. This is a valuable confirmatory finding as many of the studies that inform the databases used for taxonomic classification of 16S rDNA data are based on studies of humans or mice, and therefore there is a potential for bias when using these databases to characterise the microbiota of non-biomedical models due to the disproportionate distributions of

unclassified sequences. It has been suggested that the high proportion of Proteobacteria in young chickens is attributable to the animal husbandry (Rychlik, 2020). Interventions employed to prevent vertical transmission of pathogens to young birds also prohibit the seeding of newly hatched chickens with the microbiota of maternal birds, with only limited potential for transmission of maternal bacteria on the eggshell (Rychlik, 2020; Veelen et al., 2018). Alternatively, empirical studies have shown that in germ-free chickens colonized with the microbiota of mature feral chickens in early life the proportion of Proteobacteria had increased by 9 days old, relative to the inoculum, and had fallen to a level below that of the inoculum by 18 days old (Thomas et al., 2019). Reviews of the published literature and a unique approach that compared the gut microbiome of ~900 vertebrate species suggest that on average birds have higher proportions of gastrointestinal Proteobacteria than mammals (Grond et al., 2018; Song et al., 2020). This indicates that factors other than successional maturation of the gut microbiota contribute to balance of Proteobacteria in the gut of juvenile chickens, for example the availability of oxygen and/or maternal immunity (Song et al., 2020).

1.8 Objectives

The objectives of this study were as follows:

1. Describe any changes in microbiota composition associated with in-feed GOS in terms of changes in the taxonomic composition of resident caecal microbiota. In collaboration with trials performed to measure established indices of broiler productivity describe the capacity of GOS materials to reproducibly modulate the gut microbiota of broiler chickens.

2. Determine the effect of *Campylobacter jejuni* challenge on the gut microbiota in collaboration with studies of the host immune response. These studies should take into account the effect of bird development and assess the applicability of intervention such as GOS-supplementation of feed or phage therapy.

1.9 Outline of results chapters

Chapter 3 describes how dietary GOS affects the microbiota and innate immunity of the gastrointestinal tract of broiler chickens. This chapter describes work, published in Richards et al. (2020), that identifies relationships between GOS-responsive *Lactobacillus* spp., zootechnical performance and host immunity. This work provides an insight into the influence of host:microbe:diet interactions on systemic physiology.

Chapter 4 describes the differential response of host innate immunity and changes in the caecal microbiota that accompany *C. jejuni*-challenge of broiler chickens at either 6 or 20 days-old. Results presented in Connerton et al. (2018) indicate that host interactions with *C. jejuni* are dependent on bird age and/or development. Results presented in Lafontaine et al. (2020) expand upon these findings by describing the interactions between dietary GOS and *C. jejuni* in broiler chickens. This work informs future applications of prebiotics or the synergistic use of prebiotic-probiotic combinations to reduce the level of campylobacters colonizing broiler chickens.

Chapter 5 reports on the effect that *in vivo* phage biocontrol of *C. jejuni* has on the ecology of gastrointestinal microbiota. The results indicate selective reduction of campylobacters does not result in gastrointestinal dysbiosis, which may be extrapolatable to other models of targeted phage therapy.

CHAPTER 2. METHODOLOGY

2.1 Overview

There is a crisis of reproducibility in microbiology (Casadevall et al., 2016; Ravel and Wommack, 2014). Laboratory bench work can be difficult to precisely report due to the awkwardness of describing in prose what can be a matter of instinct, for example when to quench a reaction. Analysis of molecular sequence data, such as 16S rDNA amplicons, is almost uniquely placed in biological science to overcome these difficulties using computational scripted approaches (Schloss, 2018, 2019). It has been the experience of the author that reproduction of published results for many microbiota studies from the accompanying methodology is more troublesome than reproducing wet-laboratory based studies. Reproduction of results is commonly impeded by sequence data not being easily accessible from freely available repositories or sequence data being made available with incomplete metadata, for example the sample IDs are frequently omitted. Additionally, there is often an absence of reporting on what analytical packages were selected or what commands and/or parameters were used.

In line with the policy of most scientific journals we have published DNA sequence data and complete metadata for each of the articles reported in this thesis; for Richards et al. (2020) we have also released the zootechnical data. The results of microbiota analysis presented here were generated using scripted workflows written in the R programming language (R Core Team, 2014) and the mothur pipeline (Schloss et al., 2009). In addition, for both Richards et al. (2020) and Lafontaine et al. (2020) figures reporting zootechnical and host gene expression data were plotted using R. To make our findings reproducible we have adopted a literate programming approach to make the scripts more readily understandable and made scripts freely available on GitHub (https://github.com/pjrichards).

I believe that reporting our scripted workflows and releasing all DNA sequence data alongside the methodologies reported in peer-reviewed articles will facilitate ready reproduction of experimental procedures and analysis. In addition to the specific methodologies detailed in the accompanying manuscripts this chapter serves to summarize the general methods adopted in the course of the research.

2.2 Summary of methodology of microbiota analysis

DNA was isolated from caecal contents using the MoBio PowerSoil kit (now Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions. This kit has been shown to isolate greater yields of gDNA from human stools than other widely available kits (Kennedy

et al., 2014) and is routinely employed to study the chicken gut microbiota (Johnson et al., 2018; Sakaridis et al., 2018). The V4 regions of the bacterial 16S rRNA genes were subsequently PCR amplified using the primers 515f and 806r (Caporaso et al., 2011) with AccuPrime™ *Pfx* DNA polymerase (ThermoFisher Scientific, Loughborough, UK). Amplicons were then sequenced on the Illumina MiSeq platform (Illumina, Cambridge, UK) using 2 x 250-bp cycles. The 515f and 806r primers are the most widely used primers for microbiota profiling and were chosen primarily so we could seamlessly adopt the Schloss laboratory MiSeq SOP (Kozich et al., 2013), which describes how to quality filter 16S rRNA gene sequences and then curate them using the mothur pipeline (Schloss et al., 2009).

2.3 Summary of experimental procedures involving chickens

The studies reported in this thesis were approved by the Local Ethics Committee of the University of Nottingham and performed under Home Office licence. Newly-hatched male Ross 308 broilers (Aviagen UK Ltd., Midlothian, UK) were procured from a commercial hatchery (P.D. Hook Ltd., Bury St. Edmunds, UK). Birds were reared under high-biosecurity, ACDP2 containment conditions in agreement with the code of practice for experimental animal housing and checked at least twice daily for signs of disease, and three times post infection. Co-housing of experimental birds allows for uncontrolled mixing of the gastrointestinal microbiota between individuals and potentially continual ingestion and shedding of non-colonizing organisms. The penning arrangements of birds in each of the studies reported here are as follows. For the results presented in Connerton et al. (2018) and Lafontaine et al. (2020) on arrival weight-matched birds were randomly allocated to either control or GOS diet groups and separately co-housed on wood shavings. In the trial that explored the effect of 'early' colonization, chickens were challenge with *C. jejuni* at 6 days-old and then isolated in independent cages. In the corresponding trial that explored the effect of 'late' colonization, chickens were challenged with *C. jejuni* at 20 days-old and then also isolated in independent cages. The birds from which populations of caecal bacterial are reported in Richards et al. (2020) were the unchallenged birds from the 'early' trial, which were grouped by diet and co-housed until they were isolated at 6 days-old. For the microbiota reported in Richards et al. (2019), birds were isolated from day-of-hatch and randomly assigned to a treatment group at 14 days-old.

In all trials feeding regimes were harmonized with standard commercial practice for intensively-raised chickens. Wheat-based feed was provided *ad libitum* as a starter crumb (0 – 10 days), grower pellets (11 – 24 days) and finisher pellets (25 – 35 days). Water was also made freely available. Experimental feed was supplemented GOS prebiotic by inclusion

of Nutrabiotic™ (GOS, 74% w/w dry matter; Dairy Crest Ltd., Davidstow, UK). Nutrabiotic™ contains a mixture of monosaccharides (glucose and galactose) and oligosaccharides (DP2 to DP8).

Birds were euthanized in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Post mortem a total of ~1 g of caecal content was collected from all birds by separating the pouches from the gastrointestinal tract and removing content from the proximal open end. Equal quantities of digesta were collected from both pouches and stored in a cryovial. A similar mass of caecal content was additionally collected from all *C. jejuni*-challenged birds, plus a subset of unchallenged birds, and transferred to a sterile, pre-weighed plastic universal tube. The same approach was used to collect illeal content, though in some instances lower yields of content were available. Strict aseptic technique was employed to prevent cross-contamination of samples, by both other regions of the gut and other birds. Content was collected from unchallenged birds to check for cross-contamination. Collected samples were stored on wet ice and transferred to the laboratory (< 1h) where gastrointestinal content in cryovials were immediately stored at -80°C until required for gDNA isolation. Universal tubes containing gastrointestinal content were weighed and gently resuspended 1:10 (w/w) in Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, UK) to be used for enumeration of campylobacters.

2.4 Summary of bacteriology and bacteriophage methods

2.4.1 Preparation of growth and storage media

Unless otherwise stated, all growth and storage media were prepared using reverse osmosis (RO) water. After complete solution, media were sterilised by autoclaving at 121°C and 15 psi for 15 minutes on a liquid cycle. For solid agar media, molten agar was initially tempered to 45 - 50°C before addition of any supplements or antibiotics. Plates were poured inside a laminar flow cabinet. After solidification, the agar plates were stored at 4°C for up to four weeks. All other chemicals were stored at room temperature after autoclaving and cooling, unless otherwise indicated.

2.4.2 Nutrient broth No.2

Nutrient broth No.2 (Oxoid Ltd.; Lab-Lemco powder 10.0 g l^{-1}, peptone 10.0 g l^{-1}, NaCl 5.0 g l^{-1}) was prepared according to manufacturer's instruction (25 g in 1 litre of RO water). After sterilisation, it was stored at room temperature for up to eight weeks.

2.4.3 Blood agar (BA)

Blood agar base No. 2 (Oxoid Ltd.; proteose peptone 15.0 g l^{-1}, liver digest 2.5 g l^{-1}, yeast extract 5.0 g l^{-1}, NaCl 5.0 g l^{-1}, agar 12.0 g l^{-1}; pH 7.4 ± 0.2 at 25°C) was prepared according to manufacturer's instruction (40 g in 1 litre of water). Following sterilisation, media was cooled to 45 - 50°C before addition of 5% (v/v) defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK).

2.4.4 MacConkey agar

MacConkey Agar No.3 (Oxoid Ltd.; peptone 20 g l^{-1}, lactose 10 g l^{-1}, bile salts no.3 1.5 g l^{-1}, NaCl 5 g l^{-1}, neutral red 0.003 g l^{-1}, crystal violet 0.001 g l^{-1}, agar 15 g l^{-1}) was prepared to the manufacturer's instructions (51.5 g suspended in 1 litre of water).

2.4.5 *Campylobacter* blood-free selective agar (modified CCDA-Preston)

Campylobacter blood-free selective agar (Oxoid Ltd.; nutrient broth No.2 25.0 g l^{-1}, bacteriological charcoal 4.0 g l^{-1}, casein hydrolysate 3.0 g l^{-1}, sodium deoxycholate 1.0 g l^{-1}, ferrous sulphate 0.25 g l^{-1}, sodium pyruvate 0.25 g l^{-1}, agar 12.0 g l^{-1}; pH 7.4 ± 0.2 at 25°C) was prepared according to manufacturer's instruction (45.5 g in 1 litre of water). For *Campylobacter* quantification an additional 2% (w/v) of bacteriological agar No.1 (Oxoid Ltd.) was added to prevent swarming of cells. For the isolation of *Campylobacter* from environmental samples, 1 vial of CCDA selective supplement (Oxoid Ltd.; 16 mg of cefoperazone and 5 mg of amphotericin B) was dissolved in sterile RO water and aseptically added to 500 ml of sterilised CCDA after cooling to 45 - 50°C.

2.4.6 NZCYM basal agar

NZCYM broth (BD, Wokingham, UK; pancreatic digest of casein 10.0 g l^{-1}, yeast extract 5.0 g l^{-1}, NaCl 5.0 g l^{-1}, casamino acids 1.0 g l^{-1}, $MgSO_4$ (anhydrous) 0.98 g l^{-1}, pH 7 ± 0.2 at 25°C) was prepared to the manufacturer's instructions (22 g suspended in 1 litre of water). Additionally, 15 g of bacteriological agar No.1 (Oxoid Ltd.) was added to the broth to a final agar concentration of 1.5% (w/v) to create NZCYM basal agar.

2.4.7 NZCYM overlay agar

NZCYM overlay agar was prepared by adding bacteriological agar No.1 (Oxoid Ltd.) to NZCYM broth (BD) to a final agar concentration of 0.6% (w/v). Following sterilisation, 5 ml volumes were aseptically aliquoted into sterile glass universal bottles and were allowed to temper in a 55°C water bath until ready for use.

2.4.8 Phosphate Buffered Saline (PBS)

Phosphate buffered saline (Oxoid Ltd.) was prepared according to the manufacturer's instructions to a pH of 7.3 ± 0.2 at 25°C. The final composition constituted: NaCl 0.8% (w/v), KCl 0.02% (w/v), Na_2HPO_4 0.115% (w/v) and KH_2PO_4 0.02% (w/v). Following sterilisation, the PBS was stored at room temperature until ready for use.

2.4.9 Maximum Recovery Diluent (MRD)

Maximum recovery diluent (Oxoid Ltd.; peptone 1.0 g l^{-1}, NaCl 8.5 g l^{-1}) was prepared by suspending 9.5 g into 1 litre of RO water. It was stored at room temperature following sterilisation until ready for use.

2.4.10 Mueller-Hinton (MH) broth

Mueller-Hinton broth (Oxoid Ltd.; dehydrated beef infusion 300 g l^{-1}, casein hydrolysate 17.5 g l^{-1}, starch 1.5 g l^{-1}) was prepared according to manufacturer's instruction (21 g in 1 litre of RO water). After sterilisation, it was stored at room temperature for up to eight weeks. Before phage titre determination the broth was supplemented to include 10mM $MgSO_4$.

2.4.11 Salt Magnesium (SM) buffer

Salt Magnesium buffer was prepared through addition of NaCl to a final concentration of 0.1 M, $MgSO_4 \cdot 7\ H_2O$ to a final concentration of 10 mM, gelatine (Sigma-Aldrich Co. Ltd., now Merck, Dorset, UK) to a final concentration of 0.01% and Tris (Sigma-Aldrich Co. Ltd.) to a final concentration of 50 mM. Prior to sterilisation the pH was adjusted to 7.5 with HCl (36.5-38.0%) and the solution filled up to a final volume of 1 litre with RO water. After sterilising, the buffer was stored at room temperature until ready for use.

2.4.12 *Campylobacter* enumeration

Suspensions of gastrointestinal content in MRD were further serially diluted (1:10) in MRD; typically up to a factor of 1 x 10^{-6}. *Campylobacter* were counted using an adaptation of the Miles and Misra method (Miles et al., 1938) by plating 5 x 10 µl aliquots of each dilution onto mCCDA agar (Oxoid Ltd.) supplemented with 2% (w/v) additional agar to reduce swarming. Incubation of campylobacters was carried out MACS microaerobic incubator (Don Whitley Scientific Ltd., Bingley, UK) filled with a microaerobic gas mixture (2% H_2, 5% CO_2, 5% O_2, 88% N_2) at 42°C. Agar plates were examined for colonies exhibiting typical *Campylobacter* morphology after 24 and 48 h, as described by the Oxoid manual (http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=SR0155&cat=&c=UK&lang=EN; accessed 26 Feb 2020)

2.4.13 *Campylobacter* bacteriophage propagation and storage

For the propagation of bacteriophages, a suitable strain of *Campylobacter* was cultured on BA under microaerobic conditions at 42°C for 18 - 24 hours. The overnight culture was then harvested into 10 ml of MH broth supplemented with 10 mM $MgSO_4$ using a sterile cotton swab and adjusted to a final concentration of approximately 8 log_{10} CFU ml^{-1}. A 100 µl volume of bacteriophage stock containing 8 log_{10} PFU ml^{-1} was then added to the cell suspension and incubated for 20 - 30 minutes under a microaerobic atmosphere (see section 2.4.12) at 42°C. During this time, 5 ml aliquots of NZCYM overlay agar were molten in a microwave and tempered to 55°C in a water bath. A volume of 500 µl of the *Campylobacter*-bacteriophage suspension was then transferred to the cooled overlay agar and mixed. This was poured onto pre-dried NZCYM plates which were allowed to set prior to incubation under microaerobic conditions at 42°C for 18 - 24 hours. Following incubation, the bacteriophage was harvested from the plates by dispensing 5 ml of SM buffer onto the surface of the top agar followed by shaking at 60 rpm aerobically overnight at 4°C. The phage suspension was recovered with a syringe and filtered through a 0.2 µm filter to remove *Campylobacter* cells and any cell debris. The phage stock was stored indefinitely at 4°C in Eppendorf tubes.

2.4.15 Titration of bacteriophages

Quantification of bacteriophage titers was performed by employing the traditional spot test assay. A fresh overnight culture of the to be tested *Campylobacter* strain was collected from a BA plate and emulsified in MH broth, supplemented with 10 mM $MgSO_4$, to a cell density of approximately 8 log_{10} CFU ml^{-1}. A volume of 500 µl of emulsified cells was added to 5 ml of molten NZCYM top agar (55°C) and poured on top of a NZCYM basis agar plate to form an even lawn. After the top agar had solidified, serial 10-fold dilutions of phage suspensions in SM buffer, which were applied as 10 µl aliquots, were spotted in triplicate onto the surface of prepared host bacterial lawns and allowed to dry. The plates were then incubated at 42°C under microaerobic conditions for 18 to 24 hours after the spots dried. Lysis zones resulting in the formation of 3 - 30 plaques were counted and the mean of the three spots was determined.

2.4.16 Determination of bacteriophage host range

Host range determination for bacteriophage isolates and their reaction to mutant strains was done using the traditional spot test assay using bacterial lawns. Bacteriophage test solutions were diluted to 7 log_{10} PFU ml^{-1} (routine test dilution) (Frost et al., 1999). After

the top agar had solidified, 5 µl aliquots of the tested phage solutions were placed on top of the soft agar layer, either individually or with a 48-pin replicator. The spots were allowed to dry with the help of a Bunsen burner flame or inside a biosafety cabinet and the plates were incubated in under microaerobic conditions at 42°C for 18 - 24 hours.

CHAPTER 3. CHARACTERIZATION OF THE ACTION OF IN-FEED MICROBIOTA-ACCESSIBLE CARBOHYDRATES ON BROILER PERFORMANCE

This section relates to the results reported in the peer-reviewed, open access article cited below and reproduced following this section (pp. 19).

Richards, P.J., Lafontaine, G.M.F., Connerton, P.L., Liang, L., Asiani, K., Fish, N.M., & Connerton, I.F. (2020). Galacto-Oligosaccharides Modulate the Juvenile Gut Microbiome and Innate Immunity To Improve Broiler Chicken Performance. *mSystems, 5*(1). DOI: 10.1128/msystems.00827-19

3.1 Summary

The findings reported in Richards et al. (2020) show that the growth rate of fast-growing broiler chickens is improved by dietary GOS compared to birds sustained on an isocaloric control diet, which was reflected in a greater weight at slaughter at 35 days old. In Richards et al. (2020) we also report that growth performance is linked to the population of *Lactobacillus* spp. in the caeca. Improved growth performance in broiler chickens fed either supplementary GOS or preparations of viable *L. johnsonii* has previously been reported by independently performed studies (Jung et al., 2008; Wang et al., 2017). By employing an inter-disciplinary, systems biology approach we have linked these observations and described a fuller picture of the host:microbe:diet interactions that manifest faster growth in chickens. Improved growth performance was validated over three independent feeding trials and the proposed involvement of *L. johnsonii* was empirically tested by dosing young birds and observing beneficial differences in subsequent growth performance relative to control birds.

3.2 Attribution of work

My contribution to this publication was analysis and reporting of microbiota data and zootechnical data. I also determined and tested the relationships between bird weight, *Lactobacillus* abundance and host gene expression data. I plotted each of the figures, contributed to the writing of the manuscript and assisted in the collection of biological samples and gathering of zootechnical data. I had no role in analysing the *Lactobacillus* genome sequence data nor in the collection and interpretation of qPCR data (*Lactobacillus* absolute abundance, host gene expression).

3.3 The influence of the chicken gut microbiota on zootechnical performance

Diet is a major factor governing gut microbiota composition in mammals (David et al., 2014; Turnbaugh et al., 2008) and the caecal microbiota of chickens has been shown to respond to crude feed composition, ie. whether the base feed ingredient is corn, soy or wheat (Lourenco et al., 2019; Mathlouthi et al., 2002). Despite this, relative to the huge number of published works on the effect of feed on all aspects of bird performance, surprisingly few studies have reported how standard basal diets affect the microbiota. This is an important subject that would surely benefit from greater research effort; especially in light of suggestions that uncontrolled modulation of the chicken gut microbiota through exposure to feed adulterants, such as mycotoxins, is a commonplace occurrence (Pont et al., 2020). More studies have focused on describing the effect of potential prebiotic feed supplements on the broiler chicken microbiota (Jung et al., 2008; Lee et al., 2016; Pourabedin et al., 2015). However, how the host responds to changes in the gut microbiota is seldom reported alongside bacterial abundance data and *vice versa*. This paucity of data means that the fundamental mechanisms underpinning any improvement in performance are mostly obscure, which can lead to the reporting of conflicting results. Potential pitfalls in an over-reliance on microbiota profiles in infection studies have been highlighted (Lichtman et al., 2016; Tiffany and Bäumler, 2019), specifically that the microbiota is under host control and reporting of shifts in bacterial abundance without concomitant observation of any changes in host-immunity will obscure bi-directional affects and confound understanding.

3.4 The relationship between the *Lactobacilli* and performance

The results of both culture-based and molecular methods reveal that the populations of *Lactobacillus* spp. colonizing the chicken gut are responsive to feed ingredients (Lourenco et al., 2019; Mathlouthi et al., 2002; Stephenson et al., 2010; Wu et al., 2014). A number of studies linking specific distributions of bacterial taxa to chicken performance have associated the genus *Lactobacillus* with sub-optimal growth performance (Johnson et al., 2018; Stanley et al., 2016; Yan et al., 2017). In agreement with the findings of Wang et al. (2017), we observed that weight at slaughter positively correlated with *L. johnsonii* biomass in the caecal lumen. It can be speculated that expanding the colonization niche for *L. johnsonii* in early life (< 10 days) can prevent future high-level colonization by *L. crispatus* through priority colonization effects (Litvak and Bäumler (2019). However, this is not supported by other studies that determined that independent or co-administration of *L.*

johnsonii and/or *L. crispatus* to broiler chickens did not affect body weight at 42 days (Taheri et al., 2010).

We have shown that the increased growth rate of broiler chickens sustained on the GOS diet is linked to changes in the *Lactobacillus* population. The association between dietary GOS and increased *L. johnsonii* abundance is obscure as *L. johnsonii* DC22.2 is unlikely to metabolize GOS *in vivo* as it lacks a LacE/F phosphotransferase system and is unable to use GOS as a growth substrate *in vitro*. However, through deliberate modulation of growth performance by exposing chickens to *L. johnsonii* DC22.2 in early life we have empirically shown that this strain is not simply a biomarker of good performance. As discussed in Richards et al. (2020), there is little support for *L. johnsonii* DC22.2 as a keystone organism in GOS catabolism. Comparative profiling of the caecal metagenome of GOS-fed and control birds might reveal which microbial bioprocesses are increased in the presence of GOS toward identification of the taxa involved and generation of testable hypotheses on the mode of action. We report significant differences in the abundance of *Lactobacillus* spp. in the caecal pouch, however, consideration of the dynamics of Lactobacillus succession in the chicken gut has led some to postulate that the crop is the origin of the *Lactobacillus* observed throughout the remainder of the gastrointestinal tract (Tannock, 2004; Walter, 2008). The composition of the Nutrabiotic™ prebiotic that was added to the GOS-supplemented diet was 74% (w/w) GOS and 26% mixed glucose and galactose monosaccharides (see Richards et al., 2020) and the increased abundance of *L. johnsonii* may be attributable to these co-purified monosaccharides. Although very low levels of glucose and galactose are likely to reach the caeca, they may be available to the Lactobacilli colonizing the crop. However, this does not preclude that the mechanism by which *L. johnsonii* improves performance is manifested in the caeca as the organism is found in appreciable numbers and heat-killed *L. plantarum* has been reported to improve the growth performance and increase IFN-β, IFN-γ and IL-12β expression in the spleen of broiler chickens (Incharoen et al., 2019).

In an effort to exclude pathogens from flocks intensively farmed chickens are commonly raised under strict biosecurity. Hence, the gut microbiota of intensively raised birds is not seeded by the actions of their parents in development as would be the case with mammals or wild/feral birds (Grond et al., 2018) and subsequent bacterial colonization of the gut is broadly due to stochastic exposure (Rychlik, 2020), unless a commercial competitive exclusion product is employed. The results reported by Richards et al. (2020) are based on broiler chickens hatched in a clean environment and raised under high-biosecurity

conditions (ACDP 2 containment). *L. johnsonii* exhibits host specificity (Guinane et al., 2011) and clustering the core-genome of L. *johnsonii* DC22.2 with that of other *L. johnsonii* strains indicates that *L. johnsonii* D22.2 is probably autochthonous (indigenous to chickens) as it is grouped with other *L. johnsonii* of poultry origin: chicken and wild turkey (*Meleagris gallopavo*) (Figure 3.1). Interestingly *L. johnsonii* strains from poultry and those of human origin or yoghurt origin (ASM886855v1) formed a distinct clade separate from strains isolated from mice or pigs. It may be speculated that the improved growth performance linked with the abundance of *L. johnsonii* is linked to evolved/selected host:microbe commensal cooperation. The merits of using autochthonous or allochthonous (remote) *Lactobacillus* spp. as probiotics have been fully and thoughtfully discussed elsewhere (Walter, 2008).

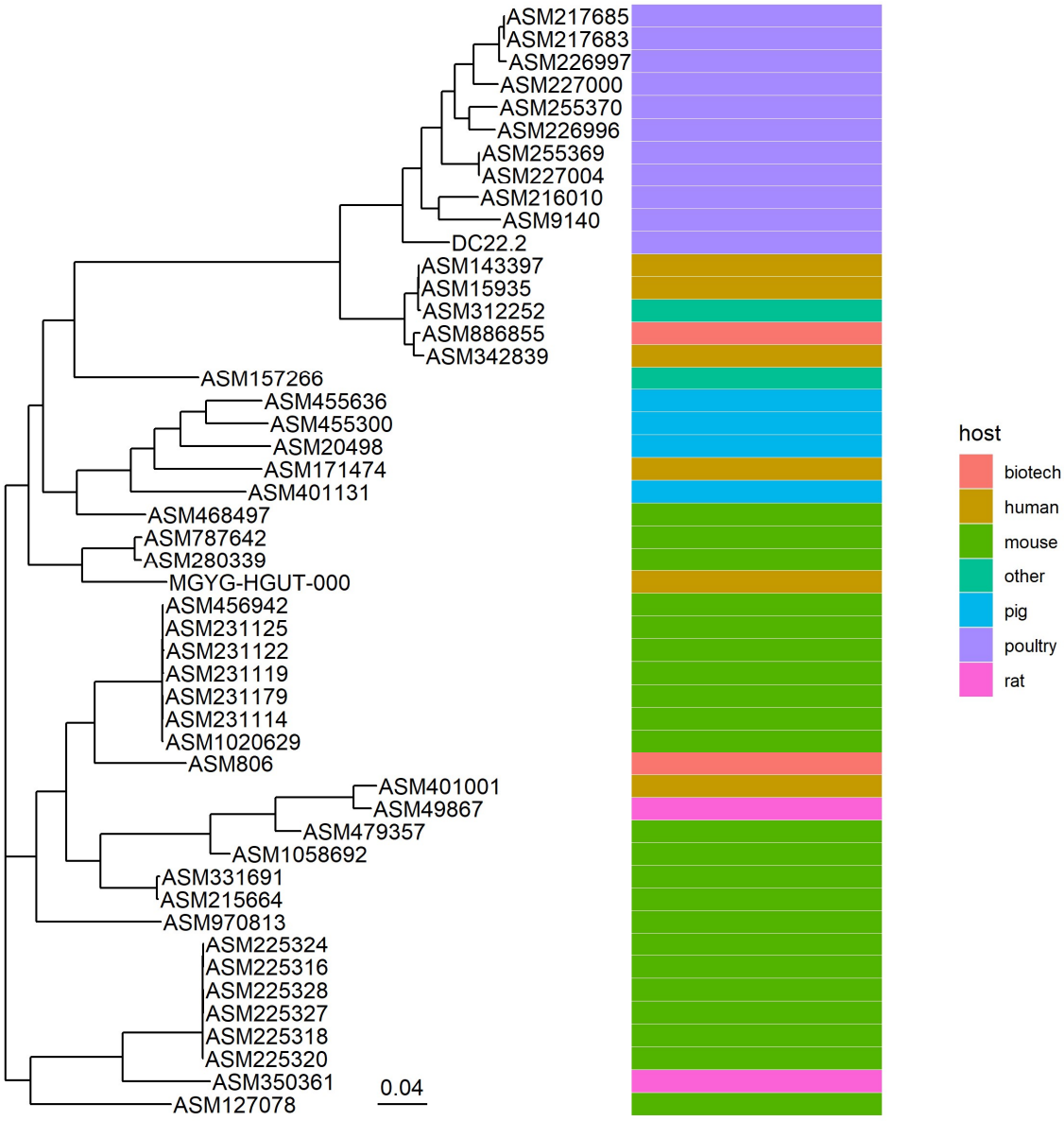


Figure 3. *Lactobacillus johnsonii* isolates exhibit host preference. All 51 *L. johnsonii* genome assemblies available were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly/organism/33959/; accessed 25-Feb-2020). One assembly was removed from the analysis as it was not of the expected size and another duplicate assembly was excluded. The *L. johnsonii* core-genomes were aligned using parsnp (Treangen et al., 2014) and visualized in R using ggtree (Team, 2014; Yu et al., 2018). Programmatic code is available from: https://github.com/pjrichards. Note that ASM216010v1 and ASM9140v1 were isolated from chickens. ASM9140v1 is *L. johnsonii* FI9785 (Ragione et al., 2004; Wegmann et al., 2009) an isolate with anti-*Campylobacter* activity (see section 3.X).

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RESEARCH ARTICLE
Host-Microbe Biology

Galacto-Oligosaccharides Modulate the Juvenile Gut Microbiome and Innate Immunity To Improve Broiler Chicken Performance

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ABSTRACT Improvements in growth performance and health are key goals in broiler chicken production. Inclusion of prebiotic galacto-oligosaccharides (GOS) in broiler feed enhanced the growth rate and feed conversion of chickens relative to those obtained with a calorie-matched control diet. Comparison of the cecal microbiota identified key differences in abundances of *Lactobacillus* spp. Increased levels of *Lactobacillus johnsonii* in GOS-fed juvenile birds at the expense of *Lactobacillus crispatus* were linked to improved performance (growth rate and market weight). Investigation of the innate immune responses highlighted increases of ileal and cecal interleukin-17A (IL-17A) gene expression counterposed to a decrease in IL-10. Quantification of the autochthonous *Lactobacillus* spp. revealed a correlation between bird performance and *L. johnsonii* abundance. Shifts in the cecal populations of key *Lactobacillus* spp. of juvenile birds primed intestinal innate immunity without harmful pathogen challenge.

IMPORTANCE Improvements in the growth rate of broiler chickens can be achieved through dietary manipulation of the naturally occurring bacterial populations while mitigating the withdrawal of antibiotic growth promoters. Prebiotic galacto-oligosaccharides (GOS) are manufactured as a by-product of dairy cheese production and can be incorporated into the diets of juvenile chickens to improve their health and performance. This study investigated the key mechanisms behind this progression and pinpointed *L. johnsonii* as a key species that facilitates the enhancements in growth rate and gut health. The study identified the relationships between the GOS diet, *L. johnsonii* intestinal populations, and cytokine immune effectors to improve growth.

KEYWORDS prebiotic, galacto-oligosaccharides, chicken, microbiome, gut health, synbiotic, innate immunity, IL-17A

The production of poultry for both meat and eggs has been increasing rapidly throughout the world (1), and the global poultry sector is expected to continue to grow as a result of growing population, rising income, and urbanization (2). In this context, animal performance and feed conversion efficiency of fast-growing birds are decisive to the economic profitability of poultry meat production. Broiler chicken production is more sustainable and has a relatively lower environmental impact than other meat-based protein production (3). There have been massive increases in the growth rate and feed efficiency of broiler chickens since the 1940s, achieved largely through selective breeding and feed optimization (4). It is generally recognized that increases in performance are slowing as the advances made possible through these approaches are reaching their biological limit (5). The inclusion of antimicrobial growth

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Prebiotic galacto-oligosaccharides improve chicken growth and gut health

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promoters (AGPs), a practice banned in the European Union (EU) since 2006, is another way in which gains in productivity have been realized. The EU ban was imposed due to increasing concerns regarding the development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota (6). Although antimicrobials are still widely used, there have been reductions in the therapeutic use of antimicrobials in poultry production, which have led to an increase in intestinal health problems (7). To mitigate the effect of antimicrobial reduction a variety of strategies has been evaluated (8). These include the addition of dietary prebiotic (9, 10), the use of phytobiotic dietary additives (11), the incorporation of beneficial enzymes in poultry feed (12), and the administration of live probiotic bacteria in various combinations of the above (13). Recent developments in sequencing technologies have led to a greater understanding of the mechanisms and effects of these treatments on the gut microbiota and the interaction with host-related functions involved in intestinal health (14, 15). It has been proposed that further improvements to broiler performance could be sought through deliberate cultivation of a beneficial gut microbiota in early development (7, 16). These bacteria are preferably autochthonous and mutualists in association with each other and their host.

Galacto-oligosaccharides (GOS) are nondigestible carbohydrates that have been shown to promote beneficial autochthonous bacteria, such as *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*. GOS are synthesized from lactose by β-galactosidase-catalyzed transglycosylation to create molecules of differing lengths and linkage types (17, 18). Several studies have reported that GOS can improve the performance of poultry and produce profound differences in desirable bacterial groups inhabiting the gut (19, 20).

Maintenance and enhancement of gut health are essential for the welfare and productivity of animals (21). In addition to nutrient digestion and absorption, the intestinal mucosa constitutes a physical and immunological protective barrier for the integrity of the intestinal tract (22). Mutualistic commensals with immunomodulatory effects (autobionts) affect the development and function of various immune cell populations, such as regulatory T cells (Tregs), Th17 T-helper cells, IgA-secreting plasma cells, natural killer (NK) cells, macrophages, dentritic cells (DCs), and innate lymphoid cells (ILCs) (23). Interleukin-17-producing $CD4^+$ T lymphocytes (Th17 cells) contribute to host defense against pathogens and maturation of the immune response at an early age. Regulatory T cells play critical roles in immune suppression (24, 25), and thus, optimum health is achieved through a balanced regulation of expression between Th17 cells and Tregs.

There is little systematic information regarding the interaction between prebiotic diet, performance, structure of gut microbiota, and host gene expression in poultry. In this study, the impact of a GOS diet was assessed in broiler chickens by comparing a cohort fed a control diet and a cohort fed a GOS diet from day of hatch until 35 days of age (da), corresponding to a typical commercial farm rearing period. Ancillary dietary trials were carried out to confirm the reproducibility of the beneficial effects. The innate immune responses to the two diets were assessed in ileal and cecal tissue biopsy specimens by quantification of the relative expression of cytokine and chemokine gene transcription. Analysis of metagenomic profiles of GOS-fed birds enabled the identification and isolation of autochthonous synbiotic organisms. Characterization of these isolates allowed an in-depth analysis of the effect of the GOS diet and synbiotic species abundance on bird performance and gut health.

RESULTS

GOS supplementation improves the growth performance of broiler chickens. Chickens fed a galacto-oligosaccharide (GOS)-supplemented diet performed better than those fed an isocaloric control diet (Fig. 1A). An increase in growth rate was apparent for the GOS-fed birds, which exhibited an increase in the mean live weight of 87.7 g/day, compared to 76.3 g/day for the control birds, calculated at between 8 and 35 days of age (da) ($P = 0.012$). Correspondingly, the mass of GOS-fed birds in trial 1 was greater than that of control birds at the slaughter age of 35 days (GOS-fed birds,

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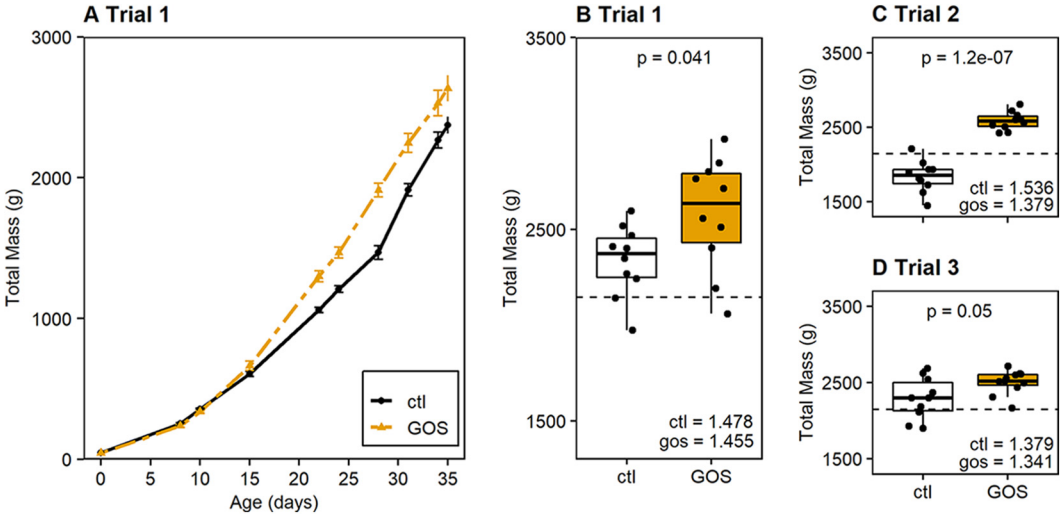


FIG 1 Galacto-oligosaccharide diet improves the growth performance of broiler chickens. (A) GOS diet trial 1 comparing median body weight of birds fed the GOS diet with that of birds fed the control (ctl) diet. Data presented are for the mass observations made for the 10 birds from each cohort that remained at 35 days and hence were recorded throughout the trial. The contemporary male Ross 308 broiler chicken performance objective weight progression (96) is indicated by the gray dashed line. Panel B shows a box-and-whisker plot of the bird weights for trial 1 at 35 da. Panels C and D show box-and-whisker plots of the bird weights at 35 da for ancillary GOS diet trials 2 and 3 to demonstrate that the birds on the GOS diet consistently achieved greater body weight at 35 da than birds provided with a calorie-matched control diet. For reference, the contemporary male Ross 308 performance objective at 35 da is indicated by a horizontal dashed black line in each panel (96). Comparisons were made of mean weights using Student's *t* test, with the corresponding *P* values reported above the diet pairs and the corresponding cumulative feed conversion ratios (FCR) indicated in the bottom right-hand side of each trial panel.

2,582 g; control birds, 2,336 g; $P = 0.041$ [Fig. 1B]). Two ancillary trials were carried out to demonstrate whether after removing the prebiotic once the microbiota was established on the GOS feed the beneficial effects on performance could be reproduced in mature birds. In these trials, the birds were either housed in individual cages (trial 2) or cohoused in 10 pens containing 3 birds (trial 3). The enhanced performance on the GOS-supplemented diet was evidenced by greater masses at 35 da in trial 2 (GOS-fed birds, 2,584 g; control birds, 1,838 g; $P < 0.001$ [Fig. 1C]) and trial 3 (GOS-fed birds, 2,501 g; control birds, 2,291 g; $P = 0.057$ [Fig. 1D]). Although trial 3 marginally failed to meet significance, the trend remained the same, with the GOS diet producing beneficial effects on growth when the birds were reared in pens. The feed conversion ratios (FCR) varied between trials but were reduced for birds on the GOS diets compared to the corresponding control groups (Fig. 1B to D). The zootechnical performance data for all trials are summarized in Table S1 in the supplemental material; they repeatedly show significantly greater weights for the GOS-fed juvenile birds over those on the control diet reared under similar circumstances at 35 da.

***Lactobacillus* spp. distinguish microbial communities colonizing the ceca of broiler chickens on GOS-supplemented diets.** Cecal bacterial communities were surveyed using 16S rRNA gene sequences. Analysis of trial 1 showed that the α-diversities of the cecal microbiota were not significantly different between GOS and control diet cohorts sampled at 8, 15, 22, and 35 da (inverse Simpson index, $P \geq 0.295$; Shannon diversity, $P \geq 0.187$ [Fig. S1A and B]). Community richness (Chao) was not significantly different throughout the trial ($P \geq 0.101$ [Fig. S1C]). Communities of cecal bacteria colonizing birds on control and GOS diets could not be distinguished on the basis of Bray-Curtis dissimilarity at any age ($P >$ Bonferroni correction for pairwise error [Fig. S1D]).

The top 10 operational taxonomic units (OTUs) with the greatest relative abundances are shown in Fig. 2A for all the birds sampled at each time point. Figure 2B shows family level taxonomy to confirm the similarity between the cecal bacterial communities of birds fed control and GOS diets. However, few OTUs were discriminative between the control and GOS diets (Fig. 2C). Two OTUs, OTU0006 and OTU0010,

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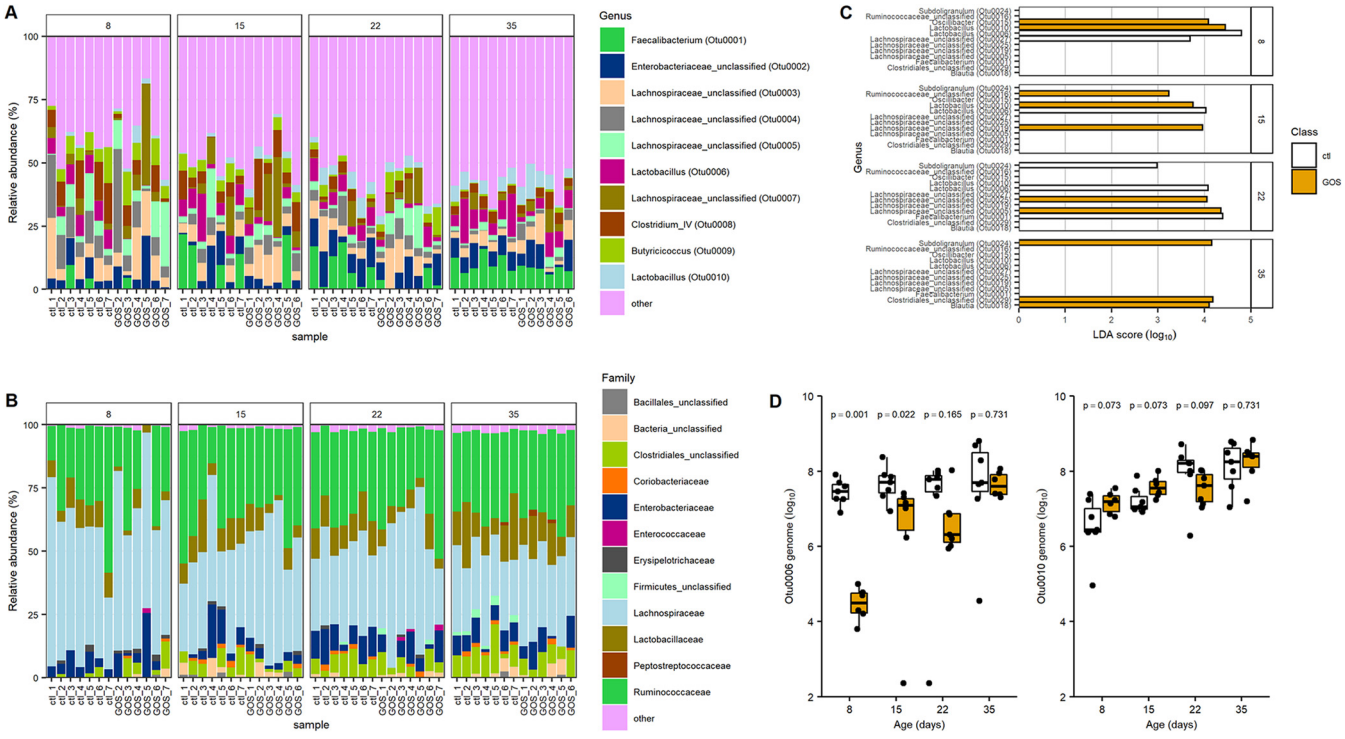


FIG 2 Dietary GOS shifts the abundance of specific taxa. Panels A and B show stacked bar charts indicating the relative abundance to OTU (A) and family (B) levels of the cecal microbiota of birds fed GOS and control diets. In three cases, the sequence data sets did not meet pre- or postprocessing quality thresholds and were removed from the analysis (GOS samples at 8, 15, and 35 da). Panel C shows the differential bacterial species identified in cecal contents from birds fed the control diet and GOS diet analyzed using linear discriminant analysis effect size (LEfSe). Prior to analysis, age-matched communities were filtered to include only OTUs at ≥1% relative abundance. The linear discriminant analysis (LDA) threshold was set at 2, and the *P* value threshold was set at 0.05. Panel D shows box-and-whisker plots of the genome copy numbers of OTU0006 and OTU0010 per gram of cecal content determined using qPCR. Data are reported for genome numbers identified in birds fed GOS and control diets. Significant differences are indicated by *P* values above the sample pairs.

identified as *Lactobacillus* spp., were discriminatory of the different diets in the early rearing period up to 15 da (OTU0006, $P \leq 0.01$; OTU0010, $P \leq 0.022$). Reads representing these OTUs accounted for 84.5% of those assigned to the *Lactobacillales* order and 86.1% assigned to the *Lactobacillaceae* family (Fig. 2B). The third major *Lactobacillus* species is represented by OTU0017, which together with OTU0006 and OTU0010 constitutes 99.95% of the *Lactobacillaceae*. Organisms exhibiting 16S rRNA gene V4 region sequence identity with the differential OTUs identified from 16S rRNA community analysis were isolated from MRS culture media. The genomic DNA sequences of these isolates were assembled from data generated on Illumina MiSeq and PacBio RSII platforms. Two representative isolates were designated *L. crispatus* DC21.1 (OTU0006) and *L. johnsonii* DC22.2 (OTU0010) based on whole-genome alignments with type strains available in public nucleotide sequence databases.

Quantitative PCR assays were developed to measure the absolute abundances of *L. johnsonii* (OTU0010) and *L. crispatus* (OTU0006) within the gut microbiota. Oligonucleotide primers were designed on the *groEL* gene sequences, as they have been frequently used to discriminate between *Bifidobacterium* strains with a high degree of sequence similarity (26, 27). Once validated, using spiked cecal samples, the technique was used to enumerate *L. johnsonii* (OTU0010) and *L. crispatus* (OTU0006) organisms within the cecal contents of control and GOS-fed birds. The genome copies of each isolate were measured throughout the rearing period. The results of these analyses confirmed the relative abundance estimates from metagenomic data in trial 1 and demonstrated that the abundances of these two OTUs show positive and negative associations with the GOS diet compared to the control diet (Fig. 2C). Notably, the abundance of *L. crispatus* (OTU0006) in the GOS-fed birds at 8, 15, and 22 da was significantly lower than in control birds ($P < 0.048$), and conversely, the abundance of *L. johnsonii* (OTU0010) was significantly greater at 8 da in the GOS-fed birds ($P = 0.001$).

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TABLE 1 Summary of the functional gene contents of *L. johnsonii* and *L. crispatus* isolates related to GOS utilization and host colonization

Gene(s)	*L. johnsonii* locus tag E6A54_	*L. crispatus* locus tag E6A57_	Function	Reference
lacS	06610[a]	07265	Lactose permease	41
lacA	06605	07260	β-Galactosidase	41
lacL/M	06620	07275	β-Galactosidase	34
	06625	07280		
lacE/F		O7225	PTS lactose transporter	34
lacG			Phospho-β-galactosidase	34
mucBP	04955	06095	Adhesion	28
	08660[a]	07570		
	09625	08180		
		01350		
fbpA	04640	05230	Adhesion	28
cbsA/B		00840	S-layer	32
apf1/2	07620		Aggregation factor	31
epsA–E	05690–05730	08695–08755	Exopolysaccharide	29
Bacteriocin leader		09005	Bacteriocin	28
hlv	02750	00185	Helveticin J	28
		05290		
		07800		
		10185		
bsh	00415	08515	Bile salt hydrolase	30

[a]Internal stop codons present.

Characteristics of the *Lactobacillus* spp. distinguishing the cecal communities. Summaries of the functional gene contents of the *L. johnsonii* DC22.2 and *L. crispatus* DC21.1 isolates with respect to GOS utilization and host colonization are presented in Table 1. *L. johnsonii* and *L. crispatus* have the capacity to colonize and compete in the host intestine with genes encoding multiple mucus binding proteins (28), fibronectin binding proteins (28), exopolysaccharide biosynthesis (29), and bile salt hydrolase (30). *L. johnsonii* carries the *apf* gene (aggregation promoting factor), which encodes a cell surface protein that has been assigned a role in cell adhesion (31). *L. crispatus* contains the *cbsA* gene, which encodes the structural protein that forms the S-layer (32, 33). *L. johnsonii* and *L. crispatus* both encode the bacteriocin helveticin J and multiple bacteriocin immunity factors.

There are ostensibly two pathways to utilize GOS that rely upon the cellular transporters LacS (lactose permease) and LacE/F (lactose phosphotransferase system); LacS permease appears to be capable of transporting GOS with degrees of polymerization of 2 to 6 (DP2 to DP6), but the LacE/F phosphotransferase is confined to DP2 lactose (34). The genome sequence of *L. johnsonii* DC22.2 suggests that the isolate could be impaired in GOS utilization. The *lacS* permease gene has a stop codon at the 17th position, which would require that the protein be initiated from an internal AUG with the loss of the first 31 amino acids compared to the majority of database homologues. In contrast, *L. crispatus* DC21.1 retains functional *lacS* and *lacA* genes to facilitate the use of GOS.

To assess the ability of the *L. crispatus* and *L. johnsonii* isolates to utilize GOS in axenic culture, the organisms were cultured in MRS basal medium containing DP2+ GOS in the absence of monosaccharides. Cultures were incubated for 72 h under anaerobic conditions at 37°C in basal medium with either DP2+ GOS (0.5% [wt/vol]) or glucose (0.5% [wt/vol]) as a positive control or sterile water instead of the carbon source as negative control (blank). Figure 3 shows growth of the isolates and *Lactobacillus* type strains indicated by the measurements of optical density at 600 nm (OD_{600}) corrected for the negative control. *L. crispatus* DC21.1 utilizes GOS, showing increased growth over that recorded for *L. fermentum* ATCC 33323 in a parallel culture. Under these conditions *L. johnsonii* does not efficiently use GOS, which is consistent with the putative gene content but at odds with the differential abundance observed in the ceca of GOS-fed birds.

In-feed GOS effects on gut architecture. Hematoxylin-eosin (H&E) stains of ileal histological sections did not exhibit any significant differences in heterophil infiltration

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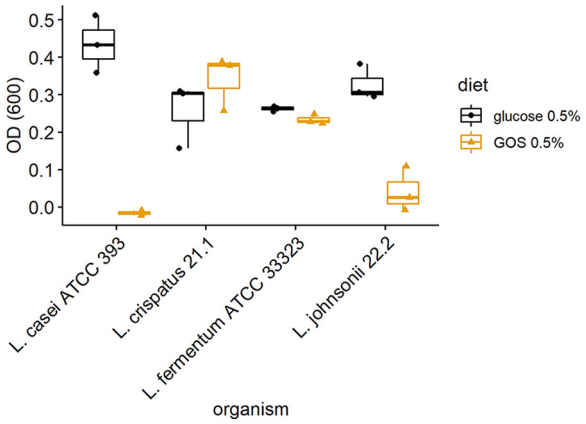


FIG 3 *In vitro* growth of lactobacilli on galacto-oligosaccharides. Utilization of DP2+ GOS by *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 isolates compared with that by *Lactobacillus* type strains is shown. Anaerobic growth was recorded from OD_{600} measurements after incubation with basal medium that was subtracted from that obtained by incubation with basal medium plus DP2+ GOS or glucose as a carbon source.

or inflammatory characteristics between control and GOS-fed birds at any time in the experiment. The measurements of villus length and crypt depth indicate that GOS-fed juvenile birds at 15 da had longer villi ($P = 0.05$) and deeper crypts ($P = 0.02$) than birds on the control diet (Table 2). However, these differences did not result in a difference in the villus/crypt ratio. At 22 and 35 da, there were no significant morphometric differences recorded. Goblet cell densities of villi from control and GOS-fed birds were evaluated from periodic acid-Schiff (PAS)-stained (neutral mucin-producing) ileal sections (Table 2). Greater densities of goblet cells were observed from GOS-fed birds sampled throughout trial 1, with significant differences recorded at days 8 ($P = 0.04$), 15 ($P = 0.002$), and 22 ($P = 0.04$).

In-feed GOS modulates host immune response. The immune responses were assessed in ileal and cecal tissues of trial 1 by quantification of the relative expressions of cytokine and chemokine genes representing the major inflammatory pathways of chickens (35). The relative expressions of the anti-inflammatory cytokine interleukin-10 (IL-10) and proinflammatory Th17-associated cytokine IL-17A were profoundly modulated in juvenile birds in both ileal and cecal tissues (Fig. 4; Table 3). In ileal tissues from GOS-fed birds, cytokine expression was marked by upregulation of IL-17A (fold change [FC] = 83; corresponding probability [*Padj*] = 0.002) at 8 da (Fig. 4A; Table 3) and downregulation of IL-10 (FC = 0.02; *Padj* = 0.002), while IL-17F remained unchanged

TABLE 2 Ileal gut morphometrics: villus length, crypt depth, and goblet cell density

Diet or parameter and age (days)	Villus length (μm)	Crypt depth (μm)	Ratio villus/crypt	Goblet/mm^2
Control diet				
8	475 ± 64	75 ± 7	6.3 ± 0.9	1,457 ± 347
15	615 ± 70	103 ± 11	6.0 ± 0.5	1,198 ± 140
22	747 ± 167	126 ± 20	5.9 ± 0.4	1,378 ± 290
35	731 ± 110	121 ± 17	6.0 ± 0.3	1,267 ± 243
GOS diet				
8	505 ± 63	79 ± 7	6.0 ± 0.8	1,707 ± 212
15	676 ± 93	113 ± 13	5.9 ± 0.5	1,450 ± 247
22	687 ± 118	118 ± 18	5.8 ± 0.3	1,616 ± 360
35	789 ± 162	126 ± 19	6.2 ± 0.6	1,394 ± 218
P value				
8	0.25	0.15	0.90	0.04
15	0.05	0.02	0.81	0.002
22	0.26	0.25	0.30	0.04
35	0.46	0.24	0.28	0.16

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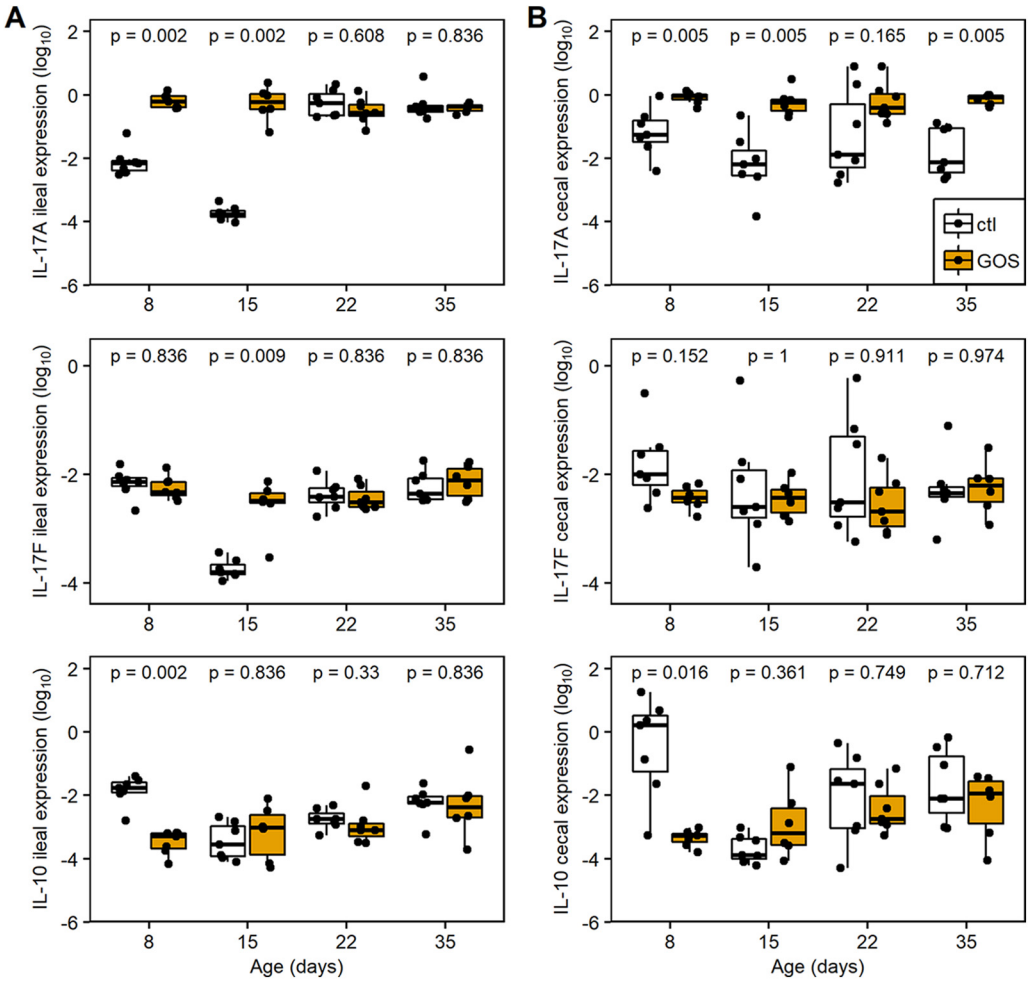


FIG 4 Changes in expression of IL-17A, IL-17F, and IL-10 from ileal (A) and cecal (B) tissues. Relative gene expression was determined by quantitative RT-PCR from total RNAs extracted from cecal tissues for GOS-fed birds (gold) and compared to the expression from birds on the control diet (black). Expression of the gene of interest (GOI) relative to the housekeeping gene (HG) is presented as box-and-whisker plots of data from 7 independent birds determined from 3 technical replicates. The GOS-fed birds analyzed were reduced to 6 for the 15- and 35-da time points due to qPCR data not meeting quality thresholds. The housekeeping genes were the GAPDH and RPL4 genes. The data are recorded as $\log_{10}$-transformed $2^{-\Delta CT}$ values. The ΔC_T was calculated (C_T GOI $-$ C_T HG) for each sample; significant differences between $2^{-\Delta CT}$ values of the control and GOS diet cohorts of birds are indicated by *P* values above the sample pairs calculated using the nonparametric Wilcoxon rank sum test with Benjamini-Hochberg false-discovery rate correction.

(*Padj* = 0.836). At 15 da, the relative upregulation of ileal IL-17A (FC = 2841; *Padj* = 0.002) and IL-17F (FC = 19; *Padj* = 0.009) was recorded as a consequence of a reduction in expression in the control birds, while expression in the GOS-fed birds remained similar to that at 8 da (Fig. 4B). In cecal tissues the GOS-fed birds were also marked by downregulation of IL-10 (FC = 0.0001; *Padj* = 0.016) at 8 da and upregulation of IL-17A at 8, 15, and 35 da (FC ≥ 12; *Padj* = 0.005).

At 22 da in GOS-fed birds, proinflammatory chemokine ChCXCLi-1 was increased in the ileum (FC = 16; *Padj* = 0.03) and, conversely, reduced in the ceca (FC = 0.05; *Padj* = 0.07). These observations indicate that the GOS diet or concomitant shifts in the gut microbiota do not drive induction of proinflammatory responses such as IL-1β or Th1-associated gamma interferon (IFN-γ) cytokines, while the reduction of IL-4, a marker for the Th2 pathway, was transient and limited to the ileum at 15 da (FC = 0.1; *Padj* = 0.05).

Figure 5 shows the relative expressions of the IL-17A, IL-17F, and IL-10 genes in the ileum and ceca of 8-da chicks on GOS or control feed. Birds on the GOS and control diets were clearly differentiated on the basis of these data and demonstrated a

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TABLE 3 Modulation of intestinal cytokine and chemokine responses to dietary GOS in broiler chickens[a]

	Ileum								Cecum							
	8 da		15 da		22 da		35 da		8 da		15 da		22 da		35 da	
Cytokine or chemokine	FC	*Padj*	FC	*Padj*	FC	*Padj*	FC	*Padj*	FC	*Padj*	FC	*Padj*	FC	*Padj*	FC	*Padj*
IFN-γ	0.5	0.8	1	0.8	1	0.8	3	0.8	0.5	0.6	7	0.2	2	0.9	1	0.9
IL-1β	0.6	0.7	0.3	0.5	0.8	0.9	5	0.5	0.6	0.9	2	0.9	0.4	0.9	3	0.9
IL-4	0.1	0.2	0.1	0.05	2	0.9	3	0.2	1	0.9	5	0.9	2.0	0.9	1	0.9
IL-10	0.02	0.002	2	0.8	0.6	0.3	2	0.8	0.001	0.02	6	0.4	0.5	0.7	0.4	0.7
IL-6	2	0.7	1	0.7	0.3	0.8	0.3	0.2	0.04	0.5	13	0.1	0.3	0.5	1	0.6
ChCXCLi-1	0.7	0.8	2	0.4	16	0.03	2	0.4	0.9	0.9	1	0.9	0.05	0.07	1	0.9
ChCXCLi-2	2	0.4	4	0.2	15	0.1	2	0.4	1	0.7	3	0.5	0.1	0.1	1	0.5
IL-17A	83	0.002	2841	0.002	0.6	0.6	0.6	0.8	12	0.005	88	0.005	11	0.1	60	0.005
IL-17F	0.8	0.8	19	0.009	0.8	0.8	1.1	0.8	0.2	0.1	0.7	1	0.3	0.9	1	0.9

[a]Cytokine and chemokine gene expression is recorded as fold change (FC) for GOS-fed birds relative to the control diet birds at the same age calculated as $2^{-\Delta\Delta CT}$. Corresponding probabilities (*Padj*) were calculated from $\log_{10}$-transformed $2^{-\Delta CT}$ values using nonparametric Wilcoxon rank sum tests adjusted with Benjamini-Hochberg false-discovery rate correction.

reduction in the variation in transcription of these specific cytokines in the ceca of birds provided with GOS feed. The increase of IL-17A transcription in the ileal tissues of GOS-fed birds at 8 da coincides with reduced expression of IL-10 (Fig. 5A), whereas IL-17F transcripts were unaffected (Fig. 5B and C). Similarly, the in-feed inclusion of GOS resulted in higher levels of IL-17A and reduced levels of IL-10 transcription in cecal tissues (Fig. 5D) without modulation of IL-17F transcription (Fig. 5E and F). These data suggest differential regulation of IL-17A and IL-17F and that IL-17A responds to in-feed GOS as a component of the Th17 immune response in the ceca, which coincides with the greater relative abundance of *L. johnsonii* (OTU0010).

Abundance of *L. johnsonii* in the cecal lumen positively correlates with bird growth performance. Correlations between abundances of the *Lactobacillus* isolates with bird weight were analyzed by combining all the data for 35-da birds from trials 1, 4, 5, and 6, which represent a range of performance outcomes. These data show a clear positive relationship between body mass and the *L. johnsonii* genome copy number

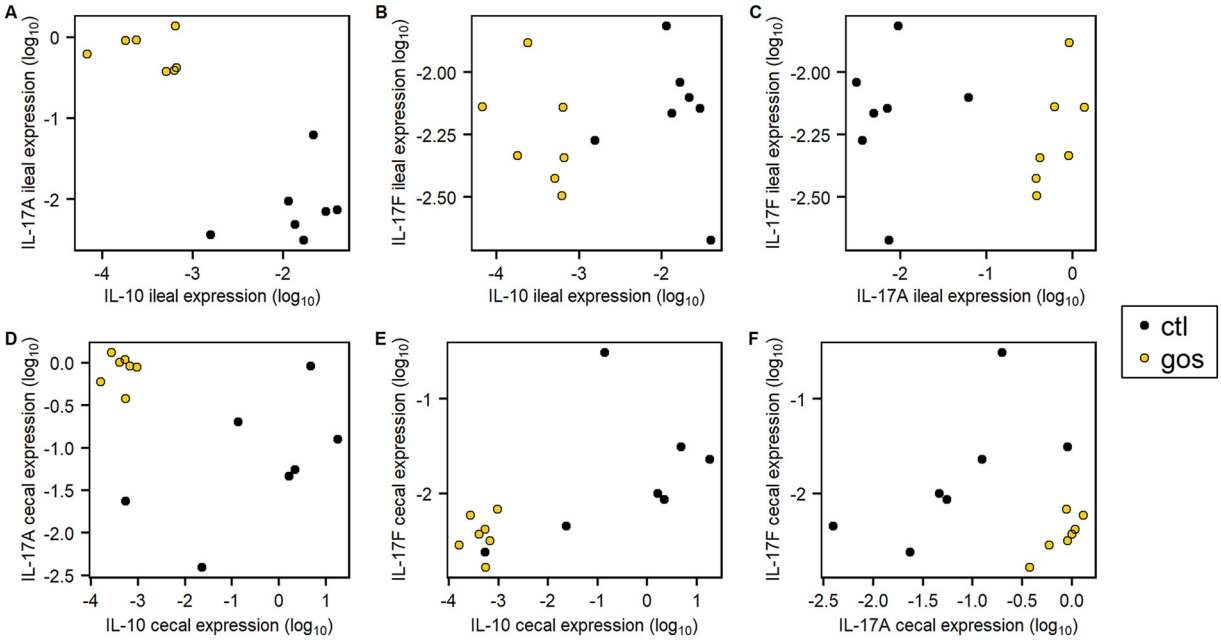


FIG 5 Differential cytokine gene expression in juvenile cecal and ileal tissues. Shown is juvenile IL-17A, IL-17F, and IL-10 differential gene expression relative to those of GAPDH and RPL4 at 8 da in ileal (A, B, and C) and cecal (D, E, and F) tissues of birds on the GOS diet compared to those on the control diet. The $2^{-\Delta CT}$ was determined as indicated in the legend to Fig. 4, and the $\log_{10}$-transformed expression values were plotted against each other ($n = 7$).

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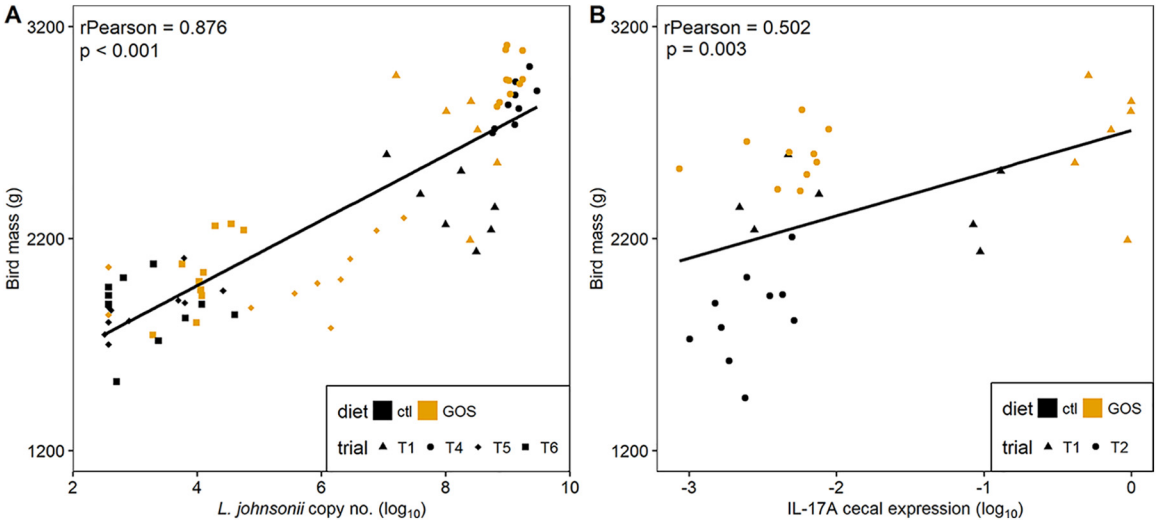


FIG 6 Correlation of growth performance to *L. johnsonii* abundance and IL-17A expression. Panel A shows the Pearson correlation of bird mass at 35 da against $\log_{10}$ *L. johnsonii* gene copy number per gram of cecal contents determined by quantitative PCR from DNA extracted from cecal contents. Panel B shows the Pearson correlation of the bird weights at 35 da with the cecal IL-17A transcription levels expressed as $\log_{10}$-transformed $2^{-\Delta CT}$ values. Gene expression was determined by RT-qPCR from total RNAs extracted from cecal tissues.

determined by quantitative PCR (qPCR) from the cecal microbiota (Pearson's $r = 0.876$; $P \leq 0.001$ [Fig. 6A]). A significant negative correlation between *L. crispatus* genome copy number and mass was also observed (Pearson's $r = -0.763$; $P \leq 0.001$ [data not shown]). The abundances of *L. crispatus* and *L. johnsonii* were further analyzed for any relationship with the expression of IL-17A, IL-17F, and IL-10 that we observed to exhibit differential expression on the GOS diet. Figure 6B shows the correlation noted with the expression of IL-17A and *L. johnsonii* genome copy number (Pearson's $r = 0.502$; $P = 0.003$). These results together strongly suggest that *L. johnsonii* acts as a key species promoted by GOS to improve growth performance and prime a Th17 immune response.

Modulation of cecal lactobacilli and bird growth performance. To examine if shifts in the cecal abundance of lactobacilli of juvenile broiler chickens can modify growth performance, we administered either *L. crispatus* DC21.1 or *L. johnsonii* DC22.2 (8 $\log_{10}$ CFU) by cloacal gavage to chicks at 6 da. Cloacal gavage has the advantage of allowing cecal colonization without the impact of upper intestinal transit and accompanying losses in the effective dose of the colonizing bacteria (36). Figure 7A shows marked shifts in the cecal abundance ratios of *L. crispatus*/*L. johnsonii* (competitive indices calculated as the ratios of the genome copy numbers per gram of cecal content) at 35 da in favor of the *Lactobacillus* spp. administered compared to the nontreated controls for birds on control or GOS diets. Extreme differences in the cecal abundance of *L. johnsonii* corresponded with disparate differences in the weights of mature birds at 35 da. The mean body weight of the birds with low cecal *L. johnsonii* abundance (administered with competitive *L. crispatus*) fed on the control diet was 1.86 ± 0.16 kg, compared with 2.83 ± 0.11 kg for the birds with high cecal *L. johnsonii* abundance ($P < 0.001$). Figure 7B and C show the respective correlations between body mass and *L. johnsonii* genome copy number from the cecal microbiota of birds administered either *L. johnsonii* DC22.2 (Pearson's $r = 0.353$; $P = 0.038$) or *L. crispatus* DC21.1 (Pearson's $r = 0.504$; $P < 0.001$). These data provide further evidence for the positive relationship between the cecal abundance of *L. johnsonii* and growth performance. The impact of the early exogenous introduction of *L. crispatus* DC21.1 was to reduce the abundance of *L. johnsonii*, which coincided with a reduction in the mean body mass of the birds at 35 da. Although the provision of in-feed GOS under these circumstances led to an increase in the relative abundance of cecal *L. johnsonii* and improved body masses at 35 da, the birds in the *L. crispatus* treatment groups did not develop a comparable

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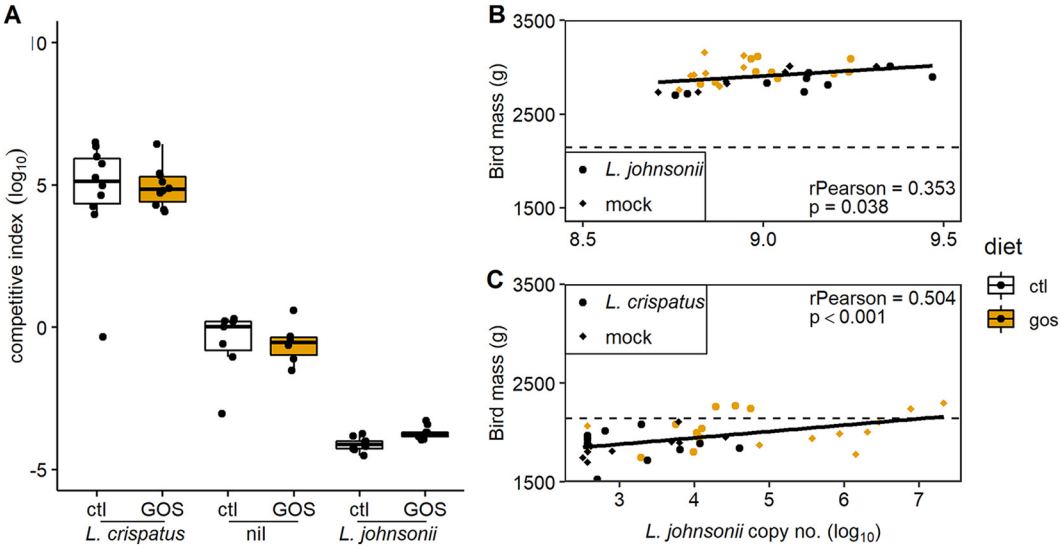


FIG 7 Modulation of the juvenile cecal microbiota. Panel A shows the competitive indices (*L. crispatus*/*L. johnsonii* ratios) at 35 da of broiler chickens either nontreated or administered 8 log_{10} CFU of either *L. crispatus* DC21.1 or *L. johnsonii* DC22.2 by cloacal gavage at 6 da. Competitive indices were calculated as the ratios of *L. crispatus* (OTU0006) to *L. johnsonii* (OTU0010) genome copy numbers per gram of cecal content determined by qPCR. Panel B shows the Pearson correlation of bird mass at 35 da against *L. johnsonii* gene copy number per gram of cecal content in birds administered *L. johnsonii*. Panel C shows the Pearson correlation of bird mass at 35 da against *L. johnsonii* gene copy number per gram of cecal content in birds administered *L. crispatus*. The dashed horizontal line represents the contemporary male Ross 308 performance objective (96).

relative abundance of *L. johnsonii* or achieve the weight at 35 da observed for mock-treated birds (Fig. 7C). Collectively, these data indicate *L. crispatus* is a competitor of *L. johnsonii* that affects differences in the compositional development of the cecal microbiota. Early shifts in the juvenile microbiota have a profound effect on the weights of market-ready broiler chickens. Figure 7C shows that the impact of dietary GOS is greatest on the weaker-performing birds administered *L. crispatus*, with increased body weight and increased cecal *L. johnsonii* abundance relative to those on the control diet due to expansion of the niche available to the resident bacteria.

DISCUSSION

Improvements in the growth performance together with improved health are key goals in broiler chicken production. Studies of broiler chickens aimed to establish productive intestinal microbiota have highlighted compositional shifts in the microbiota that discriminate between birds with opposing zootechnical parameters, but these can vary between experimental trials (15). Several families of bacteria have been reported from the intestinal communities of chickens to show positive or negative associations with feed efficiency: *Bacteroides*, *Enterobacteriaceae*, *Clostridium*, *Ruminococcus*, *Faecalibacterium*, and *Lactobacillus* (15, 37–39).

The inclusion of galacto-oligosaccharides in broiler feed resulted in an enhanced growth rate relative to those of chickens on carefully matched control diets reared under identical conditions. Prebiotic galacto-oligosaccharides have previously been reported to improve the performance and intestinal architecture and stimulate intestinal defenses of neonatal pigs (40). However, a GOS-supplemented diet fed to chickens increased fecal populations of bifidobacteria and lactobacilli but did not improve zootechnical performance (19). In contrast, we observed a significant improvement in performance of the GOS-fed broiler chickens that was also accompanied by changes in the intestinal microbiota. Differences in the abundance of specific members of the *Lactobacillus* genus in the cecal microbiota of juvenile birds on control and GOS diets were observed. *Lactobacillus* isolates were recovered from the cecal contents of these juvenile birds. Among these, specific isolates were identified that shared sequence identity with the OTUs displaying differential abundance in the microbiota of birds

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consuming either the GOS or the control diet. Whole-genome sequence alignments allowed the identification of *L. johnsonii* isolate DC22.2, prevalent in the ceca of GOS-fed birds, and *L. crispatus* isolate DC21.1, exhibiting greater abundance in control birds.

Lactobacilli need to import GOS (degree of polymerization of 2 to 6) and lactose since the enzymes required to break down the substrate are generally cell bound. Two principal pathways accomplish import: LacS lactose permease or the LacE/F phosphotransferase system, where the latter appears to be restricted to DP2 lactose (34). The dependence of LacS for the utilization of GOS with a DP of >2 was first established in *L. acidophilus* (41). Intestinal *Lactobacillus* species that form the "acidophilus complex" include *L. crispatus*, *L. johnsonii*, and *L. helveticus*, which show conservation of the *gal-lac* clusters (41). While the *lacS* gene is often present, the copresence of *lacS* and *lacA* (encoding β-galactosidase) appears to be associated with the ability to utilize GOS. *L. johnsonii* isolates exhibit host-specific differences, where human and porcine sources frequently possess the *lacS* and *lacA* genes but the poultry isolate FI9785 appears to be deficient due to genome rearrangements (42, 43). However, isolate FI9785 does contain orthologues of *lacE*/*F*. Converse to this, *L. johnsonii* DC22.2 has retained *lacS* and *lacA* but has lost the *lacE*/*F* genes. The *L. johnsonii* DC22.2 *lacS* permease gene contains a stop codon that would require initiation from an internal AUG with the loss of the first 31 amino acids based on comparisons with database homologues. However, notable exceptions to this are *L. pasteuri* and *L. gallinarum*, which, respectively, initiate translation at the corresponding position or 13 codons downstream. The syntenic position that harbors the lactose phosphotransferase-encoding genes in the *L. johnsonii* poultry isolate FI9785 (42) features a deletion in *L. johnsonii* DC22.2 that preserves the *lacR* gene encoding the repressor but dispenses with all the functional components. Paradoxically, in our experiments *L. johnsonii* DC22.2 did not efficiently utilize GOS *in vitro* but represented a greater differentially abundant component of the cecal microbiota of GOS-fed birds, which exhibited improved zootechnical performance. In contrast, *L. crispatus* DC21.1 can utilize GOS, but this did not provide a competitive advantage in GOS-fed chickens; rather, the reverse appears to be true. It seems unlikely that *L. johnsonii* DC22.2 can compete for the GOS substrate directly with *L. crispatus* DC21.1, which suggests that *L. johnsonii* DC22.2 benefits from the metabolic capability of another member of the cecal microbiota. The presence of GOS provides the trophic selection for members of the cecal community required to support autochthonous *L. johnsonii*. Indeed, a hallmark of the acidophilus complex gene contents is the absence of the biosynthetic pathways necessary to produce essential nutrients such as amino acids, purine nucleotides, and cofactors and therefore a reliance on effectively importing nutrients generated by the intestinal milieu (44, 45). Prebiotic selection *in situ* may well be a more reliable way of achieving a beneficial microbiota than directly providing dietary probiotics, as the response will be congruent with the metabolic capabilities of the resident community. Prevailing environmental conditions may alter the content and composition of the intestinal microbiota and therefore the outcomes of prebiotic selection. For example, members of the *Bifidobacterium*, *Christensenella*, and *Lactobacillus* genera have been reported to feature in the intestinal communities of chickens on GOS diets (19, 46, 47). However, removing GOS from the diet at 24 da and feeding the control finisher diet did not alter the performance improvement associated with feeding GOS to the juvenile birds or the succession of *L. johnsonii* in the process. This would suggest that the impact of juvenile prebiotic feed is lifelong. Recently, Slawinska et al. (48) have reported the delivery of galacto-oligosaccharides *in ovo*, which resulted in an increase in the relative abundance of *Bifidobacterium*-specific 16S rRNA PCR-amplifiable sequences from the cecal contents of mature birds at 42 da, implying that the composition of the mature microbiota can be programmed in early development.

The route to establishing a beneficial microbiota notwithstanding, we observed an increase in the abundance of *L. johnsonii* in GOS-fed birds that correlated with improved performance. *L. johnsonii* is an established probiotic with a variety of reported effects when administered to humans and animals. For example, *L. johnsonii* isolate

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N6.2 has been shown to have immunomodulatory effects in animal experiments and to protect diabetes-prone rats from developing the disease (49, 50). In another study, *L. johnsonii* was shown to attenuate respiratory viral infection via metabolic reprogramming and immune cell modulation (51). *L. johnsonii* LB1 expresses a bile salt hydrolase active against tauro-beta-muricholic acid (T-β-MCA), a critical mediator of farnesoid X receptor (FXR) signaling that is important in maintaining metabolic homeostasis (52). In broiler chickens, administration of an *L. johnsonii* isolate has been reported to improve growth performance (53). Subsequently, it was reported that meat from *L. johnsonii*-treated birds had higher nutritional value and the birds showed resistance to the development of necrotic enteritis (54, 55). Similar results were obtained by administering *L. johnsonii* LB1 to piglets to improve performance and reduce diarrhea (56). The administration of *L. johnsonii* FI9785 to broiler chickens has also been reported to reduce colonization by *Clostridium perfringens*, *Escherichia coli* O78:K80, and *Campylobacter jejuni*, which have a significant impact on poultry production (57, 58). *Lactobacillus crispatus* is also a recognized probiotic but is better known for its activity against recurrent urinary infections, bacterial vaginosis, and vaginal candidiasis (59). However, it should be noted that *L. crispatus* is commonly reported as a major constituent of the chicken microbiome (46, 60).

Shifts in the *Lactobacillus* spp. in response to GOS are accompanied by changes in the expression of cytokines and chemokines that have the potential to prime innate intestinal immune systems and enhance pathogen resistance. However, unchecked low-grade proinflammatory responses can cause tissue damage and inefficient feed conversion (61). Lactic acid, for example, is a by-product of glycolytic pathway of immune cells that can affect local T cell immunity by inhibiting T cell motility and inducing the change of $CD4^+$ cells to a Th17 proinflammatory T cell subset, which leads to IL-17 production and chronic inflammation (62). However, lactic acid is also the homofermentative product of lactic acid bacteria such as *L. johnsonii*, the action of which in the gut has recently been reported to promote the expansion of intestinal stem cells, Paneth cells, and goblet cells (63). Coincident with the increased abundance of *L. johnsonii*, we observed significantly greater ileal goblet cell densities from juvenile birds on the GOS diet. We also observed an increase in IL-17A and a decrease in IL-10 gene expression in juvenile birds on the GOS diet. IL-17A has been proposed to promote the maintenance of intestinal epithelial cell integrity based on observations that IL-17A inhibition exacerbates colitis in a mouse model, which leads to weakening of the intestinal epithelial barrier (64). In contrast, IL-17F knockout mice are reported to be protected against chemically induced colitis, whereas IL-17A knockout mice remain sensitive (65). Moreover, IL-17F-deficient mice showed an increase in the colonic abundance of *Clostridium* cluster XIVa organisms that promote beneficial regulatory T cells and the expression of β-defensins 1 and 4 (65). Extrapolating from these data, we propose that the increased expression of IL-17A we observed without impact on IL-17F in GOS-fed chickens will promote gut health, a prerequisite for improved commercial production.

The induction of host Th17 responses in ileal and cecal tissues by an indigenous symbiont is reminiscent of the Th17 stimulation brought about by adherent segmented filamentous bacteria (SFB) described for mice (66–68). The tight adherence of SFB to epithelial cells was observed to accelerate postnatal maturation of intestinal mucosal immunity by triggering a Th17 response (69). The observation of an intestinal Th17 response to tightly adherent symbiotes was extended to the human symbiont *Bifidobacterium adolescentis* in mice (70). However, the response arose through transcriptional program distinctly different from that observed for SFB, suggesting that intestinal Th17 responses are maintained by parallel sensor and signaling pathways. The upregulation of IL-17A in juvenile chickens fed a GOS diet may also support gut immune maturation through Th17 cell stimulation. In chickens, lactobacilli are recognized as adherent to the intestinal tract epithelium from crop to ceca. In addition to established cell adhesion factors, such as the expression of a fibronectin binding protein, it is

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proposed that *L. johnsonii* co-opts alternative cell surface-associated structures to cell adhesion roles; these include GroEL, elongation factor Tu, and lipoteichoic acid (71–74).

Juvenile chickens have been reported to exhibit a transient IL-17 induction during the development of the natural microbiota (75). The study suggested that in the absence of IL-22, proinflammatory Th17 induction did not result in intestinal tissue damage. It is possible that IL-17 has a role in the codevelopment of the microbiota and innate immunity in chickens, which is consistent with our findings that upregulation of IL-17A did not cause lamina propria inflammation. Crhanova et al. (75) also reported the outcome of *Salmonella enterica* serovar Enteritidis challenge of chickens shifts from a Th1 response (induction of IFN-γ and nitric oxide synthase) at 1 to 4 da to a Th17 response at 16 da (induction of IL-17). They conclude that a mature Th17 subset of helper T cells produced IL-17 and IL-22, which confer resistance to *S.* Enteritidis infection and damage in older birds.

Conclusion. We have demonstrated an increase in growth rate of broiler chickens in response to dietary supplementation with the prebiotic GOS. Juvenile chickens on GOS starter feed exhibited differences in the cecal abundance of key species of *Lactobacillus* compared to those on control feed. Differences in the cecal microbiota in early development correlated with the composition of the mature cecal microbiota and performance outcomes. The provision of dietary GOS increased the density of goblet cells populating ileal villi in the developing chicken gut. Goblet cell increases were accompanied by significant differences in the villus height and crypt depth at 15 da, a period when transitions in the development of the chicken microbiota from a juvenile to a mature composition are observed (76–78). We have demonstrated a significant correlation between the market weight of chickens at 35 da and the cecal abundance of a specific *L. johnsonii* isolate identified as differentially abundant in the juvenile microbiota of GOS-fed birds. *L. johnsonii* abundance also shows a positive correlation with IL-17A gene expression. *L. johnsonii* is an established probiotic that has been demonstrated to have beneficial effects when applied in poultry production (53–55, 57, 58). Several modes of action have been proposed for probiotic strains of *L. johnsonii*, but underlying these is the multimodal ability of the species to affect epithelial gut cell adherence (71–74), which we propose will induce the expression of IL-17A. By taking a system-wide approach we have, for the first time, established mechanistic links between prebiotic selection of an autochthonous synbiotic species, increased IL-17A expression, and the development of the gut in healthy animals.

MATERIALS AND METHODS

Ethical approval. Experiments involving the use of birds were subjected to an approval process under national guidelines by the United Kingdom Home Office. Work on this project was approved under United Kingdom Government Home Office project licensing ASPA 86. All project licenses are reviewed internally by the University Ethics Committee prior to submission to the Home Office. This includes the scrutiny of animal welfare, ethics, and handling.

Experimental birds. Commercial male Ross 308 broiler chicks were obtained as hatchlings (PD Hook, Oxfordshire, UK). Birds were housed in a controlled environment under strict conditions of biosecurity. Temperatures were as outlined in the *Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes* (79). Birds were provided feed and water *ad libitum*. Feeds were formulated on a least-cost basis and to meet the requirements set out in the *Ross 308 Broiler Nutrition Specifications 2014* (80) and prepared by Target Feeds Ltd (Shropshire, UK). The diet regime was as follows: the control diet group was sustained on a wheat-based diet provided as a starter crumb for 0 to 10 days of age (da), grower pellets for 11 to 24 da, and finisher pellets for 25 to 35 da. The starter diet contained wheat (59.9% [wt/wt]), soya meal (32.5% [wt/wt]), soyabean oil (3.65% [wt/wt]), limestone (0.6% [wt/wt]), calcium phosphate (1.59% [wt/wt]), sodium bicarbonate (0.27% [wt/wt]), the enzymes phytase and xylanase (dosed according to the manufacturer's instructions; DSM Nutritional Products Ltd., Basel, Switzerland), and a vitamin mix containing salt, lysine hydrochloride, DL-methionine, and threonine. The grower and finisher diets increased the wheat content at the expense of soya meal by 2 and 5% (wt/wt), respectively. GOS was provided as Nutrabiotic (GOS, 74% [wt/wt] dry matter) (Dairy Crest Ltd., Davidstow, Cornwall, UK). Galacto-oligosaccharide preparations contained a mixture of monosaccharides (glucose and galactose) and oligosaccharides (DP2 to DP8). The disaccharide lactose, a reactant in the manufacture of galacto-oligosaccharides, is not a galacto-oligosaccharide; all other disaccharides and longer oligosaccharides (DP3+) are considered to be galacto-oligosaccharides and nondigestible. The starter feed was supplemented with 3.37% (wt/wt) GOS and isocaloric adjustments made in the wheat (54% [wt/wt]) and soybean oil (4.88% [wt/wt]) contents. The grower and finisher feeds contained 1.685% GOS with

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respective adjusted wheat contents of 57.7% (wt/wt) and 63.3% (wt/wt) and soybean oil contents of 6.14% (wt/wt) and 6.22% (wt/wt). The final feeds were isocaloric (metabolizable energy including enzyme contribution) and contained the same crude protein levels and Degussa poultry digestible amino acid values (lysine, methionine, methionine plus cysteine, threonine, tryptophan, isoleucine, valine, histidine, and arginine). The feed formulations are listed in Table S2.

Chickens were euthanized by either exposure to rising CO_2 gas or parenteral barbiturate overdose followed by cervical dislocation according to schedule 1 of the UK Animals (Scientific Procedures) Act. The birds were weighed before tissue and intestinal contents were sampled postmortem. Ileal tissues were sectioned from approximately 3 cm distal to Meckel's diverticulum, and cecal tissues were collected from the distal tips of the ceca. Intestinal tissues were immediately frozen in liquid nitrogen for subsequent RNA isolation or preserved in 10% (wt/vol) neutral buffered formalin (Fisher Scientific, Loughborough, UK) for histological assessment. Intestinal contents were collected and stored at −80°C until DNA isolation.

Trial designs. (i) GOS diet trial 1. On the day of hatch, chicks were randomly assigned to either a control diet or a GOS-supplemented diet for the duration of the experiment. Two groups of 35 birds were kept in pens from day of hatch until day 6, when all birds were caged independently until euthanasia of 7 birds at sample time points 8, 15, and 22 days of age (da) to obtain intestinal contents and tissue biopsy specimens. Birds were weighed and feed consumption was recorded at least weekly from the start of the experiment at the day of hatch until then end of the study at 35 da. Growth rates between 8 and 35 da were determined for 10 birds remaining at the end of trial and for which feed consumption and live weights were recorded over the entire trial period. Feed conversion ratios (FCR) were calculated as a ratio of the cumulative feed consumed to the weights of the birds.

(ii) Ancillary GOS diet trials 2 and 3. To advance the study, we modified the experimental design to establish if removing the prebiotic feed after the microbiota had been established in the juvenile birds could reproduce the beneficial effects on performance observed for mature birds. These studies used the starter and grower feed formulations listed in Table S3, but both GOS treatment and control birds in ancillary trial 2 and ancillary trial 3 were fed the control finisher diet 25 to 35 da. The organization of trial 2 was the same as that of trial 1 ($n = 10$), while for trial 3, the birds were cohoused on wood shavings in 10 pens of 3 birds and wing tagged to identify individual birds instead of individual caging. Feed consumption was measured per pen and calculated as the average per bird for each pen ($n = 10$).

(iii) Cloacal gavage trials 4, 5, and 6. On the day of hatch, chicks were randomly assigned to either the control or GOS-supplemented diet. At 6 da, axenic suspensions of either *L. crispatus* DC21.1 or *L. johnsonii* DC22.2 containing between 7.4 and 7.8 $\log_{10}$ CFU in 0.1 ml of MRD (Oxoid, Basingstoke, UK) were administered to chicks by cloacal gavage. Control groups were mock administered with MRD alone. Cloacal gavage was performed using a blunt narrow-nosed syringe to stimulate reverse peristalsis. Postgavage, experimental groups of 20 birds were housed in pairs and maintained on GOS or control diet ($n = 10$). The birds were fed either the control diet throughout or the GOS diet until 24 da and then switched to the control until the end of the trial at 35 da. Feed consumption was measured per pen and calculated as the average per bird for each pen.

Identification of lactic acid bacteria. Cecal contents from each individual bird were serially diluted in MRD (Oxoid) and spread (0.1 ml) onto the surfaces of MRS (Oxoid) plates. The MRS plates were incubated under anaerobic conditions for 48 h at 37°C. The numbers of lactic acid bacterial colonies were recorded, and examples of distinct, well-isolated colonies were subcultured for identification and storage at −80°C. Multiple isolates from MRS plates were examined by microscopy using the Gram stain. Genomic DNAs were prepared from selected isolates showing different cell and colony morphologies using a GenElute bacterial genomic DNA kit (Sigma Aldridge, Gillingham, UK). Identification to presumptive species level was carried out by performing PCR amplification of 16S rRNA gene sequences using the primers 27f and 1522r (Table S3) (81, 82) and DNA sequencing of the products following cleanup (Wizard SV gel and PCR cleanup system; Promega, Southampton, Hampshire, UK) using dye terminator chemistry (Eurofins, Ebersberg, Germany). The 16S rRNA gene V4 region sequences were matched to the OTU clusters outputted from microbiome analysis. The genome sequences of *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 were assembled using CLC Genomics Workbench 10.0.1 (Qiagen, Aarhus, Denmark) using a combination of data generated from the Illumina MiSeq and PacBio RSII platforms. The *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 cultures were deposited at the National Collection of Industrial Food and Marine Bacteria (NCIMB) under the respective accession numbers 42771 and 42772.

***In vitro* growth of *Lactobacillus* on galacto-oligosaccharides.** To determine the ability of *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 to utilize GOS *in vitro*, a purified Nutrabiotic GOS (74% [wt/wt] dry matter) containing a DP2+ lactose fraction was prepared using high-performance liquid chromatography (HPLC), with an Imtakt Unison UK-Amino (aminopropyl stationary phase) column (ARC Sciences, Oakham, UK) with acetonitrile-water mobile phase, to remove monomeric sugars (glucose and galactose) and lactose (IPOS Ltd., Huddersfield, UK). A reduced-carbon-source medium based on MRS broth with the omission of glucose was prepared as a basal medium (pH 6.7). One liter of the medium contained 10 g of tryptone (Oxoid), 5 g of yeast extract (Oxoid), 10 g of Lab-Lemco powder (Oxoid), 1 ml of sorbitan mono-oleate (Tween 80), 2 g of dipotassium hydrogen phosphate, 0.5 g of sodium acetate $3H_2O$, 2 g of diammonium hydrogen citrate, 0.2 g of magnesium sulfate $7H_2O$, and 0.05 g of manganese sulfate $4H_2O$ (from Fisher Scientific unless otherwise stated). Each experiment was carried out using the basal medium with addition of DP2+ lactose GOS (0.5% [wt/vol]), together with a positive control, containing glucose (0.5% [wt/vol]) and a negative control with sterile water instead of the carbon source. The bacterial cultures were grown on MRS plates and suspended in the modified MRS medium to a density of 8 $\log_{10}$ CFU/ml (OD_{600} of approximately 1.5). The suspension was diluted 1 in 100 into the growth medium. The

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assay was carried out in triplicate with 3 technical replicates per biological replicate together with a set of uninoculated negative controls as blanks (0.2 ml in microtiter plates). The plates were covered and incubated at 37°C for 72 h under anaerobic conditions, with shaking. The OD_{600} obtained from growth on the basal medium, without the addition of a carbon source, was subtracted from the value of the growth on the selected carbon source.

Histology. Tissue samples fixed in a 10% formalin solution were dehydrated through a series of alcohol solutions, cleared in xylene, and embedded in paraffin wax (Microtechnical Services Ltd., Exeter, UK). Sections (3 to 5 μm thick) were prepared and stained with either modified hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) using standard protocols. After staining, the slides were scanned with the NanoZoomer digital pathology system (Hamamatsu, Welwyn Garden City, UK). Measurements of villus height and crypt depth were made using the NanoZoomer digital pathology image program (Hamamatsu). Ten well-oriented villi per tissue section of 4 birds from each diet group at each sampling time were scanned at 40× resolution for each tissue sample. Villus height was measured from the tip of the villus to the crypt opening, and the associated crypt depth was measured from the base of the crypt to the level of the crypt opening. The ratio of villus height to relative crypt depth was calculated from these measurements. Goblet cells were enumerated from ileal sections stained with PAS. In one case the histology section did not fulfill the quality criterion of 10 well-oriented villi and was omitted from the analysis.

RNA isolation and RT-qPCR of cytokines and chemokines. RNAs were isolated from cecum and ileum tissue biopsy specimens using a NucleoSpin RNA purification kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol, with the following modifications. Tissue samples were homogenized with the kit lysis buffer and 2.8-mm ceramic beads (MO BIO Laboratories Inc., Carlsbad, CA) using TissueLyser II (Qiagen, Hilden, Germany). Subsequently, total RNA was extracted as described in the protocol with a DNase I treatment step as per the manufacturer's instructions. Purified RNAs were eluted in nuclease-free water, validated for quality and quantity using UV spectrophotometry (Nanodrop ND-1000; Labtech International Ltd., Uckfield, UK), and stored long term at −80°C. RNAs with OD_{260}/OD_{280} ratios between 1.9 and 2.1 were deemed high quality; the sample ratios had a mean of 2.12 ± 0.01. Reverse transcription (RT) was performed with 1 μg of RNA, SuperScript II (Invitrogen Life Technologies, Carlsbad, CA), and random hexamers as described previously (76).

Quantitative PCR was performed with cDNA templates derived from 4 ng of total RNA in triplicate using SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific, UK). The RNA level of expression was determined by qPCR using the Roche Diagnostics LightCycler 480 (Hoffmann La Roche AG, Switzerland). The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RPL4, IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, ChCXCLi-1, and ChCXCLi-2 (83–86) are presented in Table S3. Cytokine and chemokine transcript levels and fold change were calculated according to the manufacturer's recommendation using the $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ methods, where the *CT* is the PCR cycle threshold and ΔCT corresponds to the difference between the *CT* values of the cyto/chemokine gene and the reference housekeeping genes to give normalized transcript levels, and $\Delta\Delta CT$ is the difference between transcript levels of birds on the GOS and control diets (87). The means of triplicate C_T values were used for analysis, where target gene C_T values were normalized to those of the housekeeping GAPDH and 60S ribosomal protein L4 (RPL4) genes.

Microbiome analysis. DNA was isolated from cecal contents using the MoBio PowerSoil kit (now Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions. For microbiome analysis the V4 regions of the bacterial 16S rRNA genes were PCR amplified using the primers 515f and 806r (Table S3) (88). Amplicons were then sequenced on the Illumina MiSeq platform using 2 × 250-bp cycles. The 16S rRNA gene sequences were quality filtered and clustered into OTUs in Mothur (89, 90) using the Schloss lab. MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP, accessed 10 May 2018 [91]). Batch files of Mothur commands used in this study are available at https://github.com/PJRichards/Richards_GOS_broiler. Postprocessing rarefaction curves were plotted to assess sampling effort (Fig. S2).

Quantitative PCR enumeration of lactobacilli. Quantitative PCR protocols to enumerate *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 organisms from intestinal contents were developed by designing primers specific for the *groEL* gene sequences of these bacterial strains (Table S3). Real-time qPCR quantification of *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 was performed with 1 μl of cecal content DNA (15 to 150 ng) using SYBR green master mix with the Roche Diagnostics LightCycler 480. The amplification conditions were denaturation at 95°C for 5 min followed by 45 cycles of 95°C for 15-s denaturation and 60°C for 1-min annealing. The fluorescence signals were measured at the end of each annealing step. Melting curves were generated by heating the samples from 65°C to 97°C at a ramp rate of 0.11°C per s. The data obtained were plotted against a standard curve generated with 5-fold serially diluted target bacterial DNAs. Genome copy number of target bacteria in each dilution was calculated based on its genome length and applied DNA quantity with the assumption of the mean molecular mass of one base pair as 650 Da. The method was validated first with DNA extracts from pure cultures of known CFU and then by spiking chicken cecal samples with increasing concentrations of target cells. The data were calculated as genome copy number per microliter of DNA applied in the PCR and converted into genome copy number per gram of cecal content based on the mass of cecal material and elution volume applied for DNA extraction.

Statistical analysis. Growth rate was tested for significance by measuring the weight gain for each bird between 8 and 35 days and testing the difference between the growth rates (grams per day) using Student's *t* test. Differences in bird mass at 35 days were compared using Student's *t* test.

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Competitive indices of the *Lactobacillus* species present in the ceca were calculated as the ratio of the *L. crispatus* (OTU0006) copy number per gram of cecal content to the *L. johnsonii* (OTU0010) copy number per gram of cecal content determined using qPCR.

For the microbiota β-diversity analysis, Bray-Curtis distances were tested for significance using analysis of molecular variance (AMOVA) implemented within Mothur (89). Linear discriminant analysis effect size (LefSe) (92) was also implemented within Mothur (89). Differences in α-diversity, Chao richness, and absolute abundance of *Lactobacillus* spp. were tested using Wilcoxon rank sum test. With the exception of Fig. 5, all figures were drawn using R version 3.6.1 (7 May 2019) (93) in Rstudio 1.2 (94). R scripts used to draw figures are available at https://github.com/PJRichards/Richards_GOS_broiler.

Significance tests of heterophil counts and villus and crypt measurements were performed using single-factor analysis of variance (ANOVA) with a P value of <0.05 used as the level significance. Non-normally distributed gene expression data were compared using the nonparametric Wilcoxon rank sum tests adjusted with the Benjamini-Hochberg false-discovery rate correction (95).

Data availability. The genome DNA sequences of *L. crispatus* DC21.1 appear in the NCBI database under the accession numbers CP039266 to CP039267. The genome DNA sequences of *L. johnsonii* DC22.2 appear in the NCBI database under the accession numbers CP039261 to CP039265. Raw DNA sequence data and metadata in support of 16S rRNA metagenomic analysis appear in the NCBI database within Bioproject PRJNA380214. Raw zootechnical observations are available at https://github.com/PJRichards/Richards_GOS_broiler/tree/master/zootechnical.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 2.9 MB.

FIG S2, TIF file, 2.6 MB.

TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.01 MB.

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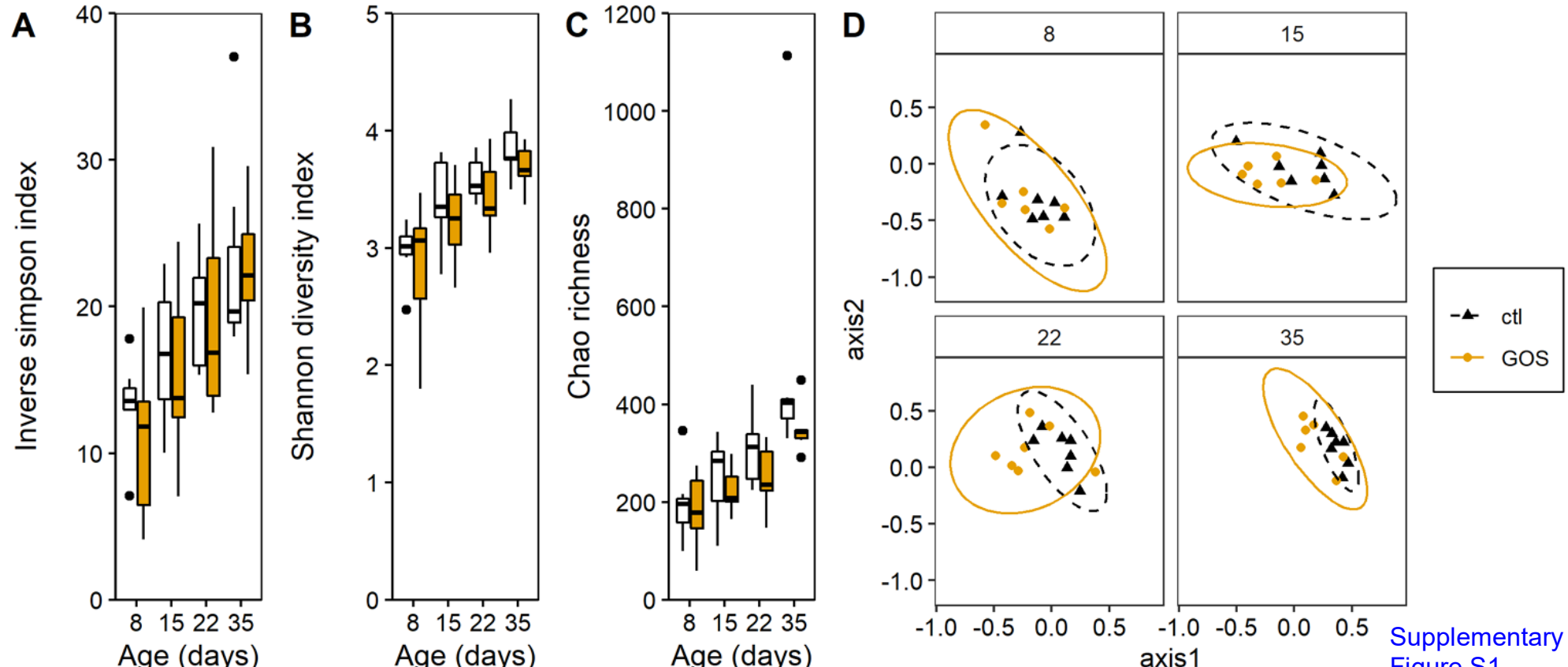
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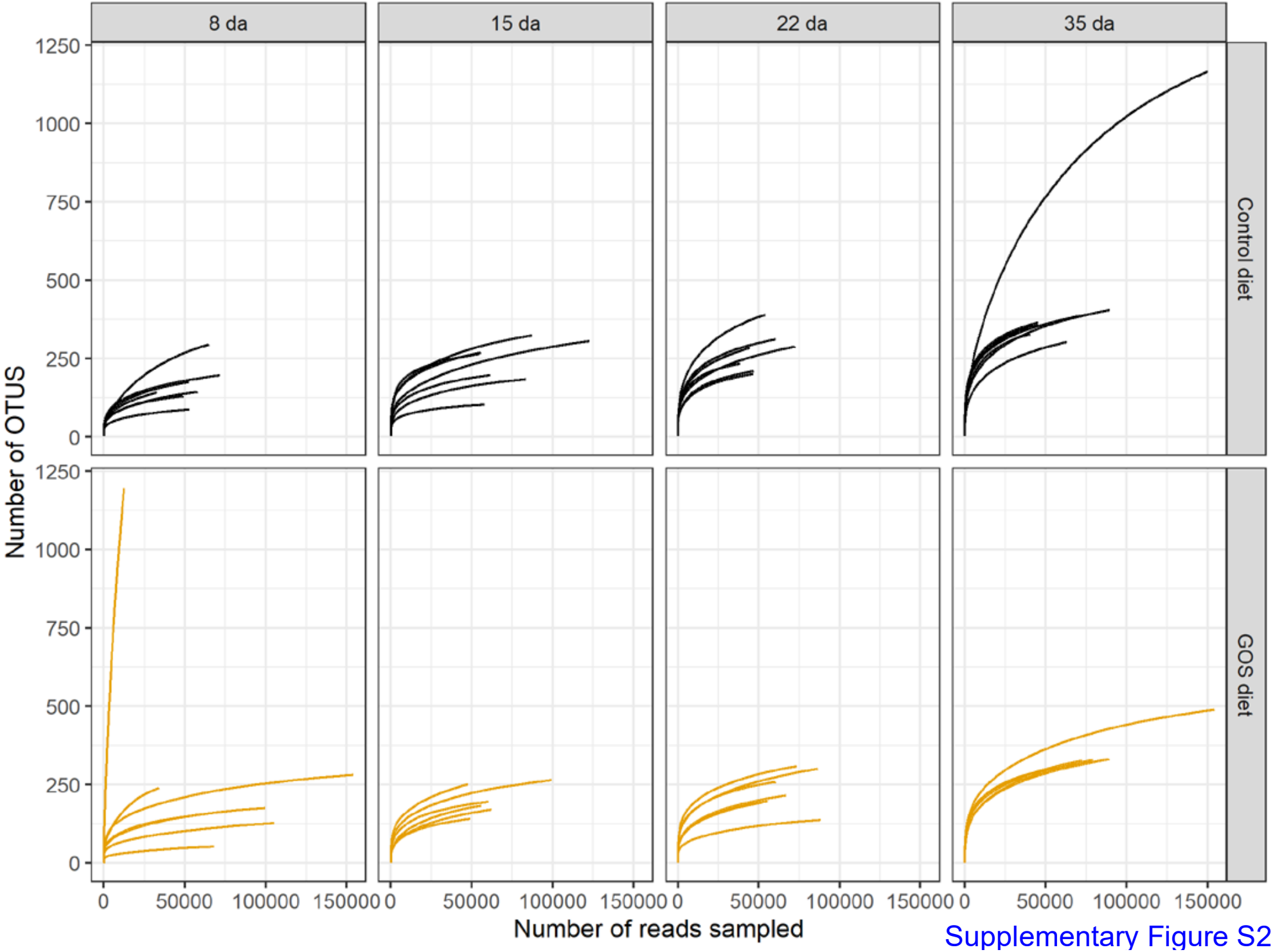
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Supplementary Figure S1



Supplementary Figure S2

Supplementary Table S1

Ross 308 Broiler chickens fed nutritionally matched control and GOS diets

Diet	Control	GOS	p-value
Trial T1 GOS treatment 0-35 da (n=10)			
Mean body mass at day of hatch (g)	45.9	44.8	0.4152118
Mean body mass at day 35 (g)	2,336	2,582	0.0409658
Mean BWG 0 - 10 da (g)	325	333	0.6632645
Feed per BWG 0 - 10 da	1.025	1.064	
Mean BWG 11 - 35 da (g)	1,987	2,238	0.0383560
Feed per BWG 11 - 35 da	1.551	1.512	
Cumulative feed intake per bird – 35da (g)	3452	3756	
FCR (including initial body weight)	1.478	1.455	
Trial T2 GOS treatment 0-24 da & control 25-35 da (n=10)			
Mean body mass at day of hatch (g)	43.7	41.2	0.1171804
Mean body mass at day 35 (g)	1,838	2,584	0.0000001
Mean BWG 0 - 11 da (g)	284	320	0.0045978
Feed per BWG 0 - 11 da	1.075	1.056	
Mean BWG 12 - 35 da (g)	1,510	2,223	0.0000001
Feed per BWG 12 - 35 da	1.647	1.433	
Cumulative feed intake per bird – 35da (g)	2823	3563	
FCR (including initial body weight)	1.536	1.379	
Trial T3 GOS treatment 0-24 da & control 25-35 da (n=10)			
Mean body mass at day of hatch (g)	38.8	38.5	0.7792273
Mean body mass at day 35 (g)	2,291	2,501	0.0566421
Mean BWG 0 - 10 da (g)	247	262	0.0346623
Feed per BWG 0 - 10 da	0.996	0.995	
Mean BWG 11 - 35 da (g)	2,044	2,239	0.0873694
Feed per BWG 11 - 35 da	1.425	1.381	
Cumulative feed intake per bird – 35da (g)	3159	3354	
FCR (including initial body weight)	1.379	1.341	
Trial T4 *L. johnsonii* at 6da, GOS treatment 0-24 da & control 25-35 da (n=10)			
Mean body mass at day of hatch (g)	44.6	46.0	0.1540935
Mean body mass at day 35 (g)	2,836	2,961	0.0187776
Mean BWG 0 - 14 da (g)	404	435	0.0201485
Feed per BWG 0 - 14 da	1.203	1.112	
Mean BWG 15 - 35 da (g)	2,387	2,480	0.0383560
Feed per BWG 15 - 35 da	1.411	1.398	
Cumulative feed intake per bird – 35da (g)	3874	3929	
FCR (including initial body weight)	1.366	1.326	
Trial T5 mock at 6da, GOS treatment 0-24 da & control 25-35 da (n=10)			
Mean body mass at day of hatch (g)	39.5	38.6	0.2869586
Mean body mass at day 35 (g)	1,867	2014	0.0367011

Mean BWG 0 - 11 da (g)	281	287	0.4932771
Feed per BWG 0 - 11 da	1.195	1.142	
Mean BWG 12 - 35 da (g)	1,565	1,640	0.3940673
Feed per BWG 12 - 35 da	1.667	1.587	
Cumulative feed intake per bird – 35da (g)	2823	3563	
FCR (including initial body weight)	1.577	1.456	
Trial T6 *L. crispatus* at 6da, GOS treatment 0-24 da & control 25-35 da (n=10)			
Mean body mass at day of hatch (g)	38.9	38.9	0.9719941
Mean body mass at day 35 (g)	1,869	2,032	0.0470342
Mean BWG 0 - 10 da (g)	285	290	0.6558510
Feed per BWG 0 - 10 da	1.133	1.169	
Mean BWG 11 - 35 da (g)	1,598	1,723	0.1089529
Feed per BWG 11 - 35 da	1.885	1.658	
Cumulative feed intake per bird – 35da (g)	2872	3195	
FCR (including initial body weight)	1.536	1.572	

BWG represents body weight gain. FCR represents the feed conversion ratio.

[Supplementary Table S1]

FEED COMPONENT	CONTROL: STARTER	CONTROL: GROWER	CONTROL: FINISHER	GOS 3.370%: STARTER	GOS 1.685%: GROWER	GOS 1.685%: FINISHER
WHEAT	59.999	60.716	66.319	54.016	57.719	63.324
EXT. HIPRO SOYA MEAL	32.5	30.8	25.3	33.9	31.5	26.0
LIMESTONE GRANULES	0.60	0.40	0.40	0.60	0.40	0.40
SOYABEAN OIL	3.65	5.52	5.60	4.88	6.14	6.22
LYSINE HCL	0.296	0.119	0.123	0.264	0.103	0.107
METHIONINE DL	0.362	0.263	0.231	0.366	0.265	0.232
DICALCIUM PHOSPHATE	1.59	1.28	1.12	1.61	1.29	1.13
SODIUM BICARBONATE	0.269	0.188	0.193	0.249	0.179	0.183
SALT	0.150	0.210	0.210	0.170	0.220	0.220
THREONINE	0.134	0.054	0.054	0.125	0.049	0.049
TM - Blank Premix for Broiler Formulation	0.400	0.400	0.400	0.400	0.400	0.400
NUTRABIOTIC SYRUP (GOS 74% w/w)	**0.000**	**0.000**	**0.000**	**3.370**	**1.685**	**1.685**
RONOZYME P5000 (CT)	0.030	0.030	0.030	0.030	0.030	0.030
Ronozyme WX (Xyl)	0.020	0.020	0.020	0.020	0.020	0.020

[Supplementary Table S2]

Supplementary Table 3 Primer sequences used in this study

Target gene	Primer sequence (5'-3')	Product size (bp)	NCBI Accession/name	Reference
GAPDH	F: GACGTGCAGCAGGAACACTA	343	NM_204305.1	1
	R: TCTCCATGGTGGTGA AGACA			
INF-γ	F: TGAGCCAGATTGTTTCGATG	152	NM_205149.1	1
	R: CTTGGCCAGGTCCATGATA			
IL-1β	F: GGATTCTGAGCACACCACAGT	272	NM_204524.1	1
	R: TCTGGTTGATGTCGAAGATGTC			
IL-4	F: GGAGAGCATCCGGATAGTGA	186	NM_001007079.1	1
	R: TGACGCATGTTGAGGAAGAG			
IL-10	F: GCTGCGCTTCTACACAGATG	203	NM_001004414.2	1
	R: TCCCGTTCTCATCCATCTTC			
IL-6	F: GCTCGCCGGCTTCGA	71	NM_204628.1	2
	R: GGTAGGTCTGAAAGGCGAACAG			
IL-17A	F: CATGGGATTACAGGATCGATGA	68	NM_204460.1	3

[Supplementary Table S3]

	R: GCGGCACTGGGCATCA			
IL-17F	F: TGACCCTGCCTCTAGGATGATC	78	XM_426223.5	3
	R: GGGTCCTCATCGAGCCTGTA			
ChCXCLi-1	F: CCGATGCCAGTGCATAGAG	191	NM_205018.1	4
	R: CCTTGTCCAGAATTGCCTTG			
ChCXCLi-2	F: CCTGGTTTCAGCTGCTCTGT	128	NM_205498.1	4
	R: GCGTCAGCTTCACATCTTGA			
L. johnsonii GroEL	F: GGAAAACATGGTTAAGGCTGGT	152	E6A54_02330	Present study
	R: GAGCTGCAGGAGCTTGGTTC			
L. crispatus GroEL	F: GAAGGCTATCAATGATCTTAAGG	276	E6A57_02020	Present study
	R: AGCAGAAGAGCGGCAATTGA			
16S rRNA	F: AGAGTTTGATCMTGGCTCAG	1557	TPU1	5
	R: AAGGAGGTGATCCANCCRCA		RTU8	
16S rRNA	F: GTGCCAGCMGCCGCGGTAA	292	515f	6
	R: GGACTACHVGGGTWTCTAAT		806r	

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[Supplementary Table S3]

CHAPTER 4. CHARACTERIZING THE EFFECT OF *CAMPYLOBACTER JEJUNI* CHALLENGE ON BROILER CHICKENS

This chapter relates to the peer-reviewed, open access articles cited below and reproduced at the end of this section. They are discussed together as they relate to research that was performed to better understand host:*Campylobacter jejuni* interactions (pp. 25-26).

Connerton, P.L., Richards, P.J., Lafontaine, G.M., O'Kane, P.M., Ghaffar, N., Cummings, N.J., Smith, D.L., Fish, N.M. and Connerton, I.F., 2018. The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome 6*(1), p.88. DOI: 10.1186/s40168-018-0477-5

Lafontaine, G.M.F., Richards, P.J., Connerton, P.L., O'Kane, P.M., Ghaffar, N.M., Cummings, N.J., Fish, N.M. and Connerton, I.F., 2020. Prebiotic Driven Increases in IL-17A Do Not Prevent *Campylobacter jejuni* Colonization of Chickens. *Frontiers in Microbiology* 10. DOI: 10.3389/fmicb.2019.03030

4.1 Summary

In Connerton et al. (2018) we describe the effect that *C. jenuni* challenge has on broiler chickens at different ages using a systems biology approach, which profiles corresponding changes to the gut microbiota, the host immune response, gut architecture and growth performance. Building on our observations, and those previously reported in Richards et al. (2020), in Lafontaine et al. (2020) we used similar approaches to assess the effect that dietary GOS, and corresponding modulation of innate immunity, has on *C. jejuni* colonization. These published works provide a foundation on which future works can build to make the chicken gut less hospitable to *Campylobacter* through rational feed formulation.

Salmonella enterica is a facultatively anaerobic, Gram-negative bacterium for which the molecular mechanisms used to colonize vertebrate hosts are increasingly well understood. To identify possible *Campylobacter* interventions this review will contrast the host:microbe interactions that accompany *Salmonella* colonization with what is known of *C. jejuni* colonization of chickens with a particular focus on the research outputs of my PhD studies.

4.2 Attribution of work

My work presented in Connerton et al. (2018) is in the analysis of the microbiota data and the growth performance data. I also contributed to the writing of the manuscript, drew figures 5, 6 and 7 and made additional files 3, 4, 6. My involvement with Lafontaine et al. (2020) was also in the analysis of the microbiota data, analysis of the growth performance data and contribution to the writing of the manuscript. In addition, so that the figures reported in the article could be freely reproduced I plotted figures 1, 2 and 3 using scripted approaches in R, but only figures 1 and 3 are drawn from data that I worked on. Both articles report data from the same experimental trials. I assisted in the culture-based enumeration of the *Campylobacter* bacteria. I had no role in the measurement or the analysis of host gene expression data or histological observations.

4.3 *Campylobacter jejuni* is a zoonotic pathogen associated with poultry

C. jejuni is a microaerophilic, Gram-negative bacterium that is a leading cause of diarrhoeal disease in humans (Majowicz et al., 2010; Troeger et al., 2017). *C. jejuni* is commonly associated with exposure to contaminated poultry products (Skarp et al., 2016) and although in most cases human infection is self-limiting it causes significant mortality in children in developing countries (Platts-Mills and Kosek, 2014). The epidemiology of *C. jejuni* colonization of chicken flocks is well understood (Gerwe et al., 2005; Newell and Fearnley, 2003; Newell et al., 2011); but given the importance that efficient colonization of the chicken gut with high densities of *C. jejuni* plays in transmission of infection (Rosenquist et al., 2003), frustratingly little is known of the molecular basis (Hermans et al., 2011). Chickens are highly susceptible to colonization with *C. jejuni* and it has proven more difficult to exclude *C. jejuni* from flocks than it has Salmonella (EFSA, 2019). Unlike *Salmonella* although *C. jejuni* can colonize chickens from the first day of life (Beery et al., 1988), it is not commonly reported in intensively farmed flocks until birds are three weeks of age (Evans and Sayers, 2000). Mathematical modelling approaches have also indicated that this 21-day lag period is attributable to age-dependent factors and not simply susceptibility to colonization (Conlan et al., 2011).

4.4 Competitive exclusion of *Salmonella enterica*

Newly hatched chickens are effectively colonized with high densities of *Salmonella enterica* following oral challenge, but after one week of life a similar challenge leads to less frequent colonization with lower levels of *Salmonella* (Beal et al., 2004; Desmidt et al., 1997; Varmuzova et al., 2016). Similarly, *S. enterica* serovar Enteritidis causes systemic disease in

newly hatched birds, but seldom does so in older birds (Barrow, 1991). Susceptibility of young birds to *Salmonella* colonization is linked to development of the gut microbiota and it is well established that a protective effect against *Salmonella* is generated by exposing chicks to the gut microbiota of mature birds (Nurmi and Rantala, 1973; Varmuzova et al., 2016) or commercial competitive exclusion products of mixed bacterial populations (Methner et al., 1997; Nakamura et al., 2002).

4.5 Interactions between *Campylobacter jejuni*, the cecal microbiota and the host

These observations outlined above suggest that *C. jejuni* colonization is independent of the microbiota, but challenge trials have shown that mixed cocktails of predominantly lactic acid bacteria (Cean et al., 2015; Ghareeb et al., 2012), individual *L. johnsonii* strains (Mañes-Lázaro et al., 2017) and preparations of gut microbiota from mature birds (Alrubaye et al., 2019; Gilroy et al., 2018; Mead et al., 1996) all protect chickens against *C. jejuni* colonization to some degree. In Connerton et al. (2018) we report that oral challenge of broiler chickens with a well characterized *C. jejuni* HPC5 strain (Scott et al., 2007) results in similarly high levels of cecal colonization independent of the age of the birds at exposure, although the *C. jejuni* population in birds challenged at 6 days-old took longer to reach a steady state. Reid et al. (2016) reported that colonization with *C. jejuni* induces a Th17-driven immune response in chickens and this is supported by the observations we report in Connerton et al. (2018). Although the weight gain of birds challenged with *C. jejuni* at 6 days old was slightly checked in early life, there was no indication of disease in challenged birds, eg. abnormal behaviour, diarrhoea or visible inflammation of the gastrointestinal tract. However, it must be cautioned that these trials were conducted under high-welfare, high-biosecurity conditions and may not be comparable to on-farm conditions. Proportions of Proteobacteria in both control and challenge groups were broadly unchanged throughout both trials. This is likely because the youngest birds from which cecal content was collected were 8 days-old, by which time the high proportions of cecal Proteobacteria associated with early life have already subsided. Additionally, we did not observe the blooms in the relative abundance of *Escherichia coli*, or other family Enterobacteriacea, that are promoted by the host pro-inflammatory response to *Salmonella* challenge in chickens and mice (Litvak et al., 2019; Stecher et al., 2012). *Salmonella* Enteritidis is dependent on a Type 3 secretion system (T3SS) to colonize the chicken gastrointestinal tract (Litvak et al., 2019; Porter and Curtiss, 1997). In both mouse and chicken models of *Salmonella* infection, T3SS-associated gut inflammation has been shown to generate a more favourable niche for facultative anaerobes and facilitate

colonization at high cell densities (Litvak et al., 2019; Stecher et al., 2007; Winter et al., 2010). Comparing the host response in the ceca of 3 week old broilers Setta et al. (2012) found that *S*. Entertidis and *S*. Infantis colonized at high levels and were pro-inflammatory, whereas chicken-adapted *Salmonella* Pullorum and *S.* Gallinarum, which cause terminal systemic disease, colonized the ceca at relatively low levels which corresponded with a limited pro-inflammatory response (Setta et al., 2012). Additionally, Setta et al. (2012) reported that pre-administration of vancomycin (antibiotic) to the birds did not effect the levels of *S.* Pullorum and *S.* Gallinarum in the ceca indicating that colonization resistance was not implicated in the low level of colonization. These results indicate that high levels of cecal colonization are not required for pathogenesis and that factors other than competitive exclusion likely affect the bacterial abundance in the gastrointestinal tract.

4.6 Modulating the host response

Although *C. jejuni* induces a pro-inflammatory response, there is no evidence in support of this being a mechanism by which *C. jejuni* expands its gastrointestinal niche in a similar manner to *Salmonella*. In mouse models maintenance of the low epithelial oxygen concentrations that result from colonocyte metabolism of microbiota-derived butyrate are important in suppressing *Salmonella* colonization (Rivera-Chávez et al., 2016). In chickens, dietary inclusion of butyrate, that will reinforce the barrier to colonization by providing butyrate to the colonocytes even in the absence of butyrogenic bacteria, such as Clostridia XIVa (Louis and Flint, 2009), has short-term effectiveness in reducing *Salmonella* colonization (Immerseel et al., 2005). However, in-feed butyrate was ineffective as an intervention against *C. jejuni* colonization (Deun et al., 2008).

We speculate that the adaptations for co-respiration of different electron acceptors present in *C. jejuni* allow it to compete with the cecal microbiota in the absence of niche modification by inflammation as exhibited by the the Enterobacteriacea (Lopez et al., 2016; Stel and Wösten, 2019; Winter et al., 2010). Microbiota transplantation between chickens lines with differing susceptibility to *C. jejuni* colonization did not transfer a protective effect and this suggests that colonization resistance in chickens is linked to host factors and is not only a function of colonization resistance by the microbiota (Chintoan-Uta et al., 2020). This does not exclude the fact that the activity of specific probiotic strains can exclude *C. jejuni* (Cean et al., 2015; Ghareeb et al., 2012; Mañes-Lázaro et al., 2017). In Richards et al. (2020) we observed that dietary GOS increases the abundance of *L. johnsonii* and modulates the host expression of IL-17A and IL-10, either directly or indirectly through microbially mediated bioprocesses. In Lafontaine et al. (2020) we show that the changes in the

microbiota and the host pro-inflammatory response did not affect *C. jejuni* colonization and that *C. jejuni* colonization does not effect the GOS-mediated improved growth rate.

Connerton, P.L., Richards, P.J., Lafontaine, G.M., O'Kane, P.M., Ghaffar, N., Cummings, N.J., Smith, D.L., Fish, N.M. and Connerton, I.F., 2018. The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens. *Microbiome 6*, 88. DOI: 10.1186/s40168-018-0477-5

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Connerton et al. Microbiome (2018) 6:88
https://doi.org/10.1186/s40168-018-0477-5

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RESEARCH Open Access

The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens

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Abstract

Background: Campylobacters are an unwelcome member of the poultry gut microbiota in terms of food safety. The objective of this study was to compare the microbiota, inflammatory responses, and zootechnical parameters of broiler chickens not exposed to *Campylobacter jejuni* with those exposed either early at 6 days old or at the age commercial broiler chicken flocks are frequently observed to become colonized at 20 days old.

Results: Birds infected with *Campylobacter* at 20 days became cecal colonized within 2 days of exposure, whereas birds infected at 6 days of age did not show complete colonization of the sample cohort until 9 days post-infection. All birds sampled thereafter were colonized until the end of the study at 35 days (mean 6.1 $\log_{10}$ CFU per g of cecal contents). The cecal microbiota of birds infected with *Campylobacter* were significantly different to age-matched non-infected controls at 2 days post-infection, but generally, the composition of the cecal microbiota were more affected by bird age as the time post infection increased. The effects of *Campylobacter* colonization on the cecal microbiota were associated with reductions in the relative abundance of OTUs within the taxonomic family *Lactobacillaceae* and the *Clostridium* cluster XIVa. Specific members of the *Lachnospiraceae* and *Ruminococcaceae* families exhibit transient shifts in microbial community populations dependent upon the age at which the birds become colonized by *C. jejuni*. Analysis of ileal and cecal chemokine/cytokine gene expression revealed increases in IL-6, IL-17A, and Il-17F consistent with a Th17 response, but the persistence of the response was dependent on the stage/time of *C. jejuni* colonization that coincide with significant reductions in the abundance of *Clostridium* cluster XIVa.

Conclusions: This study combines microbiome data, cytokine/chemokine gene expression with intestinal villus, and crypt measurements to compare chickens colonized early or late in the rearing cycle to provide insights into the process and outcomes of *Campylobacter* colonization. Early colonization results in a transient growth rate reduction and pro-inflammatory response but persistent modification of the cecal microbiota. Late colonization produces pro-inflammatory responses with changes in the cecal microbiota that will endure in market-ready chickens.

Keywords: *Campylobacter jejuni*, Chicken gut microbiota, Intestinal cytokine and chemokines, Pro-inflammatory response, Gut histology, Food safety

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Background

The production of poultry for both meat and eggs has been increasing rapidly throughout the world [1]. Feed conversion efficiency is of foremost importance in the economic profitability of poultry meat production, and selective breeding has resulted in fast-growing birds with reduced feed conversion ratios. The relationship between the gut microbiota and the feed conversion performance of broiler chickens has been a focus of research in recent years, with the prospect of modifying the microbiota to improve production efficiency and bird health [2, 3].

Food-borne enteritis caused by the Gram-negative spiral-shaped bacteria *Campylobacter* is a major medical and economic problem worldwide, with numbers of cases continuing to increase [4]. Poultry products are considered to be a significant source of infection to humans [5]. *Campylobacter jejuni* and *coli*, the two species responsible for most human disease, are extremely prevalent in poultry production with up to 80% of flocks harboring the bacteria (depending on the country in question), and this leads to a similarly high level of transference to poultry meat following processing [6, 7]. Consequently, much attention has focused on reducing both the incidence of *Campylobacter* in poultry flocks and the numbers of the bacteria contaminating poultry meat and thereby reducing the risk of infection to the consumer. One approach is to attempt to influence the microbiota of the gastrointestinal tract (GI). The development of affordable next-generation DNA sequencing techniques has allowed detailed investigations into the diversity of this important ecosystem and offered the possibility of relating changes in the microbiota to bird health and the efficiency of feed digestion [3].

Once hatched, the GI of chicks becomes successively colonized by *Enterobacteriaceae* (1 to 3 days of age) and *Firmicutes* (approximately 7 days of age onwards) [8]. In the absence of deliberate population of the gut with commercial microbiota preparations, colonization of the avian GI tract with specific bacterial species, belonging to the *Enterobacteriaceae* or *Firmicutes* groups, is likely a stochastic process driven by exposure to bacteria from the rearing environment (which may or may not contain *Campylobacter*) and from bacteria present in food and water. Commercial broiler chickens are typically reared in barns containing flocks of 20,000 birds or more. Chickens are coprophagic, and under commercial conditions, successful intestinal colonizing microorganisms can be dispersed rapidly throughout the flock and represent a significant source of microbiota to other flocks on the farm. *Campylobacter* is usually detected at around 3 weeks of age but rarely in younger birds. At this stage, *Campylobacter* is an efficient colonizer with the frequency of colonization increasing from 5 to 95% within 6 days [9]. This suggests that *Campylobacter* becomes "transmissible" at around 2 weeks. The question arises as to what happens with regard to *Campylobacter*, during the first 2 weeks of life, the so called lag period. It has been shown that chickens aged between 0 and 3 days of age can become infected and shed *Campylobacter* [10]. However, since the occurrence of a "lag period" is frequent, flock level evidence for early infection and shedding is limited [11]. It has been speculated that maternal antibodies provide protection from colonization by *Campylobacter* during the first 2 weeks of life but decline thereafter [12, 13]. The mechanism behind this resistance would be by prevention of proliferation of *Campylobacter* cells in the GI, rather than a specific bacteriocidal action. This might involve competition with, or inhibition by, the resident microbiota in conjunction with the immune system [14]. However, mathematical models of *Campylobacter* transmission support the contention that an age-dependent mechanism is responsible for the lag period rather than any change in susceptibility [15]. Understanding the temporal influence of *Campylobacter* colonization of broiler chickens will provide insight into impact on production parameters and has the potential to reveal strategies to reduce viable numbers on finished product and improve food safety.

Researchers have attempted to answer the question of whether *Campylobacter* is a commensal organism or a pathogen of chickens [16, 17]. The answer to this question appears to depend on the genetics of the host and varies with infecting *Campylobacter* strain [18, 19]. However, whether these factors influence broiler chickens in commercial production has been challenged [20]. The outcomes of *Campylobacter* colonization of broiler chickens appear context specific, but in practice, any combination of microorganisms that produce conditions that modify the GI microbiota and reduce performance should be considered deleterious but do not necessarily constitute a disease [21–23].

Recent research has reported changes in the chicken microbiota in response to *Campylobacter* colonization [4, 24] with evidence of modification of the β-diversity of the cecal microbiota [25]. The objective of this study was to compare the microbiota of chickens that were not exposed to *Campylobacter*, with those exposed either at a young age (6 days of age) or at the age at which birds often become positive in commercial production (20 days of age), with a view to gain a better understanding of how the timing of *Campylobacter* colonization affects the microbiome and the innate and adaptive immune response.

Methods

Trial design

The first trial (referred to as trial L; late infection) monitored the development of the chicken gut microbiota

and innate immune responses post-lag period colonization of broiler chickens by *Campylobacter jejuni* HPC5 [26, 27], at 20 days of age. Two groups of 35 birds were kept in pens until day 20 when trial L group 1 (TLG1) birds were administered with a placebo and trial L group 2 (TLG2) birds with *C. jejuni*, before being caged independently until the end of the study at day 35. Six birds from the TLG1 were euthanized for sampling at 22 days of age (da) and three at 28 and 35 da. Seven birds from the TLG2 group were euthanized for sampling at 22, 28, and 35 da. The second trial (referred to as trial E; early infection) monitored the development of the gut microbiota and innate immune responses of broiler chickens colonized early at 6 da by *C. jejuni*. Two groups of 35 birds were co-housed in pens until 6 da when trial E group 1 (TEG1) birds were administered with a placebo and trial E group 2 (TEG2) birds were administered with *C. jejuni*, before being caged independently until the end of the study at day 35. Seven birds from each group were euthanized for sampling at days 8, 15, 22, 28, and 35.

Experimental animals

Day-of-hatch male Ross 308 broiler chicks were purchased from a local hatchery and brooded in floor pens on wood shavings until the day of *Campylobacter* colonization when they were randomly assigned on the basis of weight to one of two groups and held in two separate rooms under similar environmental conditions with category two biosecurity. Welfare monitoring of the chickens was undertaken either twice every 24 h or three times post *Campylobacter* colonization. Chickens had access to feed and water ad-libitum throughout the study. Chickens were fed on a wheat-based diet provided as a starter crumb 0–10 days, grower pellets 11–24 days, and finisher pellets 23–35 days. The starter diet contained wheat 59.9% (*w*/*w*), soybean meal 32.5% (*w*/*w*), soybean oil 3.65% (*w*/*w*), limestone 0.60%(*w*/*w*), calcium phosphate 1.59% (*w*/*w*), sodium bicarbonate 0.27% (*w*/*w*) , salt 0.15% (*w*/*w*), lysine HCl 0.296% (*w*/*w*), DL-methionine 0.362% (*w*/*w*), threonine 0.134% (*w*/*w*), and the enzymes phytase and xylanase (dosed according to the instructions of the manufacturers DSM Nutritional Products Ltd. PO Box 2676 CH-4002 Basel, Switzerland). The grower and finisher diets increased the wheat content at the expense of soya meal by 2 and 5% respectively. The feed and paper liners on which the chicks were delivered were tested for *Salmonella* using standard enrichment procedures and found to be negative.

For TLG1, birds were administered a placebo of 1 ml of MRD (maximum recovery diluent; Oxoid, Basingstoke, UK) by oral gavage, and the TLG2 birds were administered 10^7 CFU *C. jejuni* HPC5, a well-characterized broiler chicken isolate, in 1 ml MRD [26, 27]. TEG1 birds were administered with a placebo of MRD by oral gavage (0.1 ml) at 6 da birds and TEG2 with 10^7 CFU *C. jejuni* strain HPC5 in 0.1 ml MRD. All feed consumed was recorded as were the body weights of the birds. Feed conversion ratios (FCR) were calculated as a ratio of feed consumed to the live weight of the birds.

Chickens were euthanized by either exposure to carbon dioxide gas or parenteral barbiturate overdose followed by cervical dislocation according to Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The birds were weighed before tissue and intestinal content samples were collected post-mortem. Ileal tissues were collected from approximately 3 cm distal to Meckel's diverticulum and cecal tissues collected from the distal tips of the ceca. Intestinal tissues were immediately frozen in liquid nitrogen for subsequent RNA isolation or preserved in 10% (*w*/*v*) neutral buffered formalin (Fisher Scientific; Loughborough, UK) for histological assessment. Intestinal contents were collected and samples used either to acquire bacterial count data or for total genomic DNA extraction.

Enumeration of bacteria from intestinal contents

Approximately 1 g of material was collected from both ceca and combined in pre-weighed universals before a 10% *w*/*v* suspension was prepared in MRD (Oxoid). *Campylobacter* were enumerated in triplicate from decimal dilutions prepared in MRD to 1×10^{-7} using a modification of the Miles and Misera technique. For each triplicate dilution set, five aliquots were dispensed onto CCDA agar (PO0119; Oxoid) prepared with the addition of agar to 2% (to prevent swarming) and with addition of CCDA Selective Supplement SR0155 (Oxoid). Plates were incubated at 42 °C in a microaerobic atmosphere (2% H_2, 5% CO_2, 5% O_2, 88% N_2) for 48 h (Don Whitley Scientific modified atmospheric cabinet, Shipley, UK). Coliforms were enumerated by application of aliquots of 100 μl from decimal dilutions of the cecal suspension to MacConkey No 3 agar (CM115; Oxoid) and incubation at 37 °C for 24 h. Lactic acid bacteria were enumerated by application of aliquots of 100 μl from decimal dilutions of the cecal suspension to MRS agar (CM0361; Oxoid) and incubation under anaerobic conditions at 30 °C for 48 h (Don Whitley Scientific anaerobic workstation). Between 30 and 300 colonies were counted on MacConkey No 3 and MRS agars, and the count per gram of cecal material was calculated by multiplying by the dilution factor.

Histology

Samples of ileum for histological assessment were examined from each bird from both trials. The fixed tissue samples were dehydrated through a series of alcohol solutions, cleared in xylene, and finally embedded in paraffin wax (Microtechnical Services Ltd., Exeter, UK).

Sections (3 to 5 μm thick) were prepared and stained with modified hematoxylin and eosin (H&E) using standard protocols. After staining, the slides were scanned by NanoZoomer Digital Pathology System (Hamamatsu, Welwyn Garden City, UK). Measurements of villus height and crypt depth were made using the NanoZoomer Digital Pathology Image Program (Hamamatsu) of 10 well-oriented villi scanned at × 40 magnification. The average of the 10 measurements was calculated per bird, from three or four birds per group, per time point. Villus height was measured from the tip of the villus to the crypt opening, and the associate crypt depth was measured from the base of the crypt to the level of the crypt opening. The ratio of villus height to relative crypt depth (V:C ratio) was calculated from these measurements. Heterophils were enumerated and any histopathological features recorded in a blind assessment of five random fields from each tissue section.

RNA isolation and RT-qPCR of the cytokines and chemokines

RNA was isolated from cecal and ileal tissue biopsies using NucleoSpin RNA isolation kit (Macherey-Nagel, GmbH & co. KG, Düran DE) according to the manufacturer's protocol with the following modifications. Tissue samples were homogenized in a lysis buffer with 2.8-mm ceramic beads (MO BIO Laboratories Inc., Carlsbad, USA) using TissueLyser II (Qiagen, Hilden, DE) prior to subsequent purification as described in the protocol. RNA was eluted in DEPC-treated water (Ambion ThermoFisher Scientific, UK) and stored at − 80 °C. RNA quality and concentration were assessed using Nanodrop ND-1000 Spectrophotometer (Labtech International Ltd., Uckfield, UK). The ratio 260/280 nm was in the range of 1.79 to 2.17 with the mean of 2. 12 ± 0.01 for all RNA samples used.

Reverse transcription was performed with 1 μg of RNA using SuperScript II (Invitrogen Life Technologies, Carlsbad, USA) and random hexamers. Quantitative PCR reaction was performed with cDNA template derived from 4 ng of total RNA in triplicate using SYBR Green Master mix (Applied Biosystems, ThermoFisher Scientific). Cytokines and chemokines fold change were calculated using the comparative cycle threshold (Ct) method established by the manufacturer [28]. The average of the triplicate Ct values was used for analysis, and the target genes Ct values were normalized to those of the housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Significance tests were calculated using ANOVA of the replicate the $2^{-\Delta Ct}$ values for each gene in the control and *Campylobacter*-colonized groups. The RNA levels of expression were determined by qPCR using the Roche Diagnostics LightCycler 480 (Hoffmann La Roche AG, CH). The primers used for qPCR of GAPDH, IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, CXCLi1, and CXCLi2 [29–32] are presented in Table 1.

DNA extraction and PCR amplification of 16S rRNA gene sequences and microbiota diversity analysis

Bacterial DNA was isolated from 0.25 g cecal content using the PowerSoil DNA Isolation Kit (MO Bio

Table 1 Primer sequence 5′-3′ for the gene expression determined by qPCR

Target gene	Primer sequence (5′-3′)	Product size (bp)	NCBI accession number	Reference
GAPDH	F: GACGTGCAGCAGGAACACTA R: TCTCCATGGTGGTGA AGACA	343	NM_204305.1	[29]
IFN-γ	F: TGAGCCAGATTGTTTCGATG R: CTTGGCCAGGTCCATGATA	152	NM_205149.1	[29]
IL-1β	F: GGATTCTGAGCACACCACAGT R: TCTGGTTGATGTCGAAGATGTC	272	NM_204524.1	[29]
IL-4	F: GGAGAGCATCCGGATAGTGA R: TGACGCATGTTGAGGAAGAG	186	NM_001007079.1	[29]
IL-10	F: GCTGCGCTTCTACACAGATG R: TCCCGTTCTCATCCATCTTC	203	NM_001004414.2	[29]
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	71	NM_204628.1	[30]
IL17-A	F: CATGGGATTACAGGATCGATGA R: GCGGCACTGGGCATCA	68	NM_204460.1	[31]
IL17-F	F: TGACCCTGCCTCTAGGATGATC R: GGGTCCTCATCGAGCCTGTA	78	XM_426223.5	[31]
ChCXCLi1	F: CCGATGCCAGTGCATAGAG R: CCTTGTCCAGAATTGCCTTG	191	NM_205018.1	[32]
ChCXCLi2	F: CCTGGTTTCAGCTGCTCTGT R: GCGTCAGCTTCACATCTTGA	128	NM_205498.1	[32]

Laboratories) according to the manufacturer's instructions. Using the isolated DNA as a template, the V4 region of the bacterial 16S rRNA gene was PCR amplified using primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGACTACHVGGGTWTCTAAT 3′) [33]. Amplicons were then sequenced on the Illumina MiSeq platform using 2 × 250 bp cycles. These sequence data are deposited in the NCBI database within the Bioproject PRJNA380214 under the SRA study SRP133552.

Prior to metagenomic analysis, sequence reads with a quality score mean below 30 were removed using Prinseq [34]. The 16S rRNA sequence analysis was performed using Mothur v. 1.39 [35]. Analysis was performed as according to the MiSeq SOP (accessed online 28/06/2017; [36]). The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database [37] for use in Mothur (available at: https://www.mothur.org/wiki/Silva_reference_files), and clustered into operational taxonomic units (OTUs) using the opticlust clustering algorithm [38]. The nearest 16S rRNA gene sequence identities to the OTUs are reported on the basis of BLASTn searches if data matches are from type cultures with a BLAST identity ≥ 99%. If not, the consensus taxonomy of the OTUs is reported as generated using the classify.otu command in Mothur with reference data from the Ribosomal Database Project (version 14) [39, 40] adapted for use in Mothur (available at: https://www.mothur.org/wiki/RDP_reference_files).

Data and statistical analysis

For the microbiota beta diversity analysis, Bray-Curtis distances were tested for significance using analysis of molecular variance (AMOVA) implemented within Mothur [38]. For alpha diversity, inverse Simpsons indices and species abundance were tested using Kruskal-Wallis test followed by Dunn's multiple comparison test with Benjamini-Hochberg p value correction within R [41, 42] using Dunn.test 1.3.4 package [43]. The Shapiro-Wilk normality test for data distribution analysis was used from within GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, USA, http://www.graphpad.com). Data processing and ordination were performed using R project. Statistical differences between *Campylobacter* and non-*Campylobacter*-colonized groups with respect to the zootechnical parameters were determined using repeated measures ANOVA implemented in Genstat release 19.1 (VSN International, UK). *Campylobacter* viable counts exhibiting a normal distribution, heterophil counts, and the villus and crypt measurements were made using single-factor ANOVA with < 0.05 used as the level significance. For microbiota data sets, non-parametric Mann-Whitney tests were performed. Linear discriminant analysis effect size (LEfSe) was used to identify differentially abundant OTUs (available at https://bitbucket.org/nsegata/lefse/overview) using a minimum cutoff of 0.05% [44]. Analysis of similarity (ANOSIM) with the Benjamini-Hochberg correction for multiple comparisons with analysis of similarity percentages (SIMPER) [45] was used to determine the contribution of each taxonomic unit to the Bray-Curtis dissimilarity of pairs of distinct sample groups using the vegan package [46] in R using a script by Andrew Steinberger (https://github.com/asteinberger9/seq_scripts) as previously reported for the interrogation of 16S rDNA OTUs [47].

Results

Growth rate and feed conversion ratio (FCR) of birds infected with *C. jejuni* HPC5

Each bird was weighed regularly throughout the experimental period to compare the growth of birds infected with *C. jejuni* HPC5 and uninfected control birds. There were significant differences between the weights of the control and experimental birds infected at 6 da (TEG; $p < 0.01$). Notably, these differences were evident at 2 and 9 days post-infection (dpi), when the control birds in TEG1 were significantly heavier ($p < 0.01$) than infected birds (TEG2). The reduced weights of the TEG2 birds at 2 dpi coincided with the observation of temporary diarrhea that resolved within 72 h. However, by the end of the rearing cycle (35 da), there was no significant difference ($p > 0.05$) in the weights of the birds infected with *Campylobacter* compared to uninfected controls (Additional file 1). In contrast, the weights of birds in TLG1 were not significantly different to those in TLG2. The cumulative FCR up to 35 days for TLG1 ($n = 8$) and TLG2 ($n = 7$) were 1.52 and 1.56 respectively while the FCR for TEG1 ($n = 10$) and TEG2 ($n = 7$) were 1.48 and 1.45 respectively. Breed performance targets for commercial broiler chickens suggest an FCR of 1.54 at 35 da.

Campylobacter jejuni colonization

All birds were culture negative for *Campylobacter* spp. until experimental infection with *C. jejuni* and control birds remained culture negative for *Campylobacter* spp. throughout the study. *Campylobacter* viable counts of the cecal contents recovered at the end of the rearing cycle were high independent of age at infection (mean *Campylobacter* density = 6.1 $\log_{10}$ CFU g^{-1}; Fig. 1a, b). The dynamics of colonization were however affected by the age at which birds were infected with *Campylobacter*. Birds from TLG2, infected at 20 da all exhibited cecal colonization with *C. jejuni* (mean 5.1 $\log_{10}$ CFU g^{-1}) at 2 dpi, with all the birds sampled at each time point thereafter ($n = 7$) showing colonization until the end of the rearing cycle at 35 da (15 dpi; Fig. 1a). Only two of seven birds sampled from TEG2 at 2 dpi had levels of

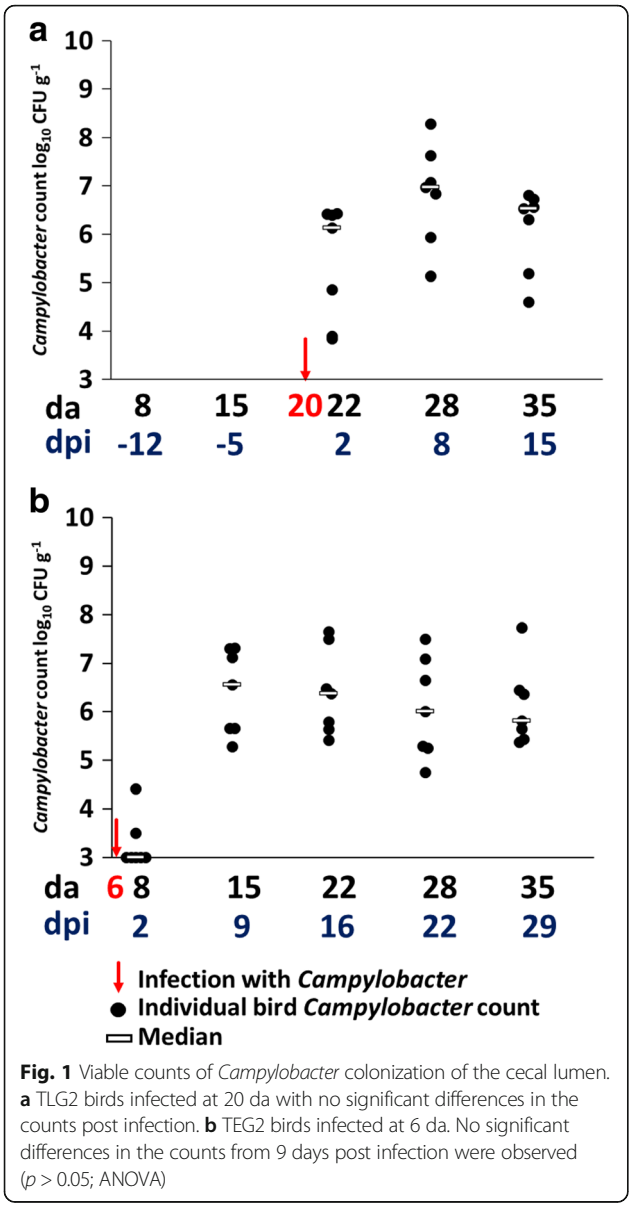


Fig. 1 Viable counts of *Campylobacter* colonization of the cecal lumen. **a** TLG2 birds infected at 20 da with no significant differences in the counts post infection. **b** TEG2 birds infected at 6 da. No significant differences in the counts from 9 days post infection were observed ($p > 0.05$; ANOVA)

Campylobacter in their ceca above the limit of detection, but by the next sample point at 9 dpi, all birds showed colonization to levels that remained similar after this stage ($p > 0.05$; Fig. 1b).

Colonization with C. *jejuni* affects intestinal villus and crypt metrics

Heterophil infiltration counts were determined in a blind assessment of formalin-fixed H&E-stained ileum sections (Additional file 2 contains typical examples) to reveal significant differences using ANOVA at 2 ($p = 0.02$) and 9 dpi ($p = 0.01$) for birds infected with *C. jejuni* at 6 da (TEG2) compared to uninfected birds but were not significant thereafter ($p > 0.05$). Heterophil infiltration at 2 and 9 dpi was accompanied by mild multi-focal villous blunting, with evidence of mild edema and villous fusion. For the birds infected at 20 da, significant increases in the heterophil counts were observed in the ileum sections of the infected birds (TLG2) at 2 ($p = 0.04$) and 8 dpi ($p = 0.01$). However, villus crypt ratios obtained from measurements taken from H&E-stained sections of the ileum, comparing uninfected TLG1 to infected TLG2 from 3 to 4 birds from each group, at each sample time point, revealed no significant difference ($p > 0.05$) between the two groups at any age. The same comparison, made with H&E-stained sections of the ileum from uninfected TEG1 and infected TEG2, showed no significant difference between uninfected and infected birds. However, when comparing the villus height and the crypt depth measurements separately, significant differences using ANOVA were noted between the infected and uninfected birds (Fig. 2). Villus length and crypt depth were reduced immediately after infection but both measurements were increased at the end of the rearing period. TLG2 birds show a significant reduction ($p = 0.0005$) in crypt depth, 2 dpi, combined with an observable, but not statistically significant ($p = 0.13$), reduction in the villus height compared to uninfected TLG1 birds. The measurements of the villi and crypts of birds in

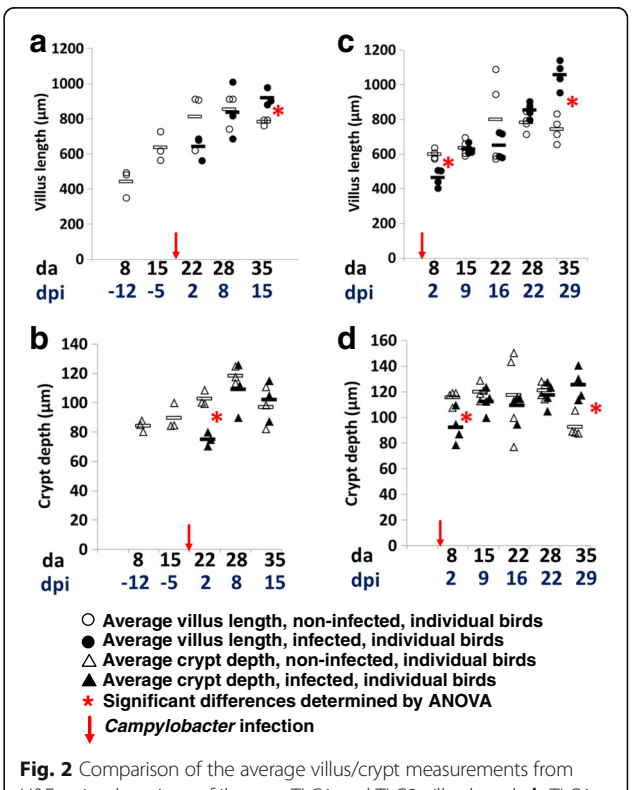


Fig. 2 Comparison of the average villus/crypt measurements from H&E-stained sections of ileum. **a** TLG1 and TLG2 villus length. **b** TLG1 and TLG2 crypt depth. **c** TEG1 and TEG2 villus length. **d** TEG1 and TEG2 crypt depth length. Measurements were taken from 10 villi or 10 crypts per bird. Three or four birds were sampled from each group for each time point

TLG1 and TLG2 were similar ($p > 0.05$) at 8 dpi, but at the final sampling point (15 dpi), the villus height from the *Campylobacter*-infected TLG2 birds was increased compared to the uninfected TLG1 birds at the same age ($p = 0.0004$) although the crypt depths were not significantly different ($p = 0.7$). The birds in TEG2 showed a similar pattern. Immediately following infection (2 dpi), the villi were significantly reduced in height ($p = 0.003$) and the crypts reduced in depth ($p = 0.02$) compared to the control birds (TEG1). However, by the next sample point (9 dpi), there was no significant difference in villus height or in the crypt depth for birds in TEG1 compared to TEG2 ($p > 0.05$). No significant differences were observed thereafter until the final sample point (29 dpi), where the villi were significantly longer ($p = 0.004$) and the crypts significantly deeper ($p = 0.008$) in the infected TEG2 birds compared to the uninfected TEG1 birds.

Effect of *C. jejuni* colonization on cytokine and chemokine gene expression

The inflammatory effect of *C. jejuni* colonization was assessed by quantification of the relative expression of cytokines and chemokine gene transcripts in ileal and cecal tissue biopsies (Figs. 3 and 4) representing major inflammatory pathways in chickens [30]. The cytokines IL-17F, IL-17A, IL-6, and IL-1β and chemokines CXCLi1 and CXCLi2, also known as ChIL-8, have previously been described as markers of the Th17 pathway. Whereas IFN-γ is related to the Th1 pathway, IL-4 is connected to the Th2 pathway and IL-10 is produced by regulatory T cells (Treg) to control the inflammatory effects of the Th cell responses. There was no significant change in the cytokine and chemokine expression in ileum tissues (Fig. 3) at 2 dpi following infection at 6 da in TEG2 birds. However, at 9 dpi, most cytokines showed a significant ($p < 0.05$) increase in expression compared to controls corresponding to the increasing levels of colonization observed in Fig. 1b. Notably, increases in IFN-γ, IL-4, and IL-17A provided evidence for activation of Th1, Th2, and Th17 pathways but these were also accompanied by an increase in IL-10. Levels of expression remained higher than controls for the majority of the *Campylobacter*-colonized birds until 29 dpi when they were reduced to similar or lower levels than control birds.

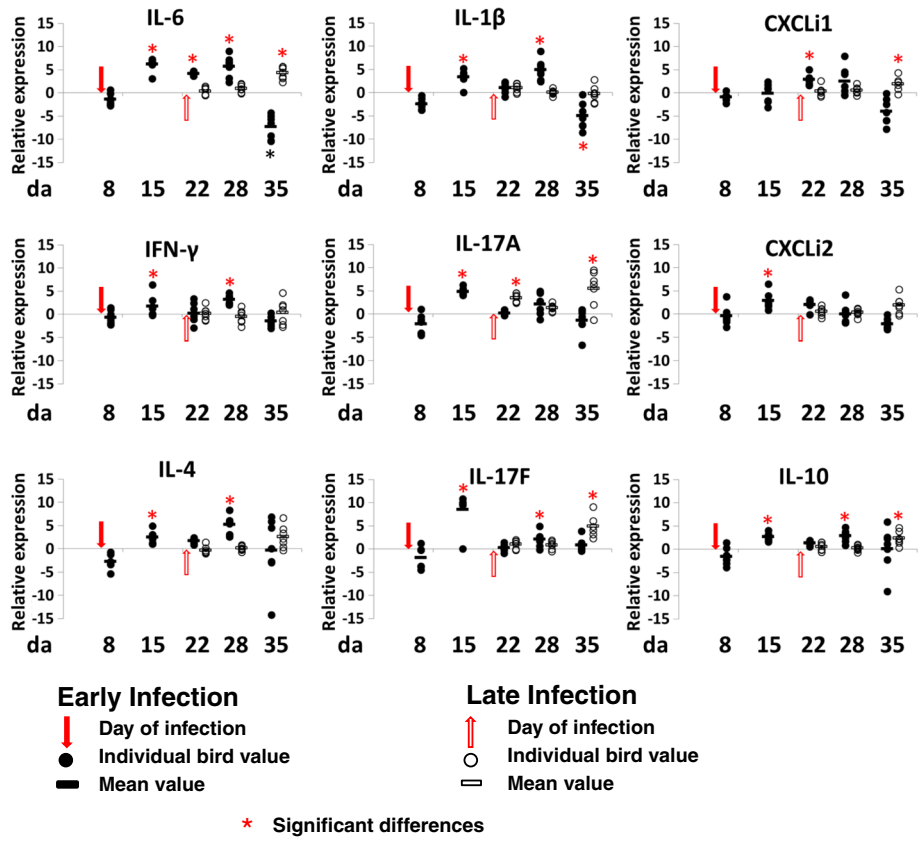


Fig. 3 Relative change in expression of cytokines and chemokines in ileum tissues. Relative gene expression represents $\log_2$ ratio infected/non-infected from qPCR of infected birds (TLG2 and TEG2) compared to expression in tissues from non-infected birds (TLG1 and TEG1). Significant differences between $2^{-\Delta Ct}$ values of the control and *Campylobacter*-colonized groups are indicated by an asterisk (ANOVA $p < 0.05$, *) for the expression of each gene at the corresponding time points

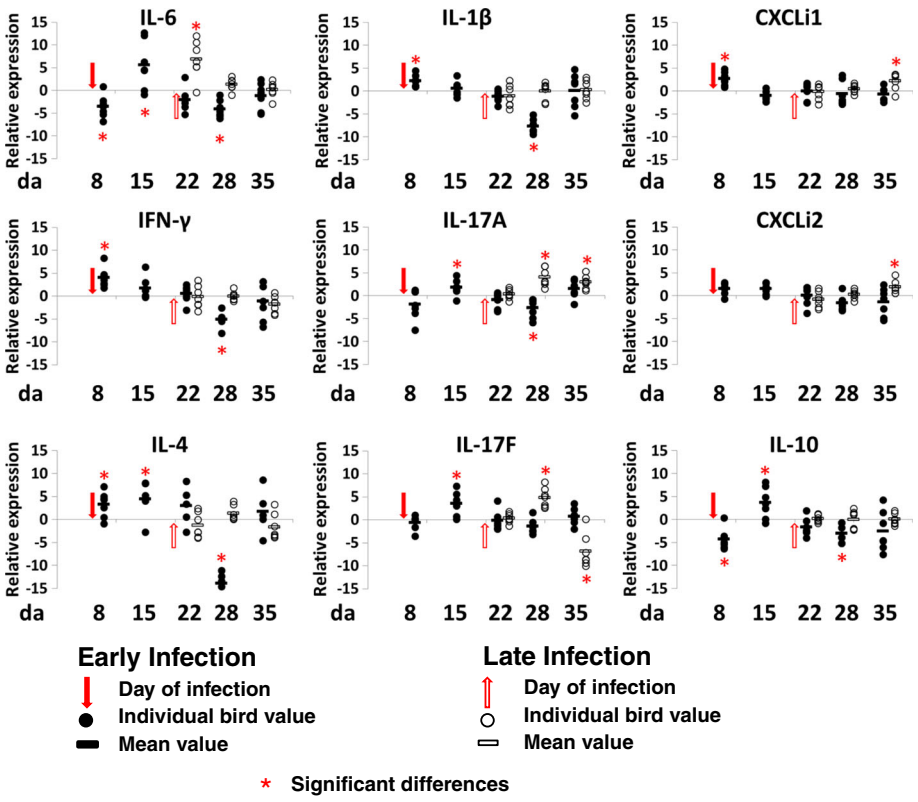


Fig. 4 Relative change in expression of cytokines and chemokines in cecal tissues. Relative gene expression represents $\log_2$ ratio infected/non-infected from qPCR of infected birds (TLG2 and TEG2) compared to expression in tissues from non-infected birds (TLG1 and TEG1). Significant differences between $2^{-\Delta Ct}$ values of the control and *Campylobacter* colonized groups are indicated by an asterisk (ANOVA $p < 0.05$, *) for the expression of each gene at the corresponding time points

Changes in cytokine expression in response to infection by *Campylobacter* at 20 da in TLG2 birds was characterized in the ileum tissues (Fig. 3) by a significant ($p < 0.05$) increase in most of the cytokine expression at 15 dpi compared to uninfected TLG1 birds, with the exception of IFN-γ and IL-1β. Prior to that time point, the level of cytokine expression was not significantly different to the non-infected birds (TLG1) at 2 dpi and 8 dpi despite a high level of *Campylobacter* colonization detected as early as 2 dpi, although the cytokine IL-17A showed a significant increase in expression from 2 dpi onwards in the TLG2 birds. Interestingly, most of the immune response markers were upregulated at an earlier stage during the infection in TEG2 birds (at 9 dpi) rather than in TLG2 birds (at 15 dpi) despite the high level of *Campylobacter* detected at 2 dpi in the TLG2 birds.

Changes in cytokine and chemokine expression in cecal tissues in response to colonization by *Campylobacter* (Fig. 4) at 6 da were characterized by significant increases in IFN-γ, IL-1β, IL-4, and CXCLi1 and a decrease in IL-6 and IL-10 at 2 dpi in TEG2 birds. A week later at 9 dpi, the expression of IL-6 was increased along with IL-17A, IL-17F, IL-10, and IL-4. By 16 dpi, their level of expression was not significantly different to the uninfected TEG1 birds, and at 22 dpi, the majority of the cytokines showed a significant ($p < 0.05$) reduction in expression compared to control birds TEG1, with the exception of IL-17F and CXCLi1. Finally, at the last time point 29 dpi, the cytokine and chemokine levels had recovered to levels not significantly different to the non-infected control (TEG1). Cecal tissues of birds infected at 20 da did not show the concerted Th1 and Th2 immune responses relative to the non-infected control birds at 2 dpi that the birds colonized at 6 da experienced. However, IL-6 showed a significant increase of 35-fold, followed by increases in the levels of IL-17A and IL-17F at 8 dpi and ultimately increased CXCLi1, CXCLi2, and IL-17A at 15 dpi. Following infection with *Campylobacter*, the immune response in the cecal tissues appears to be more focused on the Th17 pathway featuring IL-6 induction with IL-17A and IL-17F responses, as compared to that observed in the ileum tissues.

Effect of *C. jejuni* colonization on the microbiota of the cecal lumen

DNA sequencing of the V4 regions of 16S rRNA genes was used to estimate the diversity and abundance of the cecal luminal microbiota of birds from the TEG and TLG experiments. A total of 6,947,272 quality-controlled sequence reads from 107 samples were resolved in to 7646 OTUs (distance 0.03) that fall into 23 phyla. As described previously for chicken cecal microbiota, *Firmicutes* dominate with a mean abundance of 87.57% (83.89–93.91%) over all samples from 8 days of age onwards and followed by *Proteobacteria* at 6.43% (3.47–8.77%) [8, 48]. The relative abundances of these phyla for all samples are presented in Additional file 3. The sequence reads were subsampled at 16,319 reads per sample for subsequent analysis.

Figure 5a, b shows estimates of the diversity of the microbiota, presented as plots of the inverse Simpsons measure of α-diversity. The α-diversity of the cecal microbiotas from birds of TEG or TLG was not affected by *C. jejuni* colonization ($p > 0.05$). However, an age-linked increase in alpha diversity was evident for the non-colonized TEG birds between days 8 and 28 ($p = 0.0005$). Figure 5c, d shows that richness of the cecal microbial communities are generally not affected by *C. jejuni* colonization ($p > 0.05$) with the exception of a significant increase in the observed OTUs at day 28 for the *C. jejuni*-colonized TEG birds.

Bray-Curtis indices of dissimilarity demonstrate differences in species composition between communities on the basis of age and *C. jejuni* infection status. The cecal microbiota of birds infected with *Campylobacter* at 6 da (TEG2) was significantly different to age-matched controls at 2, 16, and 22 dpi ($p < 0.05$, AMOVA). Principal component analysis of these data demonstrates clustering of the data with respect to age (Additional file 4). The exception to this is the data at 15 da (9 dpi), which

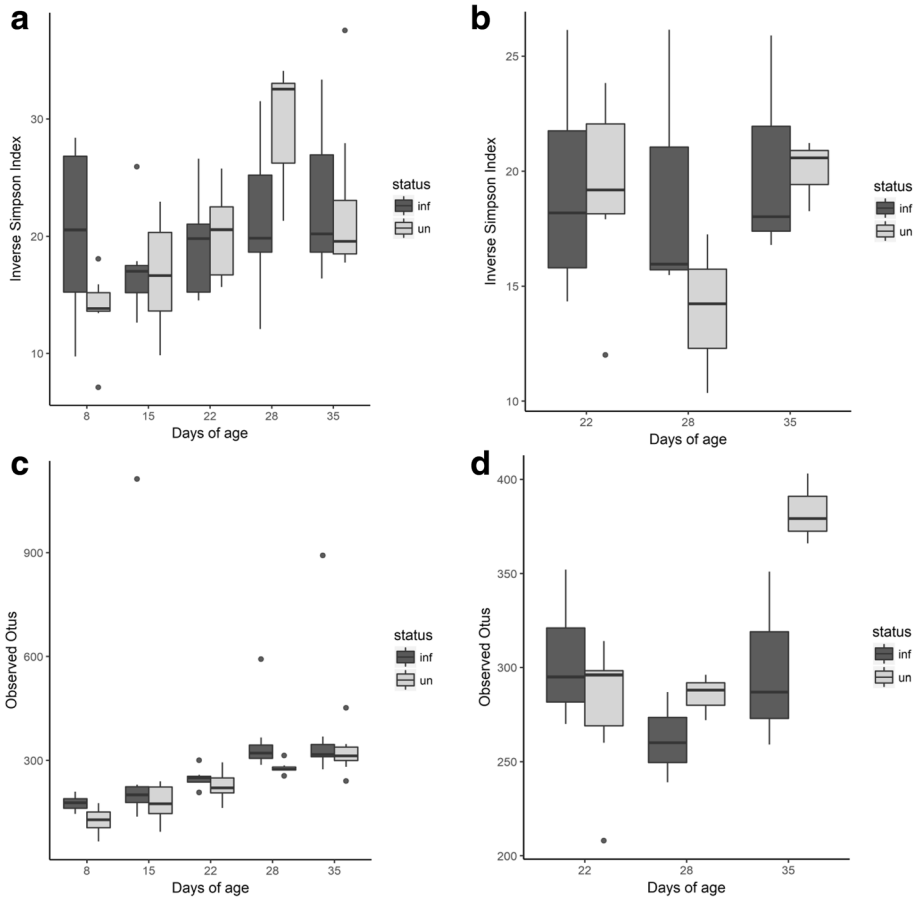


Fig. 5 Estimates of α-diversity and richness for TEG and TLG microbial communities from cecal contents. Inverse Simpsons indices (**a**, **b**) and species richness estimates (**c**, **d**) from 16,319 subsampled sequences for the non-colonized control (gray bars) and *C. jejuni*-colonized (dark gray bars) chickens. The charts are presented with respect to the age of the birds colonized at either 6 days (TEG2) or 20 days (TLG2). **a** Inverse Simpsons index of TEG1 and TEG2. **b** Inverse Simpsons index of TLG1 and TLG2. **c** OTU counts of TEG1 and TEG2. **d** OTU counts from TLG1 and TLG2. Data are presented as a box and whisker plot (in the style of Tukey). The solid black line indicates the median, and the top and bottom of the shaded boxes indicate the 25th and 75th percentiles. Whiskers indicate maximum and minimum values, unless these values exceed 1.5-fold the interquartile range. Outlying data are plotted as individual markers

exhibit similarities with either the pre- or proceeding data. The transition in the microbiota at 15 da is also marked in the microbial counts obtained for coliforms and lactic acid bacteria by a shift in the dominance of the coliform count to that of lactic acid bacteria after the 15 da time point independent of the *C. jejuni* colonization status (Additional file 5). Bray-Curtis indices indicate the microbiota of birds exposed to *Campylobacter* at 20 da (TLG2) was significantly different from uninfected birds immediately post-infection (2 dpi; $p < 0.001$, AMOVA), but could not be distinguished from controls at subsequent stages of the rearing cycle ($p > 0.05$, AMOVA).

Linear discriminant analysis effect size (LEfSe) was applied to identify differentially abundant OTUs between *Campylobacter*-infected and non-infected birds. Figure 6a shows the significant differentially abundant OTUs for the entire TEG microbiota that include the colonizing *C. jejuni* HPC5 (OTU0062) at all taxonomic levels as indicated in Fig. 6b. Only those microorganisms that are noted as type cultures and had BLASTn identities ≥ 99% are reported to species level; otherwise, the consensus taxonomies with the corresponding OTU numbers are reported. Differential abundance of members the dominant *Firmicutes* phylum was evident in response to *C. jejuni* colonization. *C. jejuni*-colonized birds exhibited increased abundance of *Lachnospiraceae* ssp. OTU0005 and OTU0022, *Blautia* ssp. OTU0023, *Ruminococcaceae* OTU0039 and OTU0071 in addition to several unclassified members of the *Clostridiales* class. In the non-colonized birds, LEfSe highlights the greater differential abundance of *Lactobacillus* OTU0008, *Anaerostipes butyraticus* OTU0009, *Clostridium* XIVa OTU0011, *Lachnospiraceae* spp. OTU0035 and OTU0027, *Clostridium* IV OTU0083, and *Enterococcus* OTU0118. The differential abundances identified by LEfSe for age-matched colonized and non-colonized birds are presented in Additional file 6. At 8 da (2 dpi), the corresponding *C. jejuni* OTU was not significantly more abundant using the 0.05% cutoff adopted for all samples, although it should be noted that viable *C. jejuni* were only detected by culture in the ceca of 2 of 7 birds from the TEG2 group at this early time point. As an alternative approach, the OTUs contributing to differences in the Bray-Curtis dissimilarity indices were identified by analysis of similarity percentages (SIMPER). Figure 6c shows box-whisker plots of the relative OTU abundances between *C. jejuni*-colonized and non-colonized birds for five OTUs identified using SIMPER (p adj < 0.05). All five OTUs coincide with those identified as differentially abundant by LEfSe.

LEfSe analysis of the TLG differentially abundant OTUs between *C. jejuni*-colonized and non-colonized birds are presented in Fig. 7a with the corresponding phylogenetic relationships in Fig. 7b. Notably, three of the OTUs identified with increased abundance in the *C. jejuni*-colonized TLG birds coincided with those from the TEG comparison: *Lachnospiraceae* ssp. OTU0022, *Blautia* ssp. OTU0023, and unclassified *Clostridiales* OTU0089. In the non-colonized birds, LEfSe identified greater differential abundance of *Eggerthella* OTU0028, *Clostridium* XIVa OTU0041, unclassified *Clostridiales* OTU0050, *Ruminococcaceae* OTU0070 and OTU0081, and *Lachnospiraceae* spp. OTU0162. Figure 7c shows box-whisker plots of the relative OTU abundances between *C. jejuni*-colonized and non-colonized birds for three OTUs identified using SIMPER (p adj < 0.05). The increased abundances corresponding to *Eggerthella* OTU0028 in the colonized birds and *Clostridium* IV OTU0056 in the non-colonized birds also feature in those identified as those responsible for the differential abundance by LEfSe for TLG. The taxon *Clostridium* XIVa (OTU0011 and OTU0041) shows differential increases in abundance in the non-colonized birds that contributes to the dissimilarity between the *C. jejuni*-colonized and non-colonized groups for TEG and TLG.

Discussion

Recent reports have linked *Campylobacter* colonization of broiler chickens with reduced economic performance in terms of an increase in cumulative FCR. Evidence for this comes from correlating poor economically performing farms with high *Campylobacter* prevalence [49] and from smaller scale experimental trials [50]. There were distinct differences in zootechnical performance between the two independent trials reported here despite similar diets and controlled housing, but these were independent of *Campylobacter* colonization. The TLG trial showed FCRs of 1.52 and 1.56 respectively for TLG1 and TLG2, whereas the TEG trial had FCRs of 1.48 and 1.45 respectively for TEG1 and TEG2. The between trial differences could not be explained by either an increase in the α-diversity or richness of the cecal microbiota.

Early infection of the birds in TEG2 resulted in significantly reduced live weights compared to control birds at 2 and 9 dpi, but this appeared to be a temporary setback that the birds recovered from, as there were no significant differences between infected and non-infected bird weights thereafter. There were no significant difference ($p > 0.05$) between non-infected TLG1 and infected TLG2 bird weights. Within trial performance differences between the infected and non-infected birds within the current study were marginal considering the limited number of birds but appear to be associated with differences in feed intake post *C. jejuni* colonization. Chickens were housed under favorable conditions in this study, so how these observations may play out in commercial settings with greater stocking densities and environmental

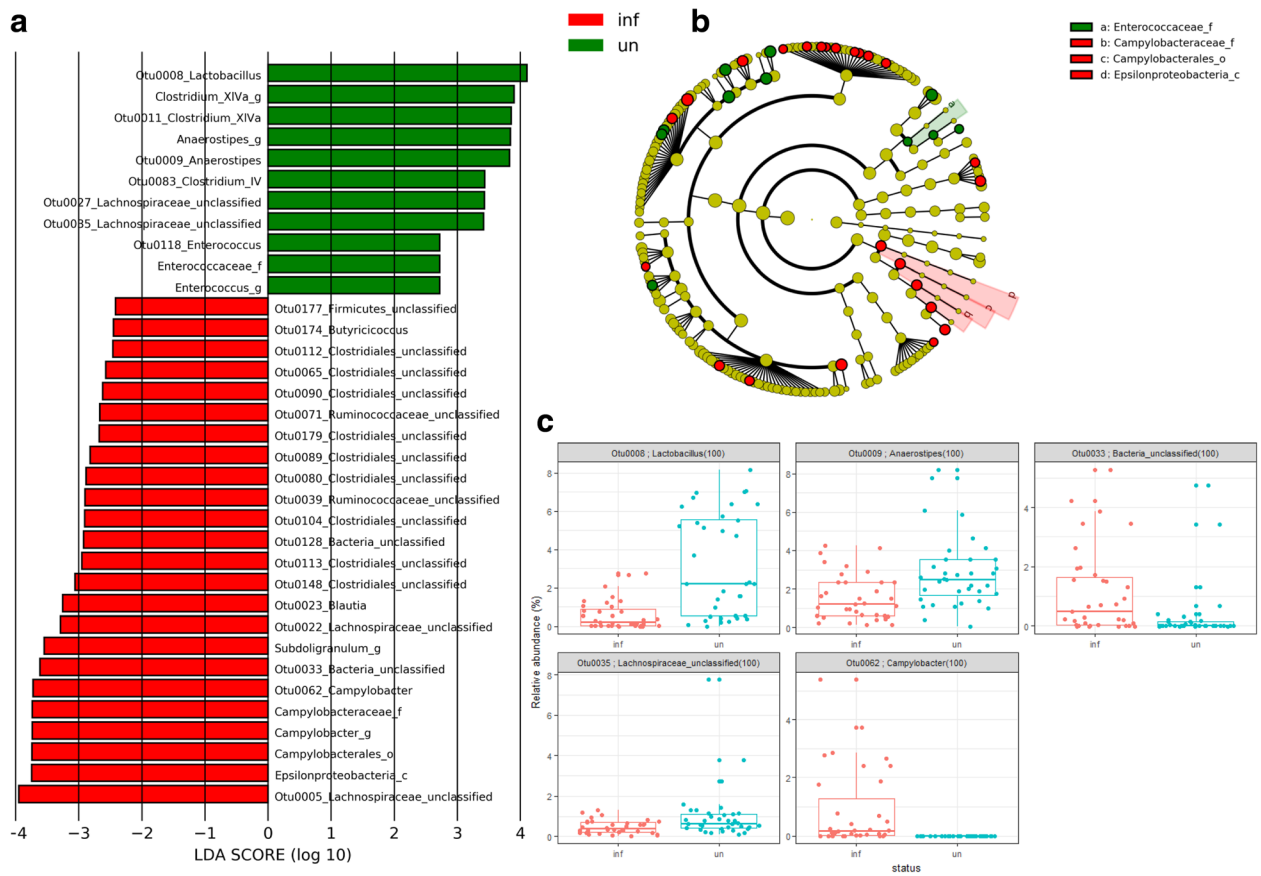


Fig. 6 Differential abundance of members of the cecal microbial communities of TEG *C. jejuni*-colonized and non-colonized broiler chickens. **a** Histogram of the LDA scores computed for features differentially abundant between *C. jejuni*-colonized broiler chickens from 6 da (denoted as "inf" by red bars) and non-colonized birds (denoted as "un" by green bars). LEfSe identifies which clades amongst those detected as statistically differential will explain the greatest differences between the communities [42]. **b** A taxonomic representation of the clades responsible for the greatest differences based on the Ribosomal Database Project [39], where red circles represent those of greater abundance in the *C. jejuni*-colonized birds, green circles for those of non-colonized birds, and yellow for non-significant differences. The diameters of the circles are proportional to the taxon's abundance. The representation highlights the presence of the differentially abundant taxanomic levels containing *Campylobacter* (_f family, _o order and _c class) as concentric arcs labeled a to c. **c** Plots of the relative abundance differences between *C. jejuni*-colonized (denoted as inf in red) and non-colonized chickens (denoted as un in blue) for TEG when calculated using ANOSIM from Bray-Curtis indices and identified by SIMPER. Each data point represents the relative OTU abundance in a single bird. The horizontal line indicates the median, and the top and bottom of the boxes indicate the 25th and 75th percentiles. Whiskers indicate maximum and minimum values with the exception of those exceeding 1.5-fold the interquartile range

challenge requires consideration. Studies of natural infection reported by Gormley et al. found no correlation between bird body weights and cecal loads at slaughter age [20]. *C. jejuni* have been reported to exhibit strain-dependent differences in the outcomes of infection [51], which could contribute to differences in *Campylobacter*-positive flock performances. Exposure to multiple *Campylobacter* strains that result in succession of the fittest is indicative of multi-factorial challenges in barn-reared birds [52], which are likely to influence flock performance and associated negative welfare indicators.

Campylobacter jejuni colonization to high levels occurred more rapidly in birds infected at the end of the lag phase (20 da; TLG2) than in birds infected at 6 da (TEG2), which exhibited low or undetectable levels of cecal colonization at 2 dpi but reached full colonization at 9 dpi. The reduced weight gain and changes in villus/crypt measurements observed were more evident in the TEG2 birds at 2 dpi than at later sample points when levels of colonization were higher, suggesting that the level of *Campylobacter* colonization was not necessarily linked to these responses. The fact that similar responses were present in all the birds despite the majority being culture negative for *C. jejuni* suggests that following administration of the bacteria, the organism is able to persist, affect shifts in the microbial community, and affect

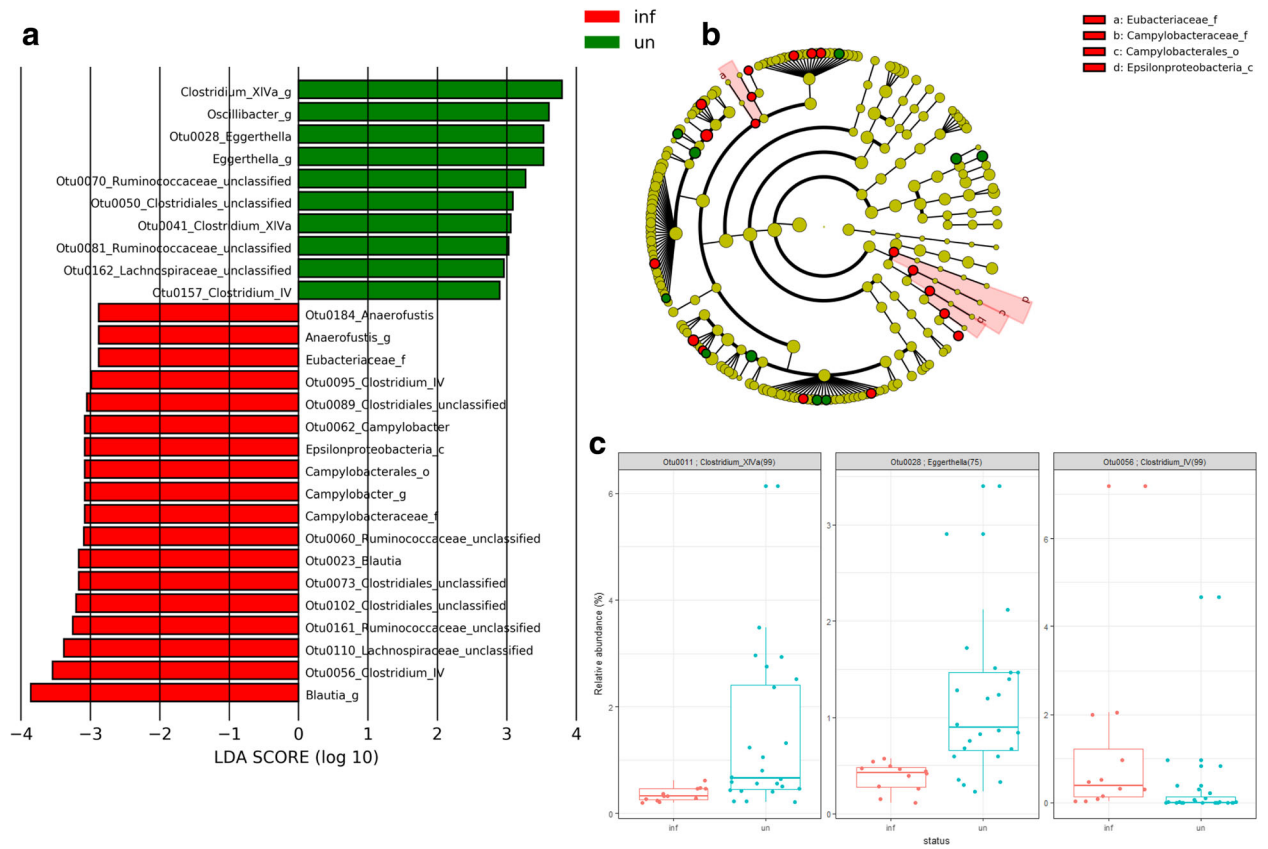


Fig. 7 Differential abundance of members of the cecal microbial communities of TLG *C. jejuni*-colonized and non-colonized broiler chickens. **a** Histogram of the LDA scores computed for features differentially abundant between *C. jejuni*-colonized broiler chickens from 20 da (denoted as "inf" by red bars) and non-colonized birds (denoted as "un" by green bars). LEfSe identifies which clades amongst those detected as statistically differential will explain the greatest differences between the communities [42]. **b** A taxonomic representation of the clades responsible for the greatest differences based on the Ribosomal Database Project [39], where red circles represent those of greater abundance in the *C. jejuni*-colonized birds, green circles for those of non-colonized birds, and yellow for non-significant differences. The diameters of the circles are proportional to the taxon's abundance. The representation highlights the presence of the differentially abundant taxanomic levels containing *Campylobacter* (_f family, _o order and _c class) as concentric arcs labeled a to c. **c** Plots of the relative abundance differences between *C. jejuni*-colonized (denoted as inf in red) and non-colonized chickens (denoted as un in blue) for TEG when calculated using ANOSIM from Bray-Curtis indices and identified by SIMPER. Each data point represents the relative OTU abundance in a single bird. The horizontal line indicates the median, and the top and bottom of the boxes indicate the 25th and 75th percentiles. Whiskers indicate maximum and minimum values with the exception of those exceeding 1.5-fold the interquartile range

physiological change, but not necessarily multiply to the extent that it can be detected by culture from cecal content. Clearly, birds at 6 da exhibit colonization resistance, which may in part be due to the presence of maternal antibodies [12, 13] that act to prevent immediate high-level colonization but are absent by 20 da. Regarding the lag phase observed in commercial production whereby flocks remain *Campylobacter* negative until the birds are 2 weeks of age, the current study indicates that chickens can become infected at any time during the rearing period but the colonizing campylobacters only multiply to the extent of being detectable and efficiently transmittable when the birds are over 2 weeks old, which lends support of the proposed mechanism of age-dependent transmission [15].

A healthy well-differentiated intestinal mucosa consists of long regular villi with high villus/crypt ratios [53]. Awad et al. [50] reported that Ross 308 birds infected with *Campylobacter* at 14 days of age (approximately half way between the two infection points described here) were found to have decreased villus height, crypt depth, and villus surface area by 21 days of age and were accompanied by changes in ion transport and barrier function compared to controls. Birds from TEG2 similarly showed a reduction in villus height and crypt depth compared to TEG1, immediately following infection (2 dpi) but by 9 dpi there was no significant difference, and by 29 dpi, the *Campylobacter* infected TEG2 birds actually had longer villi and deeper crypts than TEG1. This pattern would indicate that infection with *Campylobacter*

can result in rapid changes in villus length, which can be correlated with temporary reduced weight gain and diarrhea, perhaps due to reduced nutrient absorption. However, this was followed by a fairly rapid recovery, within 9 days and in the long term, increased villus length compared to non-infected controls. Later infection with *Campylobacter* had a significant, but less drastic effect on villus heights immediately following infection of TLG2 compared to TLG1 uninfected birds. This was followed by a rapid recovery and by the end of rearing period exhibited increased villus height and depth compared to uninfected controls, similar to the observations made for TEG2.

Infection of the gastrointestinal tract by pathogens is detected by the host immune system which then responds via a complex interconnecting system of pathways involving the innate and adaptive immune systems. Cytokines play an important part in intracellular and extracellular immunity against pathogens and also in regulating the response appropriately. In chickens, the effector T cell pathway Th17 includes IL-17A and IL-17F and is thought to be important in limiting both invasion and colonization of bacterial pathogens in the gastrointestinal tract that include *Campylobacter* [31]. Cytokine expression in response to infection by *C. jejuni* in chickens challenged at 20 da, in TLG2, confirmed the upregulation of IL-6, IL-17A, and IL-17F ($p < 0.01$) reported by Reid et al. [31], although prolonged diarrhea was not observed as reported for faster growing broiler chicken breeds [19]. All TLG2 birds showed cecal colonization with *C. jejuni* at 2 dpi (mean 5.1 $\log_{10}$ CFU g^{-1}) that was accompanied by an increase in IL-6 expression. For birds infected at 6 da, the kinetics of the response was different with no increase in IL-6 expression and largely undetectable levels of cecal *C. jejuni* colonization at 2 dpi. Instead a relative increase in IFN-γ and IL-4 were observed ($p < 0.05$), characteristic of Th1 and Th2 pathways. However, by 9 dpi, colonization of all birds was evident (mean 6.1 $\log_{10}$ CFU g^{-1}), which coincided with increased expression of IL-6, IL-17A, and IL-17F ($p < 0.01$). At 9 dpi, IL-10 expression was also notably upregulated in ileal and cecal tissues ($p < 0.05$), which may account for the subsequent suppression of the pro-inflammatory cytokines, and in particular the declines in IL-6, IL-17A, and IL-17F. Cytokine IL-10 is produced by regulatory T (Treg) cells to control Th cell pro-inflammatory responses and prevent damage to affected tissues. The differential expression of IL-10 in broiler chicken breeds has been reported to be critical to the outcome of *C. jejuni* infection in terms of inflammation and diarrhea [19]. In this context, birds infected at 20 da did not show a significant increase in IL-10 in the ceca but a response was evident in the ileum by 35 da. These tissues exhibited increased levels of IL-17A until the end of the rearing period at 35 da. *C. jejuni* generally colonize the ceca of chickens to far greater cell densities; it is therefore of interest that the chickens did not upregulate IL-10 in their ceca within the 35 da rearing period that is typical of commercial flocks. A differential effect on the persistence of the pro-inflammatory response to *Campylobacter* colonization of a popular broiler chicken breed depending on the age of the bird is of significance to the poultry industry. Late colonized birds will be subject to an on-going pro-inflammatory response, the outcome of which will likely depend on the resident intestinal microbiota.

AMOVA of Bray-Curtis indices indicate significant differences between the cecal microbiota compositions of control birds and the TEG2 group colonized with *C. jejuni* at 2, 16, and 22 dpi ($p < 0.05$). Inspection of the PCoA plots shows partition of the control bird indices at 15 and 22 da as the microbiota undergoes a transition from a juvenile to a more mature composition (Additional file 4). The timing of the shift in microbiota does not correspond with any of the programmed changes in diet. The *C. jejuni*-colonized birds also exhibit the transition at 15 da but show less variance at 22 da. The transition is also marked in the ratio of coliforms to lactic acid bacteria counts by a shift in the dominance from coliform to lactic acid bacteria after the time point independent of whether or not the birds were colonized by *C. jejuni* (Additional file 5). Any differences between the *Campylobacter*-colonized and control groups will be superimposed upon this developmental transition. Han et al. [22] examined the influence of *C. jejuni* infection with age by inoculating broiler chickens with $\log_{10}$ 4 CFU *C. jejuni* at 1, 10, 22, and 31 da and determining the colonization levels and immune functions in the colonized birds. Circulating *C. jejuni*-specific maternal antibodies were detected in control birds from 3 da but absent by 15 da, which correlates well with the transition in microbiota we observe at that time point. A recent study by Ballou et al. [8] examined the development of the layer chicken microbiome and the effect of microbial interventions in the form of administering microbial treatments of probiotic bacteria and live *Salmonella* vaccines. These authors demonstrate changes in the microbiota with treatment and suggest that the functional impact of these treatments can stimulate greater differences at 14 da rather than later. Similarly, Awad et al. [54] recently noted a transition in the cecal microbiota of broiler chickens post 14 da with a relative increase in *Firmicutes* and *Tenericutes* at the expense of *Proteobacteria*. These authors also reported changes in the abundance of the microbial communities in response to *C. jejuni* colonization at 14 da and highlighted a

reduction in *Escherichia coli* at different intestinal sites while *Clostridium* spp. showed a significant increase. Using LEfSe, we also noted that non-colonized TEG1 cecal microbiota show a greater abundance of *Enterobactericeae* compared to *C. jejuni*-colonized TEG2 at 2 dpi with relative increases in the abundance of *Clostridia* in the colonized birds (TEG2). The relative increase in the abundance of the *Enterobactericeae* was short lived with no significant differences between the age-matched samples from the non-infected group thereafter (Additional file 6).

In response to *Campylobacter* colonization, we observed variable shifts in the abundance of members of the *Clostridiales*, which are largely unclassified but feature members of the *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae* families based on the consensus taxonomies. Increases in the abundance of clostridial species have been noted in association with experimental *C. jejuni* colonization previously [25, 54] and have been postulated to arise due to the *Campylobacter* acting as a hydrogen sink that would improve growth of clostridial organisms and their competitive standing through increased fermentation, leading to increased organic acid production that can be used by the campylobacters as an energy source [55]. However, several clostridial OTUs show greater abundance in the absence of *C. jejuni*, most notably *Clostridium* XIVa that feature in the analyses of the TEG and TLG experiments, and as major butyrate-producing bacteria play a key role in maintaining metabolic and immune functions in the gut [56].

It may be argued that variations in the abundance of the *Clostridiales* are a consequence of whether they benefit to the same degree from the bourgeoning *C. jejuni* population or show a relative reduction in abundance due to competition for alternative resources. These differential responses may also be driven by the prevailing chicken immune responses provoked by the *C. jejuni* colonization. For example, the late group will have to contend with pro-inflammatory cytokine and chemokine production in the ceca while the early group will have returned to levels similar to the non-infected group. *Ruminococcus* spp. OTUs identified from mature chicken cecal contents have been correlated with increases in IL-1β and IL-6 independent of any external microbial treatment [57], and therefore, any observed difference in abundance could represent a response to changing the immune status of the bird rather than a result of any direct interaction with a new member of the microbiota. Reductions in the abundance of *Clostridium* XIVa OTU0011 in the *C. jejuni*-colonized birds notably coincide with the peak Th17 pro-inflammatory responses that relate to the time of exposure in the TEG and TLG experiments (Fig. 8).

Microbial communities from fecal samples of *C. jejuni*-colonized commercial chickens at slaughter are reported to show increases in the abundance of *Streptococcus* and *Ruminococcaceae* and decreases in the abundance of *Lactobacillus* and *Corynebacterium* [55]. Notwithstanding that *Lactobacillus* are reported to be significantly over-represented in fecal samples compared with cecal content [57], we also found a relative decrease in the abundance of *Lactobacillus* spp. OTU0008 in the ceca of TEG2 *C. jejuni*-colonized birds. *Lactobacillus* spp. OTU0008 becomes significantly reduced at 16 dpi in the early colonized birds. This specific shift in the microbiota occurs after Th17 pro-inflammatory response in TEG2 when relative IL-6 expression is reduced and appears to represent a change in the microbiota driven by *C. jejuni* populations becoming established and tolerated (summarized in Fig. 8). In the late challenge where the Th17 response persists until the end of the study, the abundance of *Lactobacillus* spp. OTU0008 is not significantly changed. *Lactobacillus* spp. are established probiotics and have been proposed as feed additives to reduce the *C. jejuni* colonization of chickens [58–61]. If *C. jejuni* and *Lactobacillus* spp. OTU0008 compete for a similar niche and/or resource, then our observations could provide a basis for the inclusion of similar or better competing *Lactobacillus* ssp. in feed post programmed pro-inflammatory challenges such as those posed by vaccination. Inclusion would also have to minimize any potential negative impact on performance observed previously [2, 3], although it should be noted that at least one species of *Lactobacillus* spp. has been proposed to enhance the performance of broiler chickens [62].

Conclusions

We have demonstrated specific increases in cytokine/chemokine expression that are consistent with a Th17 response to *C. jejuni* colonization for early and late infection experiments. However, the outcomes for the cytokine/chemokine responses differ with respect to the age of infection in that the early colonized birds return to levels not distinguishable from age-matched non-infected birds, whereas the later infection continues the show elevated IL-17A responses until the end of the study (summarized in Fig. 8). These differences do not result in lower *Campylobacter* colonization levels at the end of the study. It is evident that a sudden shift in microbiota, caused by the introduction and colonization of a highly successful enteric bacteria, would elicit an immune response but the response in itself is not necessarily an indication of pathogenic behavior. It has been suggested that the complex relationship that permits persistent, high-level cecal colonization of *C. jejuni* in its avian host without obvious pathology is a result of inefficiency within the chicken immune system combined with mechanisms that redirect the response toward tolerance [16]. Our data would suggest there are a range of

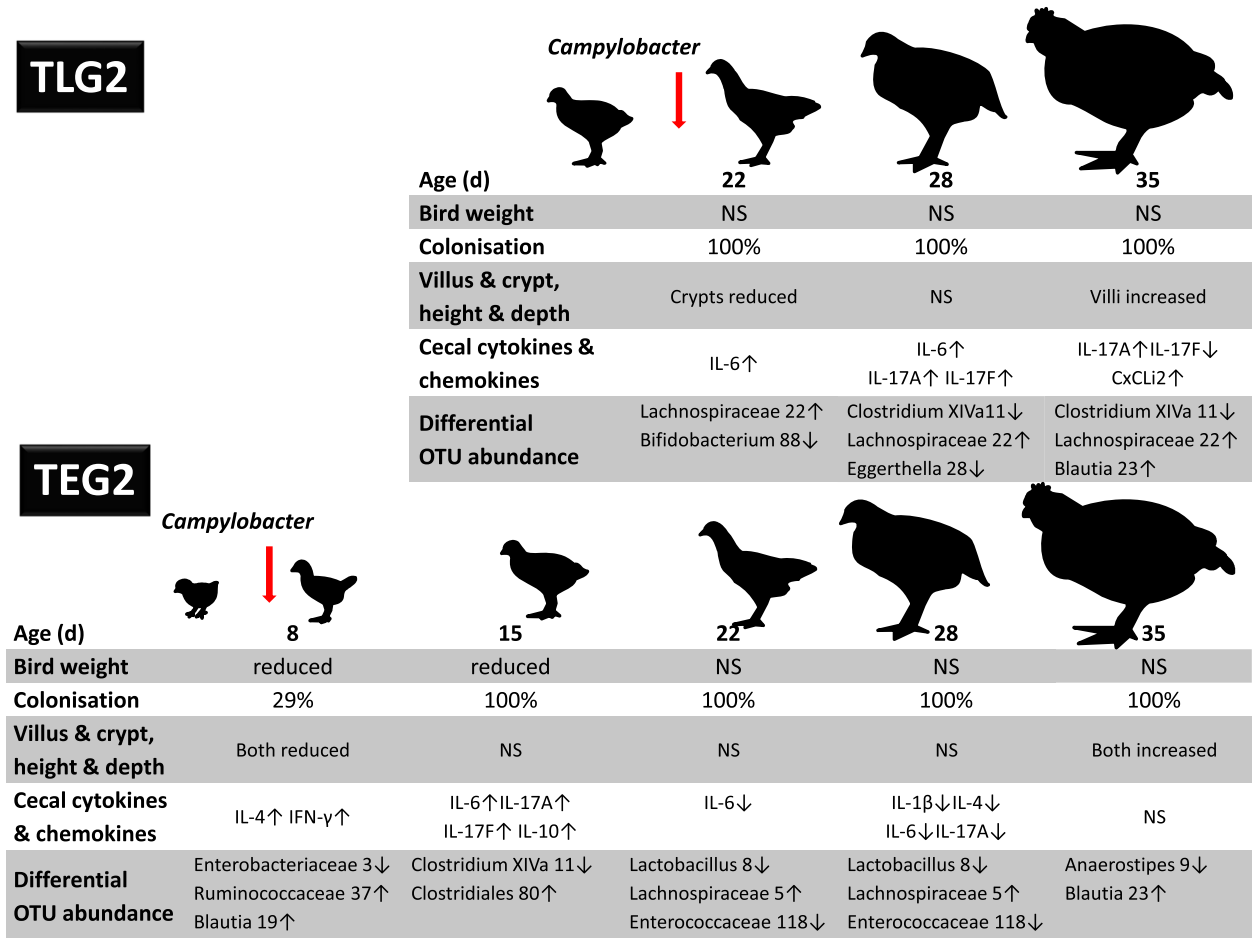


Fig. 8 Summary of age-dependent differences between *C. jejuni*-colonized broiler chickens and non-infected controls. Time dependent reductions in the mean weights of *Campylobacter*-infected chickens are indicated. Relative increases in the cecal cytokine/chemokine expression of *C. jejuni*-colonized birds compared to age-matched non-colonized control birds are marked by up arrows (↑), and down arrows indicate decreases in cytokine/chemokine expression (↓). Representative members of the cecal microbiota showing greater differential abundances between age-matched *C. jejuni*-colonized birds (↑) and non-colonized controls (↓) are indicated by their consensus genera and corresponding abundance rank identifying OTU numbers. NS indicates no significant differences between *Campylobacter*-colonized birds and non-infected controls

age-dependent chemokine/cytokine responses that are targeted to the levels of colonization, which collectively drive shifts in the resident microbial communities.

Additional files

Additional file 1: Mean weights of the broiler chickens from each experimental group. The mean live weights (SEM) of the chickens are plotted against the days of age for all experimental groups with the performance target weights for Ross 308 broiler chickens. TLG1—non-colonized control group for the late colonization experiment; TLG2—birds colonized with *C. jejuni* at day 20 for the late colonization experiment; TEG1—non-colonized control group for the early colonization experiment; TEG2—birds colonized with *C. jejuni* at day 6 for the early colonization experiment. (PDF 316 kb)

Additional file 2: Images of ileum H and E stained sections. Sections from non-infected control birds at 8 da (A), 22 da (B) and 35 da (C). Sections from *Campylobacter* infected birds in TEG2 at 2 dpi (D), 8 dpi (E) 15 dpi da (F) 28 dpi (G). The bars represent 200 μm. (TIF 8153 kb)

Additional file 3: The relative abundances 16S rRNA gene sequences of the most abundant phyla from the chicken ceca. The total read counts and the relative abundances are expressed as a percentage of the total reads for the most abundant taxonomic phyla discriminated at each sampling point over the rearing period of 35 days. (PDF 139 kb)

Additional file 4: PCoA plot of Bray-Curtis indices for the cecal microbiota of TEG. Bray-Curtis indices indicate the microbiota of birds exposed to *Campylobacter* at 6 da was different from uninfected birds at 2, 16 and 22 days post-infection by AMOVA (2 dpi; $p = 0.026$, 16 dpi; $p = 0.039$, 22 dpi; $p = 0.003$). $R^2 = 0.7$; subsample = 16,319. (PDF 94 kb)

Additional file 5: Coliform and Lactic acid bacterial counts from cecal contents. Bar charts show $\log_{10}$ CFU/g intestinal content for coliform and lactic acid bacteria counts in: A, TLG1 and TLG2 birds and B, TEG1 and TEG2 birds. (PDF 229 kb)

Additional file 6: Differential abundance of members of the cecal microbial communities in the development of TEG *C. jejuni* colonized and non-colonized broiler chickens. Histogram of the LDA scores computed for features differentially abundant between *C. jejuni* colonized broiler chickens (denoted as "inf" by red bars) and non-colonized birds (denoted

as "un" by green bars) over a 35 day rearing period. LEfSe identifies which clades amongst those detected as statistically differential will explain the greatest differences between the communities. OTUs represent individual sequences identified using BLASTn searches of type cultures with a BLAST identity ≥99%, and higher consensus taxanomic levels are indicated as _f family, _o order and _c class. Non-colonized birds were administered with 0.1 ml of carrier (MRD) by oral gavage at 6 da and colonized birds were with administered 10^7 CFU *C. jejuni* strain HPC5 in 0.1 ml MRD at 6 da. Seven birds were sacrificed from each group at days 8, 15, 22, 28 and 35 from which cecal digesta were collected and total DNAs extracted in preparation for bacterial 16S rRNA gene analysis of the bacterial communities. (PDF 1539 kb)

Abbreviations

AMOVA: Analysis of molecular variance; da: Days of age; dpi: Days post infection; FCR: Feed conversion ratio; OTU: Operational taxonomic unit; PCoA: Principal coordinate analysis; TEG: Trial early group; TLG: Trial late group

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Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and its additional information files. Sequence data are deposited in the NCBI database within the Bioproject PRJNA380214.

Authors' contributions

PLC and NJC conducted the laboratory research. PJR and IFC analyzed the microbiome data. GML collected the tissue samples and performed the qPCR experiments. PMO, NG, and IFC conducted the animal research. DLS prepared the 16SrRNA amplicons and collected the DNA sequence data. NMF and IFC designed the experiments and reviewed the data. PLC and IFC wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was carried out under license and in accordance with UK Animals (Scientific Procedures) Act 1986. All procedures were approved by the Local Ethics Committee of the University of Nottingham.

Competing interests

The authors declare that they have no competing interests.

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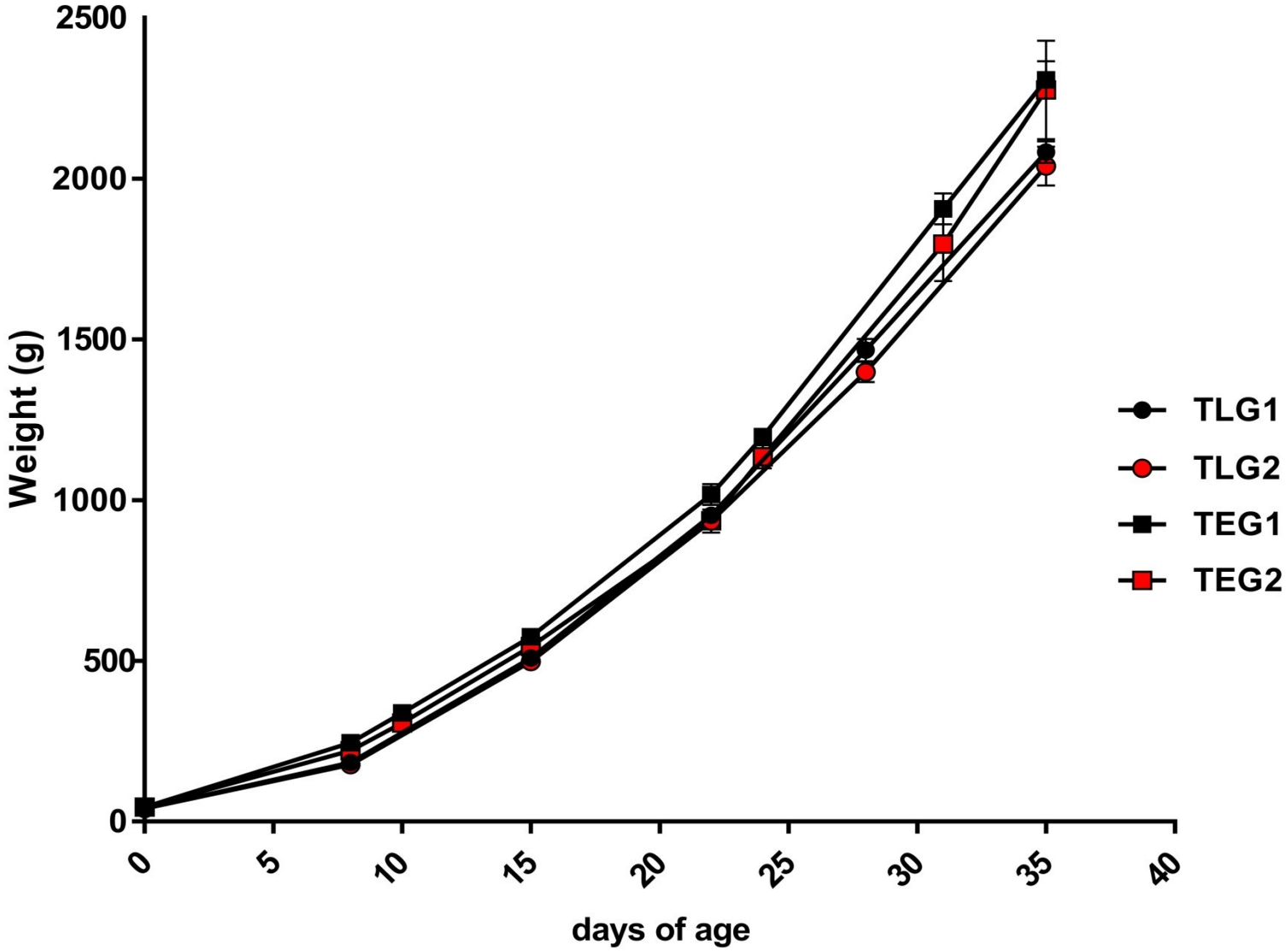
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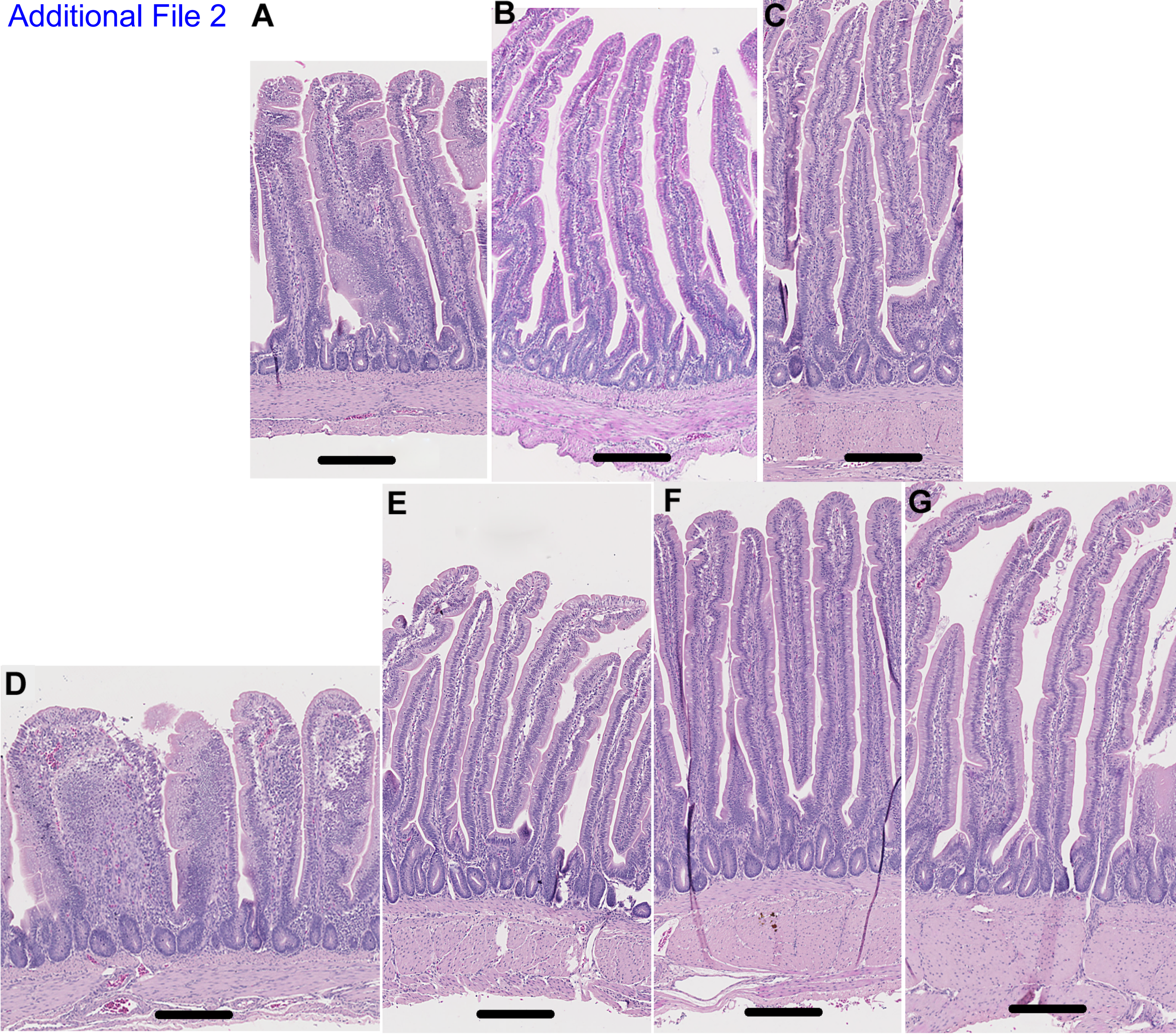
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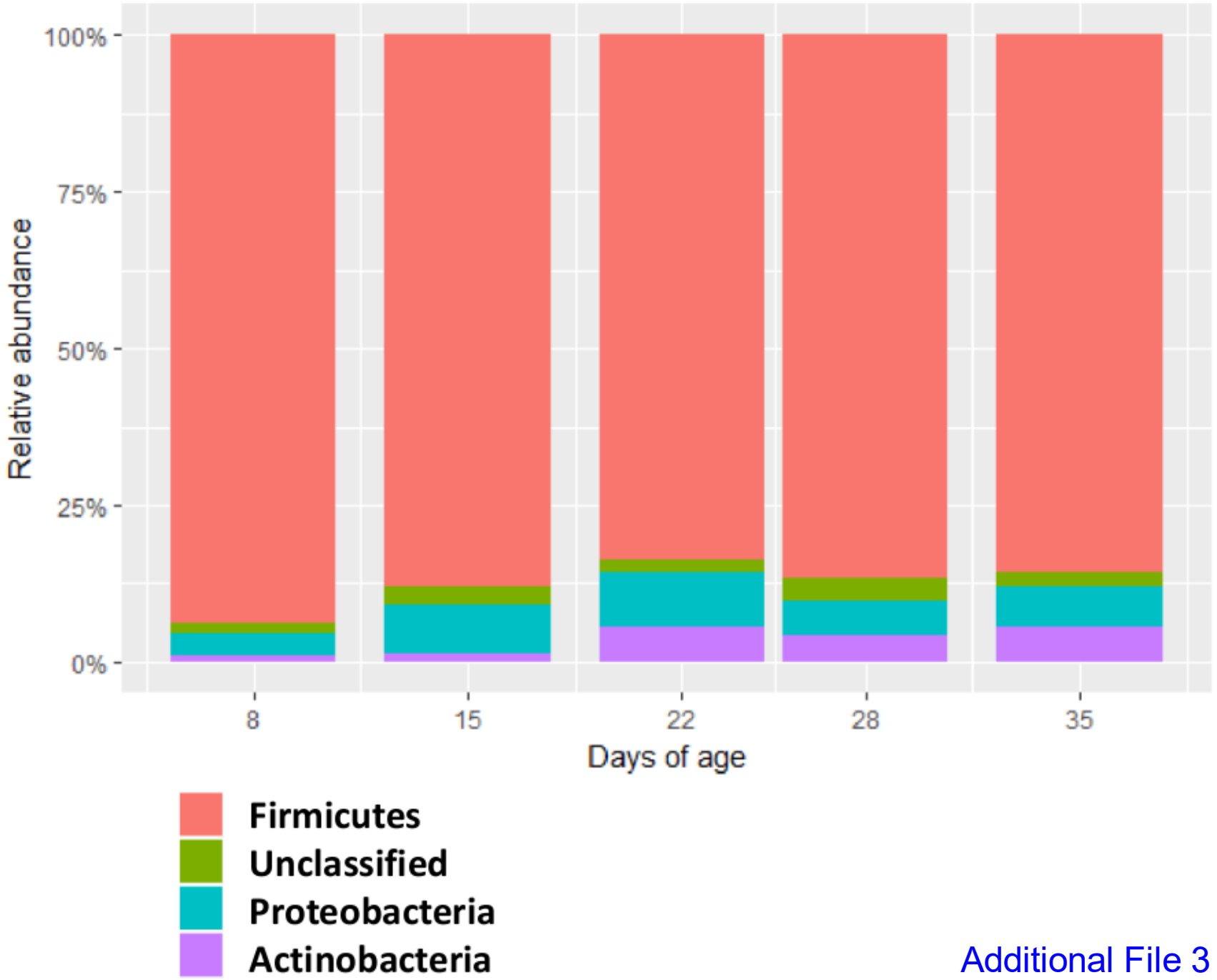
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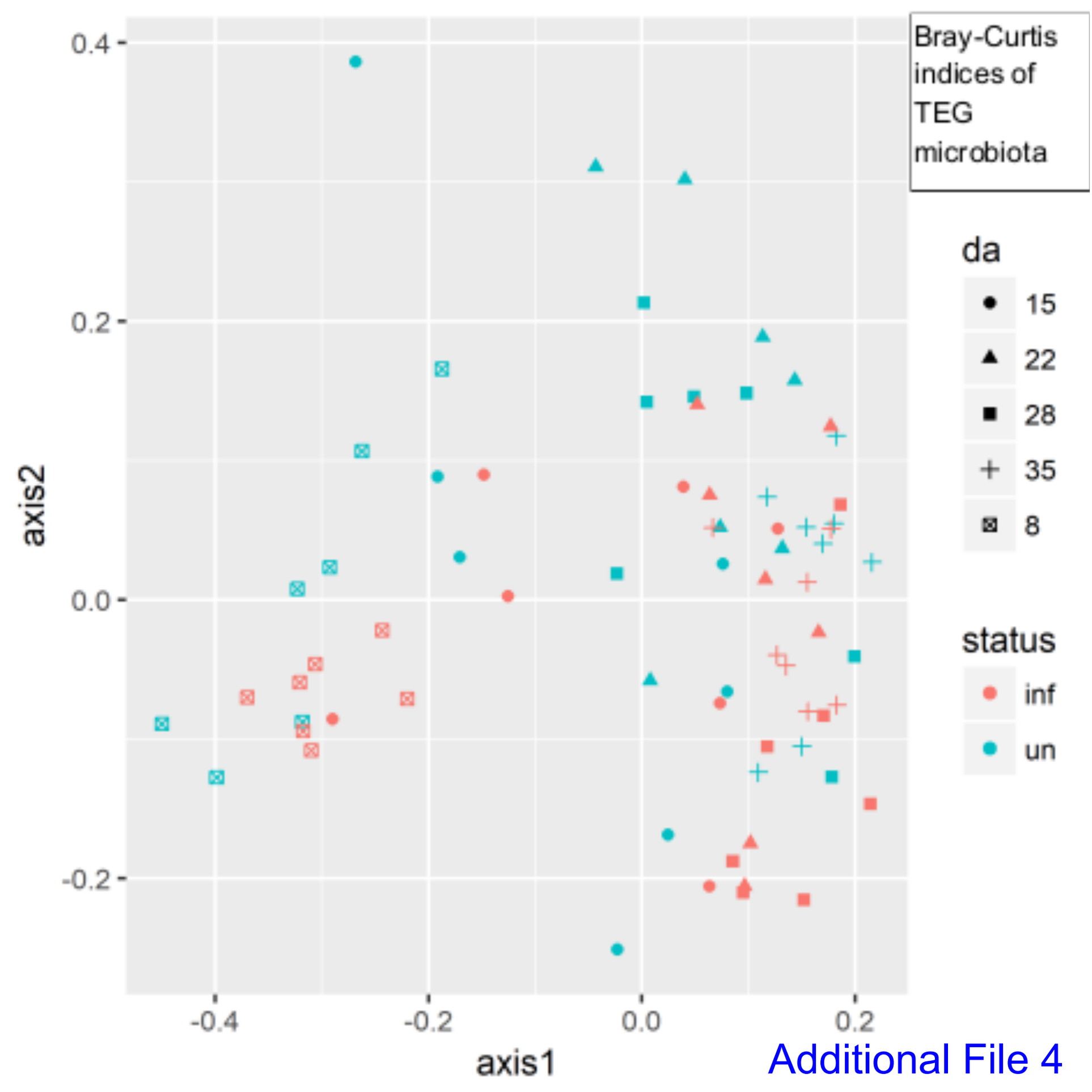
Additional File 1. Mean weights of the broiler chickens from each experimental group.
The mean live weights and SEM of the chickens are plotted against the days of age for all experimental groups with the performance target weights for Ross 308 broiler chickens. TLG1 – non-colonized control group for the late colonization experiment; TLG2 – birds colonized with C. jejuni at day 20 for the late colonization experiment; TEG1- non-colonized control group for the early colonization experiment; TEG2 – birds colonized with C. jejuni at day 6 for the early colonization experiment.

Additional File 2

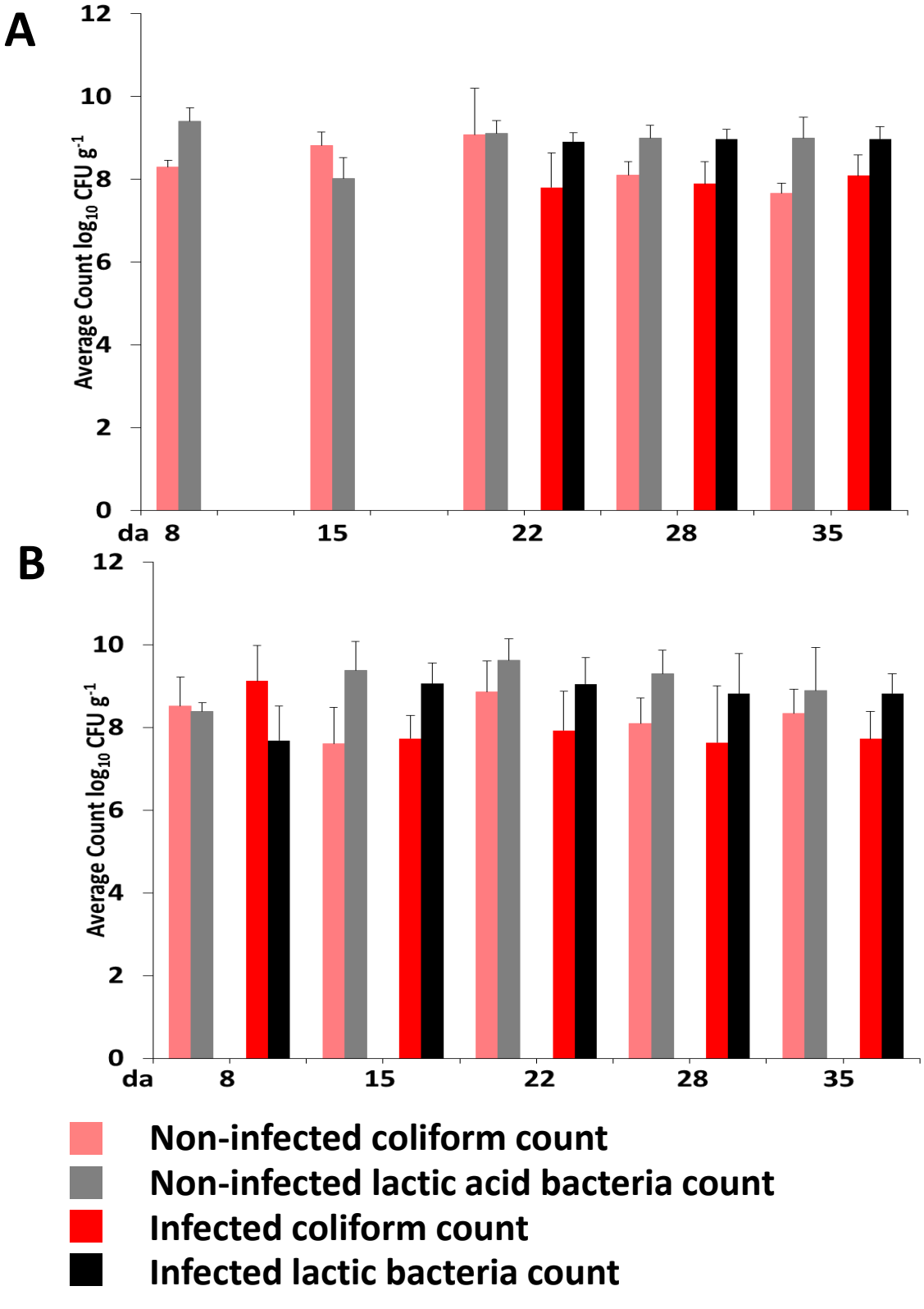




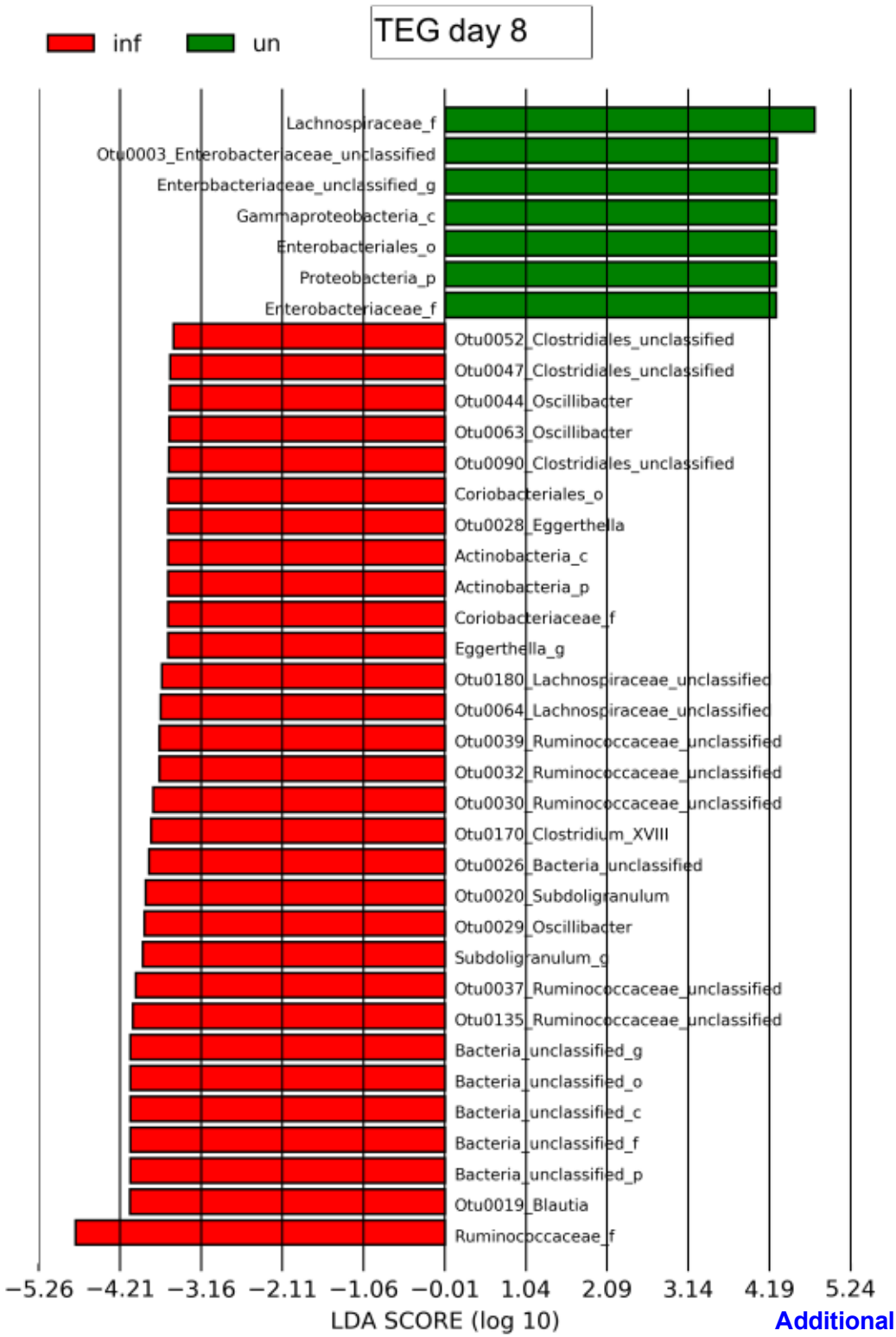
Additional File 3



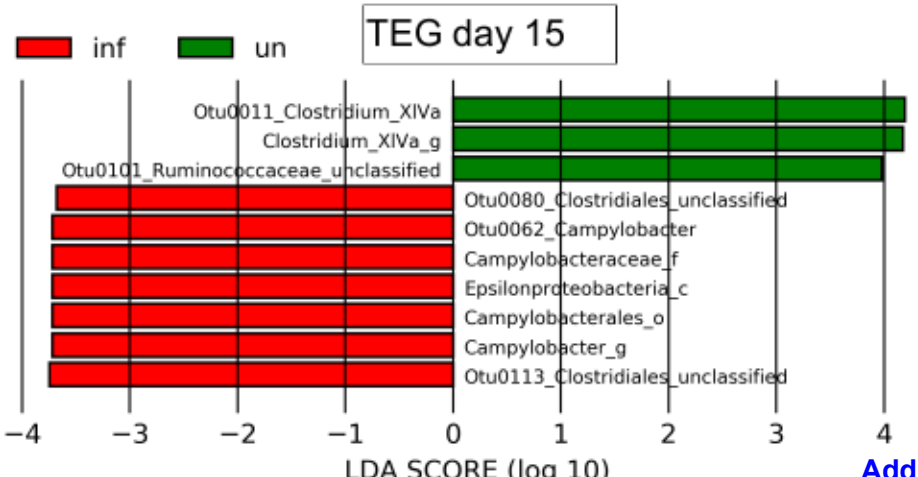
Additional File 4



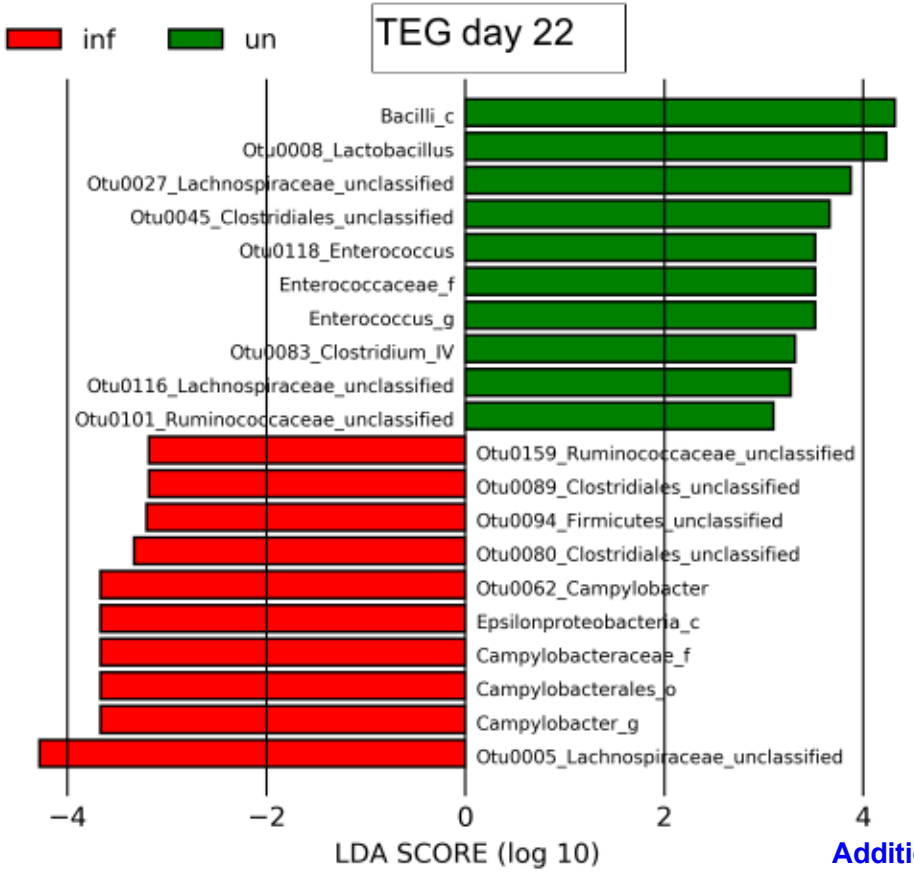
Additional File 4. Coliform and lactic acid bacteria counts in the cecal lumen. Error bars are standard deviations. A) Early cohort (TEG) infected at 6 da; B) Late cohort (TLG) infected at 20 da.



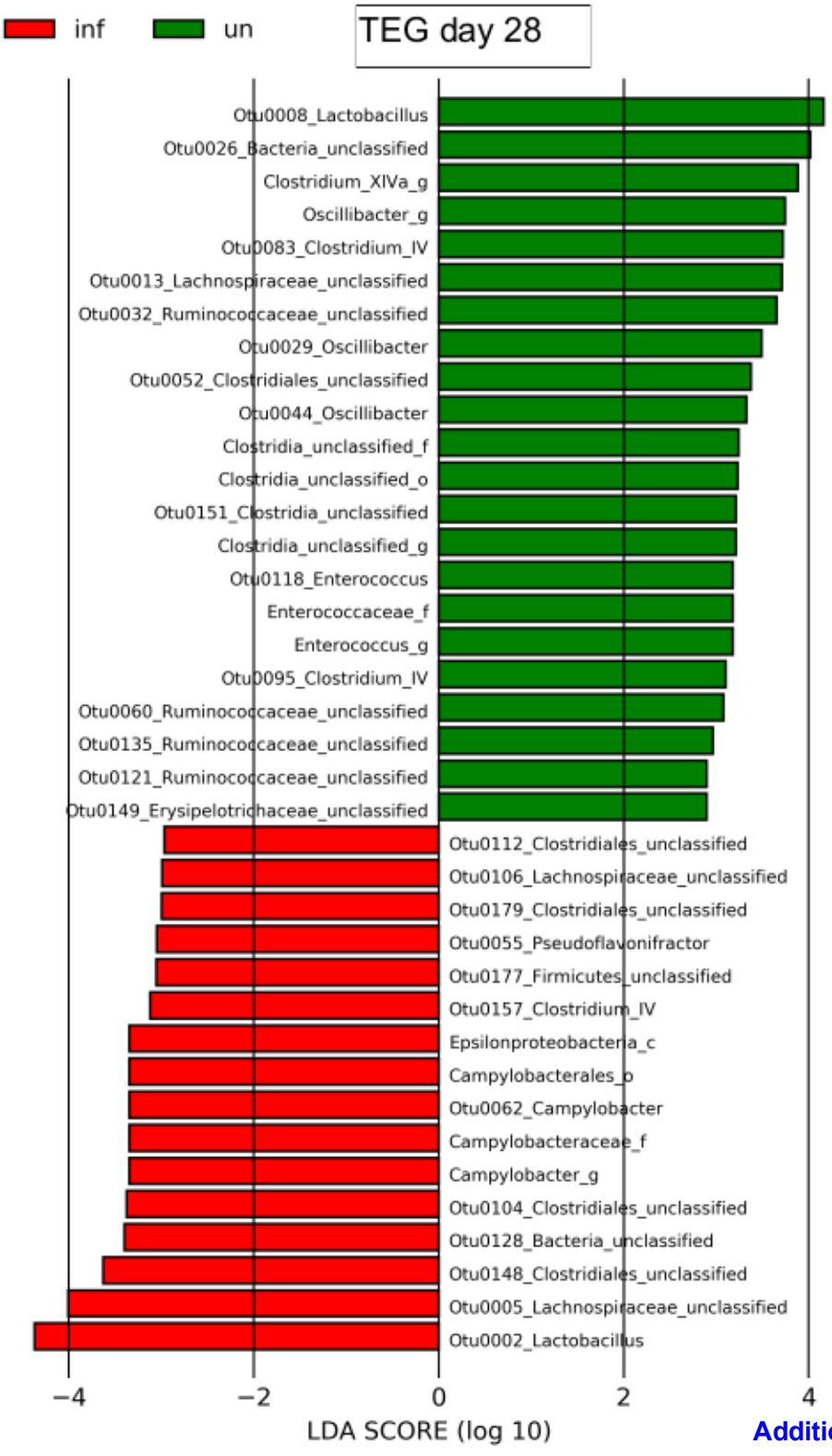
Additional file 6



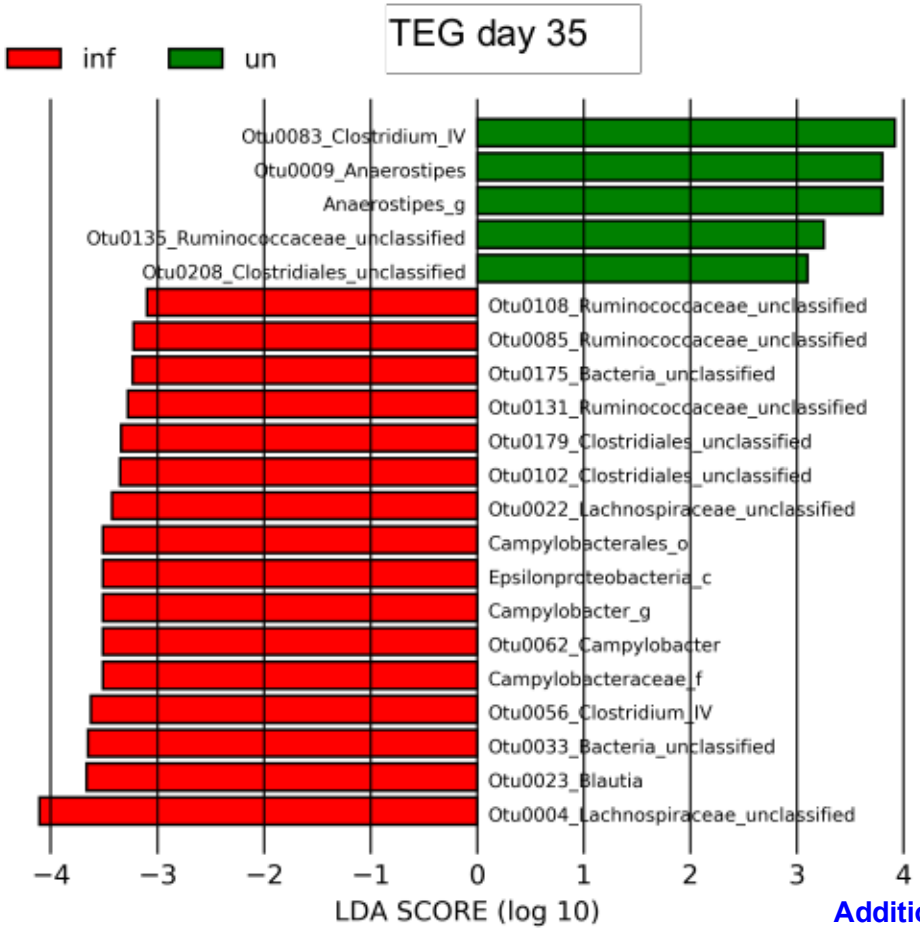
Additional file 6



Additional file 6



Additional file 6



Additional file 6

Lafontaine, G.M.F., Richards, P.J., Connerton, P.L., O'Kane, P.M., Ghaffar, N.M., Cummings, N.J., Fish, N.M. and Connerton, I.F., 2020. Prebiotic Driven Increases in IL-17A Do Not Prevent *Campylobacter jejuni* Colonization of Chickens. *Frontiers in Microbiology* 10, 3030. DOI: 10.3389/fmicb.2019.03030

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Prebiotic Driven Increases in IL-17A Do Not Prevent *Campylobacter jejuni* Colonization of Chickens

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Worldwide *Campylobacter jejuni* is a leading cause of foodborne disease. Contamination of chicken meat with digesta from *C. jejuni*-positive birds during slaughter and processing is a key route of transmission to humans through the food chain. Colonization of chickens with *C. jejuni* elicits host innate immune responses that may be modulated by dietary additives to provide a reduction in the number of campylobacters colonizing the gastrointestinal tract and thereby reduce the likelihood of human exposure to an infectious dose. Here we report the effects of prebiotic galacto-oligosaccharide (GOS) on broiler chickens colonized with *C. jejuni* when challenged at either an early stage in development at 6 days of age or 20 days old when campylobacters are frequently detected in commercial flocks. GOS-fed birds had increased growth performance, but the levels of *C. jejuni* colonizing the cecal pouches were unchanged irrespective of the age of challenge. Dietary GOS modulated the immune response to *C. jejuni* by increasing cytokine IL-17A expression at colonization. Correspondingly, reduced diversity of the cecal microbiota was associated with *Campylobacter* colonization in GOS-fed birds. In birds challenged at 6 days-old the reduction in microbial diversity was accompanied by an increase in the relative abundance of *Escherichia* spp. Whilst immuno-modulation of the Th17 pro-inflammatory response did not prevent *C. jejuni* colonization of the intestinal tract of broiler chickens, the study highlights the potential for combinations of prebiotics, and specific competitors (synbiotics) to engage with the host innate immunity to reduce pathogen burdens.

Keywords: *Campylobacter*, galacto-oligosaccharide, prebiotic, broiler chicken, innate immunity, microbiota, Th17, pro-inflammatory response

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INTRODUCTION

Campylobacter spp. are recognized as the major contributor to bacterial foodborne illness worldwide (Kaakoush et al., 2015). Campylobacterosis was the most frequently reported human zoonotic disease in the European Union in 2017 with 246,158 confirmed cases of gastrointestinal illness (EFSA, 2018). The most common species associated with human disease is *C. jejuni* (84.4%), but *C. coli* also represent a significant disease burden (9.2%; EFSA, 2018). *C. jejuni* and

Abbreviations: *C. jejuni*, *Campylobacter jejuni*; CFU, colony forming unit; FCR, feed conversion ratio; GoI, gene of interest; GOS, galacto-oligosaccharide; GIT, gastro-intestinal tract; IL, interleukin.

C. coli are referred to as thermophilic species as they can grow at 42°C, making them suited to colonize the intestinal tracts of poultry species (reviewed by Sahin et al., 2015). Poultry are a major source of campylobacters with an estimated 80% of human illness arising from poultry sources (Andreoletti et al., 2010). Source attribution estimates referenced at the point of exposure indicate 65–69% of human cases are from exposure to chicken meat (Ravel et al., 2017). Poultry meat is frequently contaminated with intestinal content harboring high levels of *Campylobacter* cells during slaughter and carcass processing, which constitutes the main risk to public health (Osimani et al., 2017). This has prompted the EU to adopt a microbiological sampling plan for broiler chicken carcasses with a limit of 1,000 CFU/g (Commission Regulation (EU) 2017/1495). Strict on-farm biosecurity measures to prevent *Campylobacter* exposure and flock colonization of broiler chickens have been implemented in many countries, but these alone do not maintain *Campylobacter*-free flocks (Newell et al., 2011). Intervention strategies have been developed aimed at reducing levels of *Campylobacter* colonization, and thereby human exposure, if the reductions can be translated on to chicken meat (Rosenquist et al., 2003; Newell et al., 2011; Sahin et al., 2015). *Campylobacter* colonization has been associated with poor flock health and performance in commercial broiler chicken production (Bull et al., 2008), although performance issues are not manifest in all circumstances (Gormley et al., 2014). The impact of *Campylobacter* colonization on bird health has been reported to vary with the broiler breed/rate of growth, stocking density, intercurrent infectious or immunosuppressive challenges and the colonizing organism (Humphrey et al., 2015; Li L. et al., 2018). It is, however, clear that *Campylobacter* colonization elicits a Th17 pro-inflammatory response in broiler chickens (Reid et al., 2016; Connerton et al., 2018). Intestinal intraepithelial lymphocytes characterized as either $CD3^{+}CD25^{+}$ cells or γδ T cells have been reported to express intracellular IL-17A in the lower intestine of chickens (Walliser and Göbel, 2018). Not all inflammatory responses lead to negative outcomes for the host. It has been questioned whether dietary anti-inflammatory additives aimed at the detrimental consequences of intestinal inflammation may actually impair necessary responses of young animals that are required to overcome the challenges present in commercial production to achieve favorable performance outcomes (Broom and Kogut, 2018). Zootechnical performance remains a key driver in the poultry industry, at the same time public and regulatory concerns are mounting regarding welfare and antibiotic use in poultry production. Although progress has been made toward reducing antibiotic use in poultry production in several countries has been reported more remains to be achieved (Speksnijder et al., 2015). It has been proposed that the rational manipulation of poultry feed formulation can improve pathogen resistance, improve production, and reduce the threat posed by zoonotic pathogens through the food chain (Kogut, 2009; Swaggerty et al., 2019). Intestinal innate immune responses to feed and pathogen challenges are strongly influenced by the gut microbiota (Kogut et al., 2018). The addition of probiotic microorganisms, prebiotics and phytobiotics in feed are approaches by which the gut microbiota of broiler chickens may be influenced (reviewed by Pedroso et al., 2013; Pourabedin and Zhao, 2015; Van Immerseel et al., 2017; Clavijo and Flórez, 2018). It is proposed that these approaches will be most effective when introduced early in life to establish a robust microbiota that benefits production (Rubio, 2019). We have recently reported that the inclusion of the prebiotic GOS in juvenile broiler feed enhances the growth and feed conversion rates of broiler chickens, increases ileal and cecal IL-17A gene expression and brings about changes in the cecal populations of key *Lactobacillus* spp. (Richards et al., 2019b). GOS represent host-indigestible carbohydrates that have been identified as promoting beneficial bacteria in humans and animals, which include *Bifidobacteria*, *Bacteroides*, and *Lactobacillaceae* (Jung et al., 2008; Hughes et al., 2017; Van Bueren et al., 2017; Tian et al., 2019).

In the present study, we have examined whether the impact of a GOS diet on host fitness, immune response and changes in the gut microbiota would support the clearance of *Campylobacter jejuni* in broiler chickens. For this purpose, we fed isocaloric GOS or control diets from hatch to 20 days of age to modulate the intestinal innate immune status and gut microbiota of broiler chickens. Two approaches were taken to determine the role of development on host response. In one experiment birds were challenged at an early stage of development at 6 days old to determine the persistence of *C. jejuni* in the modified gut environment and assess corresponding intestinal chemokine and cytokine gene expression, and the prevailing intestinal microbiota throughout the typical broiler chicken lifespan of 35 days. In a separate experiment birds were challenged at 20 days old, when campylobacters are frequently first detected in commercial flocks, with similar observations made until the trial ended when birds reached 35 days old.

MATERIALS AND METHODS

Trial Design

Two independently performed trials monitored the effect of dietary GOS on development of the gut innate immune responses and the cecal microbiota of broiler chickens challenged with *C. jejuni* HPC5 at either an early stage of development (6 days old) or late stage (20 days old), the age at which birds often become *Campylobacter* positive in commercial production. Birds were randomly assigned to either a group fed a control diet (referred to as *Campylobacter*) or to a group fed a GOS-supplemented diet (referred to as GOS + *Campylobacter*) for the duration of the experiment. In the early 6-day old challenge experiment (referred to as 6-dc), two groups of 35 birds were kept in pens from day of hatch until day 6 when all birds were administered *C. jejuni*, and subsequently independently caged until the end of the study on day 35. Birds were randomly selected (n = 7) from each diet group and euthanized prior to sampling intestinal tissues and contents at 8, 15, 22, 28, and 35 days of age (da). For the late 20-day challenge trial (referred to as 20-dc), two groups of 21 birds were similarly housed in pens until 20 days when the birds were administered *C. jejuni* and independently caged until the end of the study at 35 days. Again, randomly selected birds (n = 7) from each diet group were euthanized

for intestinal sampling at 22, 28, and 35 days. All experimental birds post challenge were maintained in independent housing to prevent the birds sharing intestinal microbiota through coprophagy, which would otherwise confound the experimental design by reducing the number of replicates.

Experimental Animals

Day-of-hatch male Ross 308 broiler chicks purchased from a local hatchery were randomly assigned on the basis of weight to control or GOS diet groups. Birds were brooded in floor pens on wood shavings until the day of *Campylobacter* challenge. Birds were housed in a controlled environment under strict conditions of biosecurity and kept under controlled light (L:D 12:12) with *ad libitum* access to food and water throughout the study. Temperatures conformed to the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes 2014. Welfare monitoring of the chickens was undertaken two or three times every 24 h post *Campylobacter* challenge. Birds in the control group were sustained on a wheat-based diet provided as starter crumb for 0–10 days, grower pellets for 11–24 days and finisher pellets for 25–35 days. The starter diet contained wheat (59.9% w/w), soya meal (32.5% w/w), soybean oil (3.65% w/w), limestone (0.6% (w/w), calcium phosphate (1.59% w/w), sodium bicarbonate (0.27% w/w), the enzymes phytase and xylanase (dosed according to the manufacturer's instructions; DSM Nutritional Products Ltd., PO Box 2676 CH-4002 Basel, CH) and a vitamin mix containing NaCl salt, lysine HCl, DL-methionine and threonine. The grower and finisher diets increased the wheat content at the expense of soya meal by 2 and 5% w/w, respectively. The 6-dc prebiotic GOS + *Campylobacter* treatment group had the starter feed supplemented with GOS from 1 to 10 days at 3.37% w/w and then 11–35 days at 1.695% w/w, whilst the 20-dc birds were fed 3.37% w/w GOS throughout the experiment. The GOS was provided as Nutrabiotic® GOS that contains 74% GOS w/w dry matter (Dairy Crest Ltd., Davidstow, Cornwall, United Kingdom). GOS preparations contain a mixture of monosaccharides (glucose and galactose) and oligosaccharides (DP2 – DP8) with the exception of lactose that is a residual component of the production process. The enzymatic synthesis of GOS produces β-(1–3) or β-(1–4) or β-(1–6)-linked galactose residues (1 to 7) with a terminal β-(1–3) or β-(1–4) or β-(1–6)-linked glucose. Isocaloric content adjustments for GOS inclusion were for the starter feed (wheat 54.0% w/w) soya meal (33.9% w/w) and soybean oil (4.88% w/w); for the grower feed (wheat 54.7% w/w) soya meal (32.2% w/w) and soybean oil (6.76% w/w); for the finisher feed (wheat 60.33% w/w) soya meal (26.7% w/w) and soybean oil (6.84% w/w). The feed and paper liners on which the chicks were delivered were found negative for *Salmonella* using standard enrichment procedures. At the time of challenge all birds were administered by oral gavage a dose of 1×10^7 CFU *C. jejuni* HPC5, a well-characterized broiler chicken isolate, suspended in MRD (Oxoid, Thermo Fisher Scientific, Altrincham, United Kingdom) in volumes of 0.1 ml for the 6-dc birds or in 1 ml for the 20-dc birds. For sample collection, birds were euthanized by either exposure to rising CO_2 gas or parenteral barbiturate overdose followed by cervical dislocation depending on bird mass in accordance with Schedule 1 of the United Kingdom Animals (Scientific Procedures) Act 1986. Ileal tissues were collected from approximately 3 cm distal to Meckel's diverticulum and cecal tissues isolated from the distal tips of the cecal pouches. Samples of intestinal tissue were immediately frozen in liquid nitrogen for subsequent RNA isolation or preserved in 10% (w/v) neutral buffered formalin (Thermo Fisher Scientific) for histological assessment. Cecal contents were collected and used either to enumerate *Campylobacter* or for isolation of total genomic DNA extraction.

Performance and Growth Rate

Live weights and all feed consumed were recorded for all birds at regular intervals from the start of the experiment until the end at 35 days. FCR were calculated as a ratio of feed consumed to the live weight of the birds. Bird growth rates were compared for each of the birds that remained at the end of the 35 days rearing period that collectively represent all the birds for which repeated measurements of the mass were recorded throughout the broiler chicken lifespan.

Bacterial Enumeration

Approximately 1 g of digesta was aseptically collected from both ceca and combined in pre-weighed universal containers before a 10% w/v suspension was prepared in MRD. *Campylobacter* were enumerated in triplicate from decimal dilutions prepared in MRD using a modification of the Miles and Misra technique (Miles et al., 1938). For each triplicate dilution set, five aliquots were dispensed onto CCDA agar (PO0119; Oxoid) prepared with the addition of agar to 2% (to prevent swarming) and with addition of CCDA Selective Supplement SR0155 (Oxoid). Plates were incubated at 42°C in a microaerobic atmosphere (2% H_2, 5% CO_2, 5% O_2, and 88% N_2 v/v) for 48 h (Don Whitley Scientific modified atmospheric cabinet, Shipley, United Kingdom).

Histology

Tissue samples fixed in a 10% formalin solution were dehydrated through a series of alcohol solutions, cleared in xylene, and embedded in paraffin wax (Microtechnical Services Ltd., Exeter, United Kingdom). Sections (3 to 5 μm thick) were prepared and stained with modified hematoxylin and eosin (H&E). After staining, the slides were scanned by NanoZoomer Digital Pathology System (Hamamatsu, Welwyn Garden City, United Kingdom). Villus height and crypt depth were recorded from operator blinded measurements collected using the NanoZoomer Digital Pathology Image Program (Hamamatsu) from histology stained slides scanned at 40× resolution for each tissue sample. Villus height was determined from the tip of the villus to the crypt opening and the associated crypt depth was measured from the base of the crypt to the level of the crypt opening. The ratios of villus height to relative crypt depth (v/c ratio) were calculated from these measurements. Dimensions for 10 well-oriented villi per tissue sample of 3 or 4 birds per diet group at each sampling time were analyzed.

RNA Isolation and RT-qPCR of the Cytokines and Chemokines

Total RNAs were isolated from ceca and ileum tissue biopsies using NucleoSpin RNA purification kit (Macherey-Nagel, GmbH & co. KG, Düren; DE) according to the manufacturer's protocol with the following modifications. Tissue samples were homogenized with the kit Lysis buffer and 2.8 mm ceramic beads (MO BIO Laboratories Inc., Carlsbad, United States) using TissueLyser II (Qiagen, Hilden, Germany). Subsequently total RNAs were extracted as described in the protocol with a DNaseI treatment step as per the manufacturer's instructions. Purified RNAs were eluted in nuclease free water, validated for quality and quantity using UV spectrophotometry (Nanodrop ND-1000, Labtech International Ltd., Uckfield, United Kingdom), and stored long term at −80°C. RNAs with OD260/280 ratio between 1.9 and 2.1 were deemed high quality, the ratios were found with a mean of 2.12 ± 0.01. Reverse Transcription was performed with 1 μg of RNA, SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, United States) and random hexamers as described previously (Connerton et al., 2018). Quantitative PCR reaction was performed with cDNA template derived from 4 ng of total RNA in triplicate using SYBR Green Master mix (Applied Biosystems, Thermo Fisher Scientific). The RNA level of expression was determined by qPCR using the Roche Diagnostics LightCycler 480 (Hoffmann La Roche AG, CH). The primers sequence for GAPDH, INF-γ, IL-1β, IL-6, IL-10, IL-17A, IL-17F, ChCXCLi1, and ChCXCLi2 (**Table 1**) were previously described (Kaiser et al., 2003; Nang et al., 2011; Rasoli et al., 2015; Reid et al., 2016). Cytokines and chemokines transcripts fold change (FC) were calculated according to the manufacturer using the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001). Averages of the triplicate Ct values were analyzed with the target genes of interest (GOI) values normalized to those of the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Microbiota Analysis

DNA was isolated from cecal content using the MoBio PowerSoil kit (now QIAGEN Ltd., Manchester, United Kingdom) according to the manufacturer's instructions. The V4 regions of the bacterial 16S rRNA genes were PCR amplified using the primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGACTACHVGGGTWTCTAAT 3′) (Caporaso et al., 2011). Amplicons were then sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, United States) using 2 × 250 bp cycles (reagent kit V2). The 16S rRNA gene sequences were quality filtered and clustered into OTUs in Mothur (Schloss et al., 2009) using the Schloss lab. MiSeq SOP[1], accessed 2018-10-05; Kozich et al., 2013). Batch files of Mothur commands used in this study are available at https://github.com/PJRichards/lafontaine_campy_gos. Raw sequences for 16S rDNA data originally reported in this article are deposited in the NCBI database within BioProject PRJNA380214 under SRA study SRP133552. Comparative 16S rDNA data from mock-challenged birds reproduced in this study was downloaded from NCBI PRJNA380214 (the FTP code is available from GitHub repository as described below). Post-processing rarefaction curves were plotted to assess sampling effort (**Supplementary Figure S1**).

[1]https://github.com/mothur/mothur

Data and Statistical Analysis

All figures were drawn and unless otherwise stated all tests for statistical significance were performed using R 3.6.1 (R Core Team, 2019) in RStudio 1.2.1 (RStudio Team, 2015). All R scripts have been made available here: https://github.com/PJRichards/lafontaine_campy_gos. Histology measurements for each diet regimen of age-matched birds were compared using ANOVA.

Zootechnical Data

Bird growth rates were compared by determining rate of growth from 15 days for each of the birds that remained at the end of the trial at 35 days, i.e., birds for which repeated measurements of the mass were recorded throughout the growing period (*Campylobacter* treatment group, $n = 7$; GOS + *Campylobacter* treatment, $n = 8$). Growth rate was determined for individual birds for the period of linear growth post-challenge (6-dc birds between 15 and 35 days; 20-dc birds between 22 and 35 days). Growth rates (g/day) were compared between cohorts using Student's *t* test. Further comparison was made between the mass of age-matched birds using Student's *t* test. *C. jejuni* viable counts were $\log_{10}$-transformed and tested for significance using Student's *t* test.

Microbiota – 16S rRNA Gene Sequence Data

Comparisons were made of α-diversity metrics (Shannon diversity and inverse Simpson's indices) generated in mothur. For the four treatment groups in 6-dc birds at 8 days (2 dpi) differences in α-diversity were tested for using ANOVA with Tukey multiple comparison of means test (*p* was adjusted for multiple comparisons). For subsequent comparisons of α-diversity at 15, 22, 28 and 35 days between two treatment groups only (*Campylobacter* and GOS + *Campylobacter*) Student's *t* test was used to test for significance. Correspondingly, comparisons of Chao richness between the four treatment groups in 6-dc birds at 8 days (2 dpi) were made using a Kruskal-Wallis test with Benjamini-Hochberg FDR correction as were subsequent comparisons at 15, 22, 28, and 35 days. Note that a randomly selected community was deleted from the GOS + *Campylobacter* treatment group at 15 days and 35 (birds 2 and 6, respectively) to even group size and allow unbiased comparisons. For 20-dc birds, comparisons of α-diversity were made using Student's *t* test and comparisons of Chao richness were made using a Kruskal-Wallis test. Differences in bacterial composition were tested for by modeling compositional population data in terms of a Dirichlet distribution and using a likelihood ratio test in DirtyGenes (Shaw et al., 2019). Differential OTUs were identified with LEfSE in mothur (Schloss et al., 2009; Segata et al., 2011).

TABLE 1 | Primer sequences for the gene expression determined by qPCR.

Target gene	Primer sequence (5′-3′)	Product size (bp)	NCBI Accession number	References
GAPDH	F: GACGTGCAGCAGGAACACTA R: TCTCCATGGTGGTGA AGACA	343	NM_204305.1	Nang et al. (2011)
INF-γ	F: TGAGCCAGATTGTTTCGATG R: CTTGGCCAGGTCCATGATA	152	NM_205149.1	Nang et al. (2011)
IL-1β	F: GGATTCTGAGCACACCACAGT R: TCTGGTTGATGTCGAAGATGTC	272	NM_204524.1	Nang et al. (2011)
IL-10	F: GCTGCGCTTCTACACAGATG R: TCCCGTTCTCATCCATCTTC	203	NM_001004414.2	Nang et al. (2011)
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	71	NM_204628.1	Kaiser et al. (2003)
IL-17A	F: CATGGGATTACAGGATCGATGA R: GCGGCACTGGGCATCA	68	NM_204460.1	Reid et al. (2016)
IL-17F	F: TGACCCTGCCTCTAGGATGATC R: GGGTCCTCATCGAGCCTGTA	78	XM_426223.5	Reid et al. (2016)
ChCXCLi-1	F: CCGATGCCAGTGCATAGAG R: CCTTGTCCAGAATTGCCTTG	191	NM_205018.1	Rasoli et al. (2015)
ChCXCLi-2	F: CCTGGTTTCAGCTGCTCTGT R: GCGTCAGCTTCACATCTTGA	128	NM_205498.1	Rasoli et al. (2015)

Intestinal Cytokine and Chemokine Transcription

Host cytokine and chemokine transcript levels were assessed by RT-qPCR of transcribed RNA isolated from ileal and cecal tissue sections. Cytokine and chemokine normalized expression was determined for each sample as $2^{-\Delta\ Cp}$ with ΔCp = Cp of GoI – Cp of housekeeping gene (GAPDH). The relative gene expression between birds fed a control (*Campylobacter*) or a GOS diet (GOS + *Campylobacter*), results were determined as a group mean FC, which was calculated from $2^{-\Delta\ \Delta\ Cp}$ with ΔΔCp = ΔCp (GOS diet) - ΔCp (average ΔCp of control). Differences between treatment groups were assessed using Wilcoxon rank sum tests with Benjamini-Hochberg FDR correction.

RESULTS

Dietary Galacto-Oligosaccharide Improved the Growth Performance of *Campylobacter jejuni*-Colonized Broiler Chickens

The aim of this research was to determine whether GOS could still act as a prebiotic and confer a growth performance advantages to broiler chickens colonized when colonized by *C. jejuni*. Chickens fed a GOS diet performed better than those fed the calorie-matched control diet (**Figures 1A,B**). Differences in the body weights were evident from 22 days for the 6-dc birds ($p \leq 0.029$) until slaughter at 35 days (mean body weights *Campylobacter* treatment = 2276 g, GOS + *Campylobacter* treatment = 2722; $p = 0.029$) (**Figure 1A**). Differences in the weights of the 20-da challenged birds were observed at 35 days (mean body weights *Campylobacter* = 2055 g, GOS + *Campylobacter* = 2341; $p = 0.004$) (**Figure 1B**). The growth rates of the 6-da challenged birds fed GOS increased in the period 15–35 days compared to the challenged chickens fed a control diet (control = mean 83.6 g/day, GOS = mean 105.1 g/day; $p = 0.0233$), and similarly for the 20-da challenged birds for the period 22–35 days (control = mean 85.3 g/day, GOS = mean 97.6 g/day; $p = 0.007$).

The cumulative FCR up to 35 days for 6-dc birds fed control diet ($n = 7$) was 1.45 and for the GOS diet ($n = 8$) was 1.42 while the FCR for 20-dc birds fed control diet ($n = 7$) was 1.56 and for GOS diet ($n = 7$) was 1.58 (data not shown). The contemporary breed performance objectives for male Ross 308 were body weight 2,283 g and FCR of 1.54 at 35 days (Aviagen Performance Objectives, 2014).

Dietary Galacto-Oligosaccharide Inclusion Did Not Prevent *Campylobacter jejuni* Colonization of Broiler Chickens

To assess the impact of dietary GOS on *C. jejuni* colonization we sacrificed birds over the rearing period to determine the cecal viable *Campylobacter* counts ($n = 7$). All birds were culture-negative for *Campylobacter* spp. until oral gavage with *C. jejuni* HPC5 at 6 days for the early-challenge cohort (6-dc) or 20 days for the late-challenge birds (20-dc). Birds challenged at 6 days (**Figure 1C**) showed incomplete colonization at 2 dpi (2/7 and 3/7 for the control and GOS cohorts, respectively), but the treatment groups all showed complete colonization with *C. jejuni* at 9 dpi. Mean colonization levels of 6.4 $\log_{10}$ CFU/g for the control diet and 5.9 $\log_{10}$ CFU/g for the GOS diet were recorded (15 days). The birds then remained colonized thereafter to the end of the 35 days rearing period with no significant differences between the colonization levels of the dietary groups at any time (**Figure 1C**). The 20-dc birds (**Figure 1D**) were all found colonized at 8 dpi with mean colonization levels of 6.8 $\log_{10}$ CFU/g for the control and 7.2 $\log_{10}$ CFU/g for the GOS diet (28 days). Viable counts of *Campylobacter* in cecal content remained high at the end of the

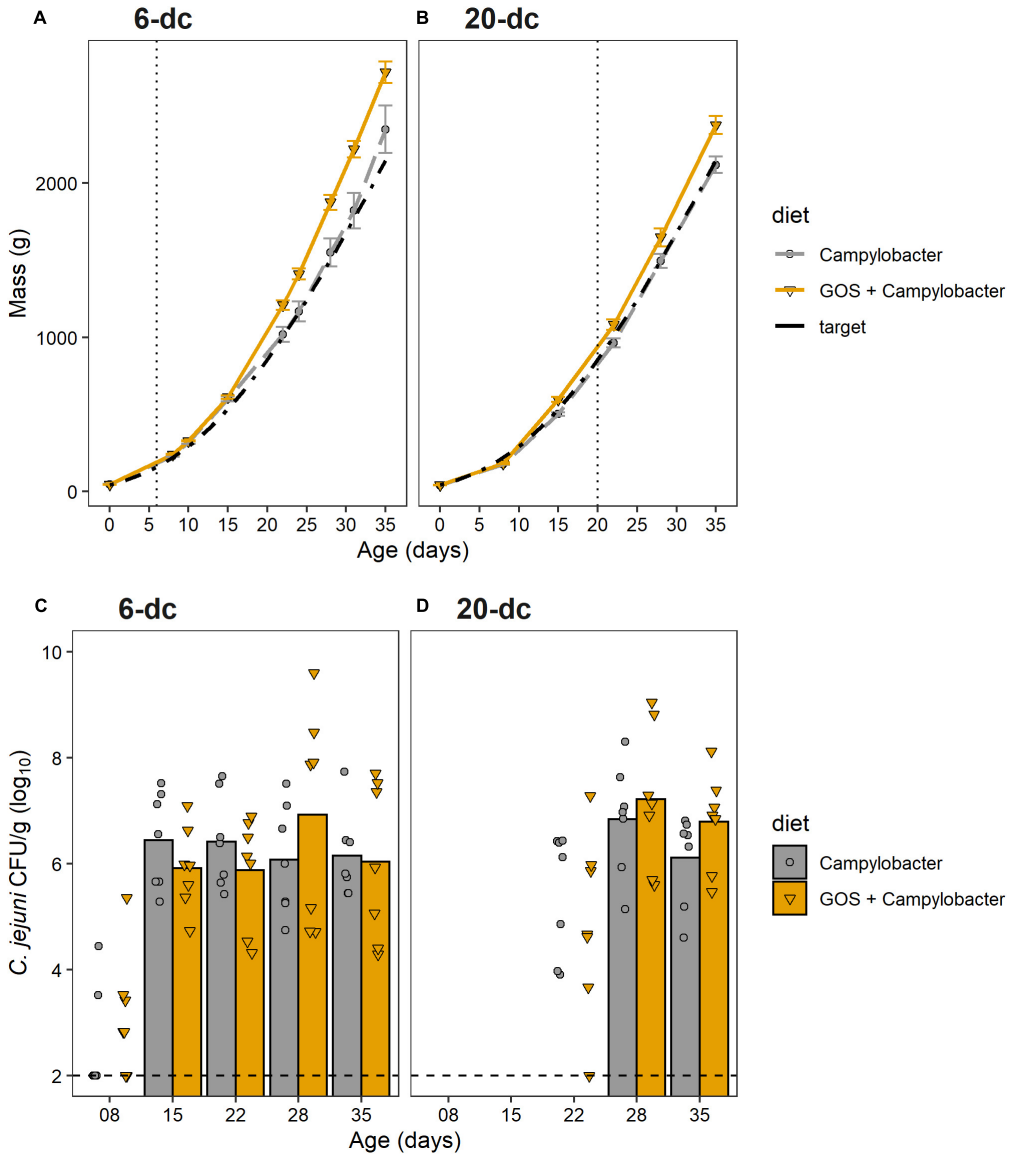


FIGURE 1 | GOS improves growth performance of broiler chickens, but does not affect *C. jejuni* colonization of the ceca. **(A,B)** Chicken live total mass from day of hatch to 35 days for 6-dc birds **(A)** and 20-dc birds **(B)**. The plots show the performances of the experimental treatments GOS + *Campylobacter* (GOS diet) *Campylobacter* (control diet) and the Ross 308 performance objective as the Target (Aviagen Performance Objectives, 2014). The dashed line indicates age at challenge. **(C,D)** Viable counts of *Campylobacter* recovered from the cecal content of 6-dc birds **(C)** and 20-dc birds **(D)**. Data markers indicate *Campylobacter* CFU isolated from individual birds. Bars indicate mean *Campylobacter* CFU, excluding cohorts where *Campylobacter* levels were below the limit of detection. There are no significant differences in the *Campylobacter* counts for the GOS diet compared to control diet post colonization ($p > 0.05$). The dashed line indicates minimum level of detection.

rearing period with no significant differences between the diets at any time, independent of the age of challenge.

Intestinal Villus and Crypt Metrics Were Affected by Dietary Galacto-Oligosaccharide Post-infection With *Campylobacter jejuni*

Villus and crypt metrics were determined from 10 well-oriented villi for 3 to 4 birds from each group in a blind assessment of formalin-fixed H&E-stained ileum sections. Measurement comparisons of the GOS and control diet groups for 6-dc birds showed greater villus length at 8 (2 dpi; $p = 0.04$) and 15 days (9 dpi; $p = 0.002$) for the *C. jejuni* colonized GOS-fed chickens compared to the *C. jejuni* colonized birds on control feed (**Table 2**). By 22 days the villus lengths of the 6-dc treatment groups were not significantly different and remained so until the end of the trial at 35 days. Comparison of the crypt depth measurements demonstrated that the GOS-fed birds at 15 days ($p = 0.005$) had significantly deeper crypts than the birds on the

TABLE 2 | Ileal histomorphometry.

	Histology measurements									
	8 days	**SD**	**15 days**	**SD**	**22 days**	**SD**	**28 days**	**SD**	**35 days**	**SD**
Villus length (μm)										
Campy (6-dc)	550	74	560	64	803	33	940	71	934	32
Campy + GOS (6-dc)	671	15	796	58	824	61	814	63	888	40
Campy (20-dc)	–		–		636	56	746	88	954	98
Campy + GOS (20-dc)	–		–		766	82	922	114	1092	124
p-value (6-dc)	0.04		0.002		0.56		0.09		0.31	
p-value (20-dc)	–		–		0.04		0.05		0.13	
Crypt depth (μm)										
Campy (6-dc)	117	12	111	7	125	8	128	5	121	8
Campy + GOS (6-dc)	110	8	133	4	128	5	132	8	122	11
Campy (20-dc)	–		–		82	16	92	24	104	22
Campy + GOS (20-dc)	–		–		106	12	102	38	128	44
p-value (6-dc)	0.32		0.005		0.65		0.49		0.69	
p-value (20-dc)	–		–		0.05		0.67		0.37	
Villus length/Crypt depth ratio (v/c)										
Campy (6-dc)	4.70	0.84	5.05	0.60	6.42	0.17	7.34	0.63	7.72	0.82
Campy + GOS (6-dc)	6.10	0.38	5.98	0.39	6.44	0.31	6.17	0.59	7.28	0.27
Campy (20-dc)	–	–	–	–	7.76	0.80	8.11	0.74	9.17	0.91
Campy + GOS (20-dc)	–	–	–	–	7.23	0.91	9.04	0.82	8.53	0.72
p-value (6-dc)	0.02		0.04		0.83		0.08		0.35	
p-value (20-dc)	–		–		0.42		0.14		0.31	

Villus length, crypt depth and ratio villus length/crypt ratios presented as mean values (±standard deviation) of the measurement of 10 well-orientated villi for 3 to 4 birds per group. Corresponding probabilities (p) were calculated using ANOVA tests, differences were considered significant at $p < 0.05$.

control diet. Differences in the villus and crypt measurements affected differences in the villus to crypt ratios at 8 days ($p = 0.02$) and 15 days ($p = 0.04$) with the GOS-fed birds exhibiting greater ratios. For the 20-dc cohorts the GOS-fed birds also exhibited significant increases in villus height compared to the control diet after *C. jejuni* colonization at 22 days (2 dpi; $p = 0.04$) and 28 days (8 dpi; $p = 0.05$). A significant increase in the crypt depth for the GOS-fed birds over the birds on the control diet was also recorded at 22 days ($p = 0.05$), but not thereafter. These differences did not result in significant differences in the villus to crypt ratios for the 20-dc experiment.

Dietary Galacto-Oligosaccharide Modulates IL-17 Transcription Post *Campylobacter* Colonization

Immuno-modulatory effects of dietary GOS on the innate immune responses of intestinal tissues from *C. jejuni*-colonized broilers were assessed. RNAs were extracted from biopsies collected from ileal and cecal tissues to enable RT-PCR quantification of cytokine and chemokine gene transcripts representing the major inflammatory pathways of chickens. Cytokines IL-17A, IL-17F, IL-6, IL-1β and chemokines ChCXCLi-1, ChCXCLi-2 (also known as ChIL-8) have previously been described as markers of the Th17 pathway (Reid et al., 2016). IFN-γ is related to the Th1 pathway and the anti-inflammatory cytokine IL-10 is largely expressed from regulatory T cells (Treg) in chickens to control the inflammatory effects of the Th cell responses.

The innate immune response of ileal tissues for the early challenged birds (6-dc) was characterized by modulation in the expression of IL-17A in *C. jejuni*-colonized birds on control feed compared to *C. jejuni*-colonized GOS-fed birds (**Figure 2A**). Following *Campylobacter* challenge IL-17A transcript levels were far greater in the GOS-fed birds (884-fold difference) than those on the control diet at 8 days (2 dpi; *p-adj* = 0.005). By 15 days the difference had declined to 12-fold due to an increase in IL-17A in the *C. jejuni*-colonized birds on the control diet (9 dpi; *p-adj* = 0.02). The rise in IL-17A continued in the birds on the control diet until 35 days such that a significant difference was recorded in favor of the control diet birds from 22 days (16 dpi; *p-adj* = 0.02), whilst the IL-17A levels were maintained in the GOS-fed birds throughout the time course. The pro-inflammatory cytokine IL-17F exhibited a significant increase in expression at 22 days (16 dpi; *p-adj* = 0.03) and 28 days (22 dpi; *p-adj* = 0.005) in the control birds. Over the transition period, the regulatory cytokine IL-10 expression was significantly greater at 15 days (9 dpi; *p-adj* = 0.05) and 22 days (16 dpi; *p-adj* = 0.03) in the *C. jejuni*-colonized birds on control diet compared to the GOS diet (GOS + *Campylobacter* treatment). For the late challenged birds (20-dc; **Figure 2B**), IL-6 was recorded as significantly greater for the *C. jejuni*-colonized birds on the control diet at 35 days (29 dpi; *p-adj* = 0.005), largely owing to a fall in the IL-6 levels of the birds on the GOS diet (GOS + *Campylobacter* treatment).

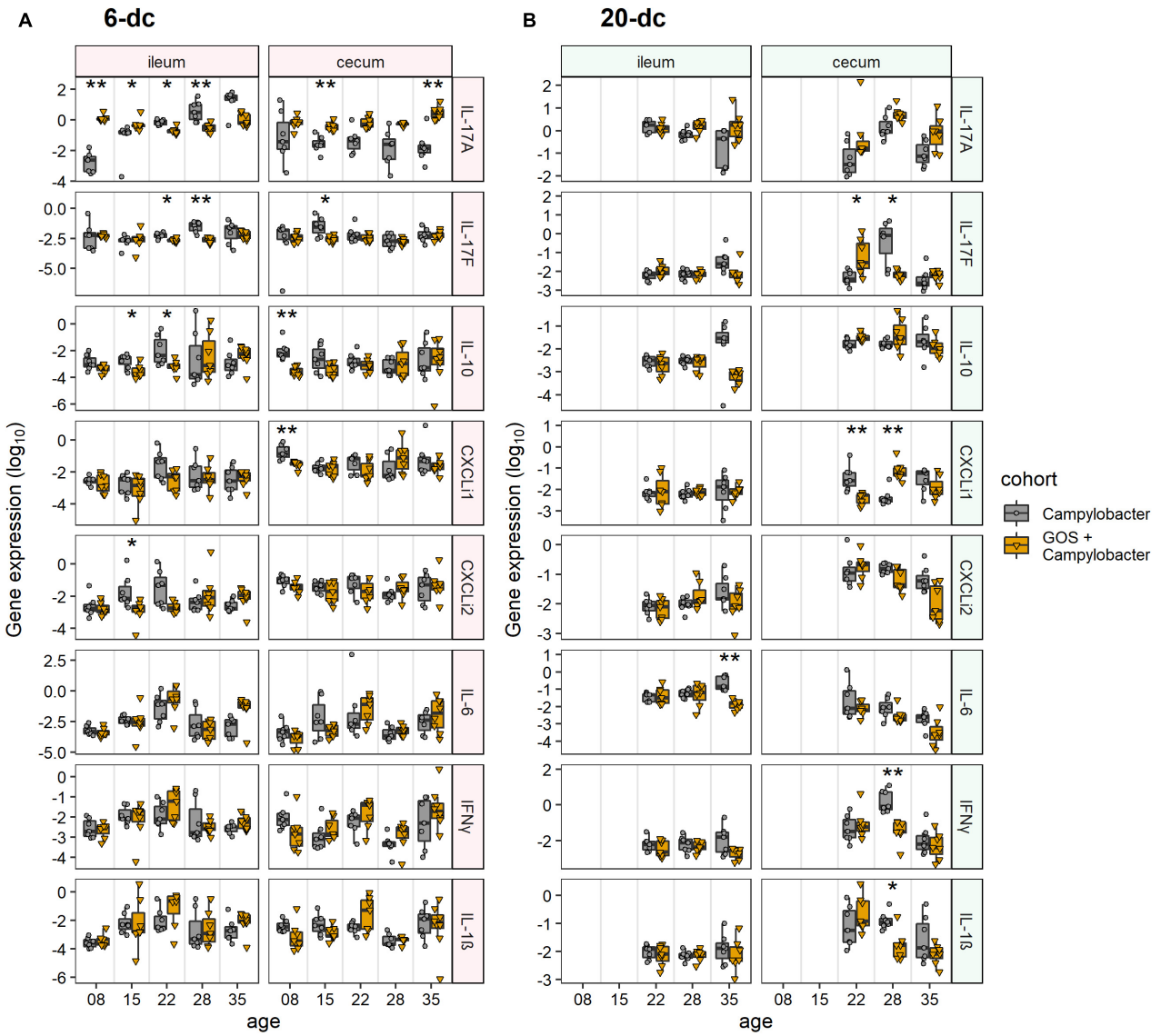


FIGURE 2 | Modulation of cecal and ileal innate immune responses to dietary GOS in *Campylobacter*-challenged broiler chickens. The figures report cytokine and chemokine normalized gene expression for 6-dc birds **(A)** and 20-dc birds **(B)** fed a control diet (*Campylobacter* treatment) or a GOS diet (GOS + *Campylobacter* treatment) recorded as $\log_{10}$ of the ratio for gene of interest/GAPDH from qPCR data of individual birds. Corresponding probabilities (*p-adj*) were calculated using non-parametric Wilcoxon rank sum tests with Benjamini-Hochberg FDR correction, where differences at *p-adj* < 0.05 were considered significant and are summarized in the plot using asterisks (**p-adj* ≤ 0.05; ***p-adj* ≤ 0.01).

In cecal tissues IL-17A expression was also maintained in the GOS-fed birds post *C. jejuni* colonization. In the early challenge experiment (6-dc) IL-17A expression levels declined in the birds fed the control diet throughout the time course with significant differences recorded at 15 days (9 dpi; *p-adj* = 0.005) and 35 days (288-fold at 29 dpi; *p-adj* = 0.01) compared to the birds on the GOS containing diet (**Figure 2A**). Pro-inflammatory IL-17F exhibited a significant increase at 15 days in the *C. jejuni*-colonized birds fed the control diet compared to the birds on the GOS diet (9 dpi; *p-adj* = 0.04). This was preceded by differential increases at 8 days in IL-10 (2 dpi; *p-adj* = 0.002) and ChCXCLi-1 (2 dpi; *p-adj* = 0.002). The late challenged birds (20-dc) featured a significant switch in the expression of IL-17F from low to high for the *C. jejuni*-colonized birds on the control diet between 22da (2 dpi; *p-adj* = 0.04) and 28 days (8 dpi; *p-adj* = 0.02), and conversely the birds on the GOS diet exhibited a high to low change in gene expression over the period (treatment GOS + *Campylobacter*). Increases in IL-17F were accompanied by significant increases in IFN-γ (8 dpi; *p-adj* = 0.005) and IL-1β (8 dpi; *p-adj* = 0.02) at 22da for the *C. jejuni*-colonized birds on the control diet. Transcription of the chemokine ChCXCLi-1 was observed to exhibit the opposite trend to IL-17F over the 22 days (2 dpi; *p-adj* = 0.005) to 28 days (8 dpi; *p-adj* = 0.005) transition, with a fall in the mean expression value for the

C. jejuni-colonized birds on the control diet compared to an increase in the *C. jejuni*-colonized GOS-fed birds (treatment GOS + *Campylobacter* in **Figure 2B**).

Galacto-Oligosaccharide -Induced Microbiota Diversity Shifts in *Campylobacter*-Challenged Birds

At 2 dpi (8 days) the α-diversity of the cecal microbiota of 6-dc birds was lower in GOS + *Campylobacter* treated birds than the *C. jejuni*-colonized birds on the control diet (treatment *Campylobacter*), as indicated by lower Shannon entropy (p = 0.046) and inverse Simpson index (p = 0.022; **Figure 3A**). This may be attributed to the dietary GOS as the inverse Simpson's index of mock-challenged birds on a GOS diet was also lower than that of the *Campylobacter* treatment birds on the control diet (p = 0.032), with the difference observed in the corresponding Shannon entropy values approaching the significance threshold (p = 0.058). Shannon entropy was also lower in GOS + *Campylobacter* treatment birds at 28 days (22 dpi; p = 0.009) and 35 days (29 dpi; p = 0.038) as presented in **Figure 3A**. There were no other observed differences in α-diversity or species richness (Chao) in 6-dc birds. In the 20-dc experiment at 22 days (2 dpi) the inverse Simpson index of the cecal microbiota of the GOS + *Campylobacter* treatment birds was significantly greater than *Campylobacter* treatment birds (p = 0.0469; **Figure 3B**). The responses recorded for the inverse Simpson index at 2 dpi for the 6-dc and 20-dc experiments show opposite trends, however, a similar trend can be noted in the higher α-diversity of 6-dc GOS + *Campylobacter* treatment birds at the same age (22 days), although these changes did not reach significance. The modulation in α-diversity at this age could be attributable to changes in host development or changes in diet formulation of grower to finisher related to husbandry.

Comparison of the phylum composition of 6-dc birds at 8 days (2 dpi) indicates that the cecal microbiota of *Campylobacter* treatment birds was significantly different from the microbiota of the GOS + *Campylobacter* treatment birds (p = 0.0003) (**Figure 3C**). Comparison of the microbiota of GOS + *Campylobacter* treatment birds with the cecal microbiota of age-matched mock-challenged birds on the GOS diet alone at 8 days (2 dpi) did not reveal any phyla-level differences (p = 0.1613), whilst mock-challenged birds on either control or GOS diets also had different phyla compositions (p = 0.0096). These data suggest that the differences in microbiota ecology are linked to dietary GOS and not *Campylobacter*-colonization *per se*. No difference in phylum-composition was determined in 6-dc birds at 15 days (9 dpi; p = 0.301), 22 days (16 dpi; p = 0.69) or 35 days (p = 0.055). However, the composition of the cecal microbiota of birds from the GOS + *Campylobacter* and the *Campylobacter* treatment groups were different at 28 days (22 dpi; p = 0.0004), which likely corresponds with the reverse in α-diversity first observed at this age. No differences were determined in the phylum-composition of 20-dc birds at 2 dpi (p = 0.742) (**Figure 3D**).

At OTU level 16S rRNA gene sequencing reads were clustered at 97% similarity, which serves as a proxy for species-level distinction. The 6-dc birds at 8 days (2 dpi) show a key differential OTU between *Campylobacter*-colonized birds on the control diet (*Campylobacter* treatment) versus *C. jejuni*-colonized birds on the GOS diet (GOS + *Campylobacter* treatment) had strong sequence similarity to *Escherichia coli* (OTU0001; 99.21%, **Supplementary Figure S2A**). OTU0001 did not discriminate the microbiota of the *Campylobacter*-challenged birds on the control diet (*Campylobacter* treatment) from the microbiota of mock-challenged birds on the GOS diet (**Supplementary Figure S2C**). In addition, previous analysis of the mock-challenged controls by our laboratory indicated that *Lactobacillus johnsonii* outcompetes *L. crispatus* in GOS-fed birds (Richards et al., 2019b). In the analysis presented here OTU0002 has strong sequence similarity with the *L. crispatus* strain (97.21%) and OTU0017 has a strong sequence similarity with *L. johnsoniii* (98.03%). In 6-dc birds at 8 days (2 dpi) both OTU0002 (*L. crispatus*) and OTU0017 (*L. johnsoniii*) are associated with dietary GOS (**Supplementary Figure S2B**). However, *L. johnsoniii* (OTU0017) continues to be associated with dietary GOS at: 15 days (9 dpi), 22 days (16 dpi) and 28 days (22 dpi). Whereas, *L. crispatus* (OTU0002) is later associated with the control diet at 15 days (9 dpi) and 35 days (29 dpi).

DISCUSSION

Concerns are growing regarding the over use of antimicrobials in animal production, and any concomitant increase in risk of antimicrobial resistance transferring to humans (Landers et al., 2012). These concerns have brought about calls for restricting antibiotic use in food producing animals (Tang et al., 2017). Although the European Union banned antimicrobial growth promoters since 2006, the practice continues in many countries and has prompted calls for a worldwide ban, particularly in the poultry and pig industries. It is becoming increasingly evident that improved on farm-performance at the expense of intestinal health and the zoonotic dissemination of antimicrobial resistance cannot continue (Awad et al., 2015, 2017; Smith et al., 2016). Under increasing economic pressure, the poultry industry is pursuing effective alternative methods to promote bird growth whilst controlling farm sources of antimicrobial resistance and zoonotic disease. Prebiotic feed additives such as GOS, a by-product of the dairy industry, have revealed potential growth-promoting effects in piglets (Alizadeh et al., 2016) and chickens (Richards et al., 2019b), whilst fructo-ologosaccharide (FOS) have been reported to reduce *Salmonella* colonization of chicks (Fukata et al., 1999). Here, we assess the impact of a GOS prebiotic on chickens colonized by *C. jejuni* at either 6 or 20 days with respect to their zootechnical performance, gut architecture and differences in intestinal cytokine and chemokine transcription (the experimental designs and corresponding data are summarized in **Figure 4**).

Two independent trials show significant differences in live bodyweights of *C. jejuni*-challenged birds, the birds fed GOS additive were significantly heavier at the typical market age of 35 days compared to a calorie-match control diet. GOS

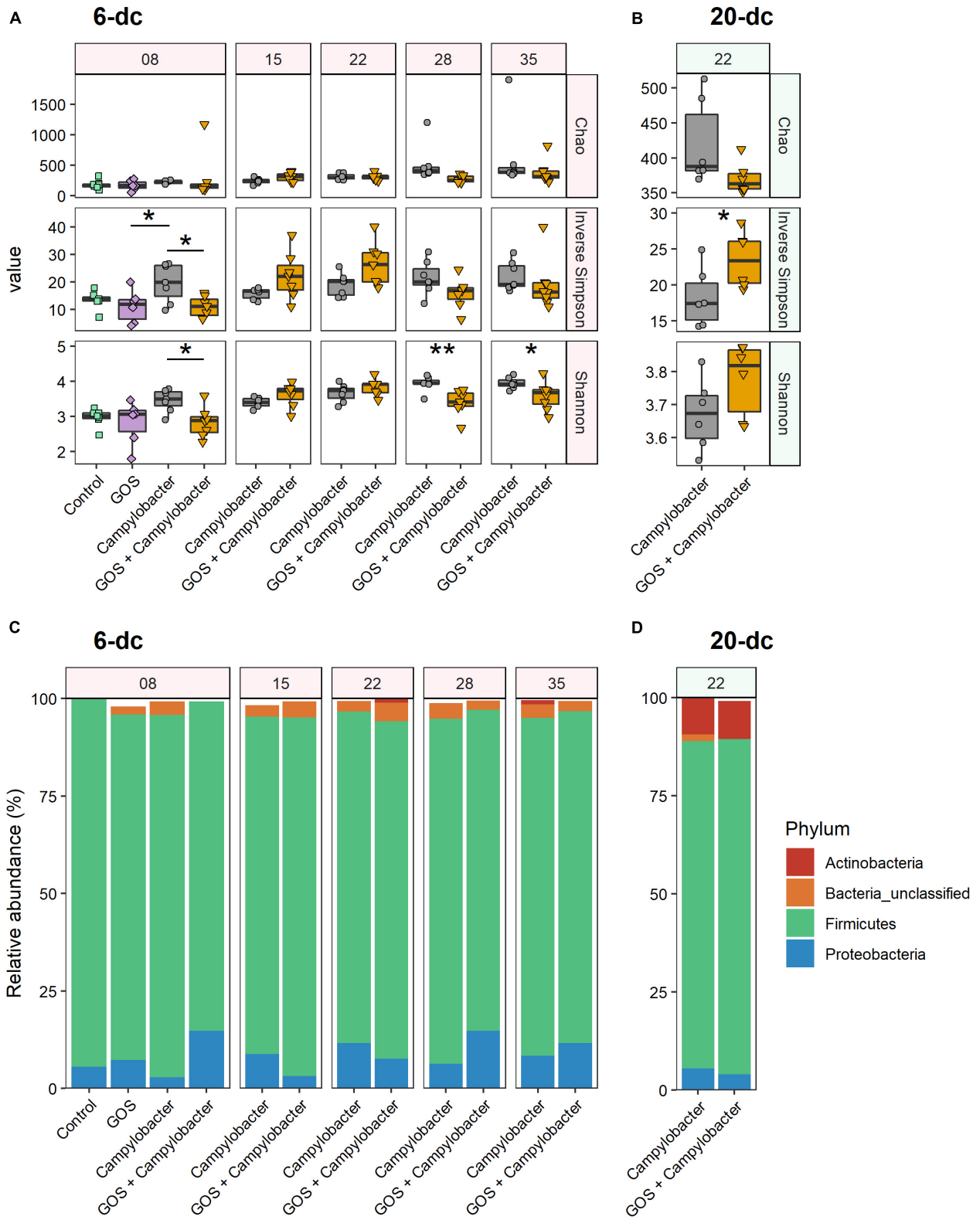


FIGURE 3 | Dietary GOS promotes differential shifts in the cecal microbiota post *Campylobacter* challenge. **(A,B)** Cecal α-diversity for 6-dc birds **(A)** and 20-dc birds **(B)** described as Chao richness, Inverse Simpson diversity and Shannon diversity (as indicated by panels on right hand side of the figure). The age of the birds are in days as indicated by the numerals in the strip at the top of the figure. Significant differences between groups are indicated by asterisks (*$p \leq 0.05$; **$p \leq 0.01$). **(C,D)** OTU relative abundance for 6-dc birds **(C)** and 20-dc birds **(D)** summarized at Phyla level.

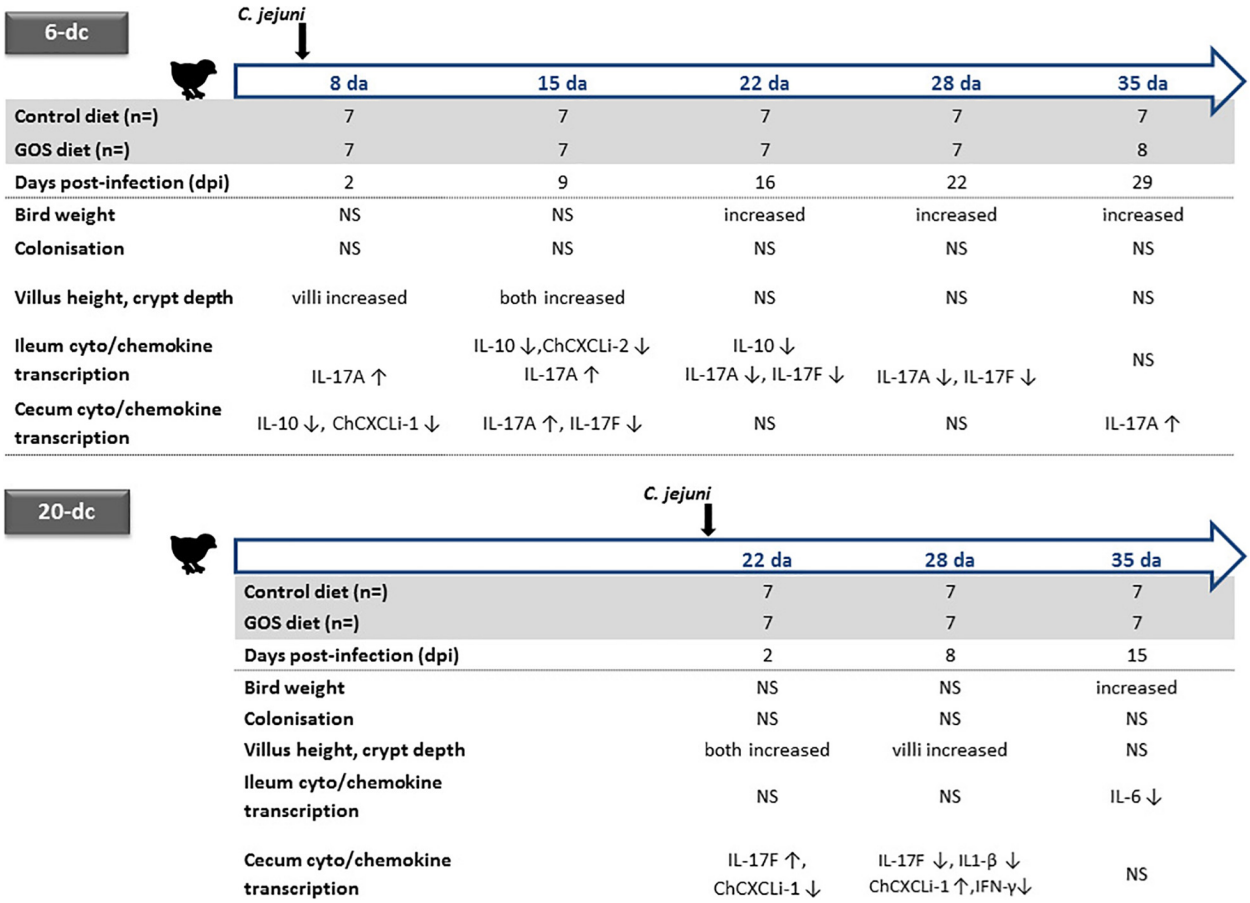


FIGURE 4 | Summary of age dependent differences for dietary GOS in *C. jejuni* colonized broiler chickens. Significant changes in the live weight, cecal *C. jejuni* colonization levels, ileal villus and crypt metrics are indicated for the GOS-fed birds compared to the birds on the control diet colonized at either 6 or 20 days of age (da) with *C. jejuni*. Relative changes in the transcription of ileal and cecal cytokines and chemokines for GOS-fed bird compared to controls are indicated for age matched birds by up arrows (↑) for increases and down arrows for decreases in cytokine/chemokine expression (↓). NS indicates no significant differences between the birds on the GOS or control diet.

supplementation improved the growth rate performance of *Campylobacter*-colonized broiler chickens irrespective of the timing of the challenge with *C. jejuni* at either 6 days or 20 days of age. However, dietary GOS inclusion did not prevent or reduce *C. jejuni* HPC5 colonization of broiler chickens GIT within the 35 days lifespan of the birds. *C. jejuni* HPC5 is a broiler chicken isolate that has been used routinely and reliably to colonize the intestinal tract of broiler chickens (Loc Carrillo et al., 2005; Scott et al., 2007; Connerton et al., 2018). In recent years several studies have employed 16S rRNA gene sequences to comprehensively document changes in the cecal microbiota that accompany *Campylobacter*-colonization of broilers. These studies have noted differences in the relative abundance of Bifidobacterium, Lactobacillaceae, Clostridium cluster XIVa and Mollicutes, with transient age related shifts in specific members of the Lachnospiraceae and Ruminococcaceae (Thibodeau et al., 2015, 2017; Connerton et al., 2018; Richards et al., 2019a). As an extension of the outputs from these studies it was recognized that transitions in the cecal microbiota were evident between 14 and 18 days that coincide with the reduced availability of maternal antibodies, and represent a window of opportunity for the entry for bacteria to bloom and new intestinal microbes to become established that can affect changes in gut health (Awad et al., 2016; Connerton et al., 2018; Ijaz et al., 2018). Early prebiotic diets offer the prospect of achieving a stable microbiota that can resist opportunist expansion or colonists. Prebiotic oligosaccharides have previously been reported to reduce the cecal *C. jejuni* colonization loads of broiler chickens, for example the use of a chicory fructan additive for 42 days in male Ross 308 birds (Yusrizal and Chen, 2003), or as the use of mannan-oligosaccharide (MOS) with male Cobb 500 birds at 34 days (Baurhoo et al., 2009). It is conceivable that improved broiler breeding programs and optimized diets will produce heavier birds faster, which will require fast-resolving methods that reduce or displace unwelcome gut bacteria.

Intestinal histomorphometric parameters are considered indicators of gut health whereby a healthy ileal mucosa should display long villi with high villus/crypt ratios. A transient increase

in villus length and crypt depth was observed over the first 2 weeks post-challenge for birds sustained on the GOS diet irrespective of the timing of *Campylobacter* challenge. Increases in the absorption surface provide a favorable environment for nutrient uptake leading to efficient feed utilization. Several studies have shown the beneficial effects of dietary additives on Ross 308 broiler chicken villus architecture and body weight gain during heat stress challenges such as prebiotic supplements (Silva et al., 2010), alpha-lipoic acid additive (Yoo et al., 2016), or probiotic mixtures (Song et al., 2014). Similarly, the data presented here suggests that nutrient absorption competence associated with the development of villi in the small intestine of birds fed the GOS diet, leads to an increase in body weight despite gut colonization by *C. jejuni*.

Intestinal mucosa constitutes a physical and immunological protective barrier for the integrity of the intestinal tract to prevent infection by pathogens and maintain an environment that can sustain a healthy and productive microbiota. However, the composition of the gut microbiota is under surveillance of the mucosal innate and adaptive immune systems (Kraehenbuhl and Neutra, 1992). Numerous immune cell populations such as regulatory T-cells (Treg), Th17 cells, IgA-secreting plasma cells, natural killer cells (NK), macrophages, dentritic cells (DCs), innate lymphoid cells (ILCs) contribute to host defense against infection with pathogenic microbes (Honda and Littman, 2012). Members of the IL-17 family of cytokines, IL-17A and IL-17F are produced by a subset of CD4 + T cells named Th17, and have more recently been associated in the gut with dendritic cells and group 3 innate lymphoid cells (ILC3) (Li S. et al., 2018). While they beneficially mediate resistance to extracellular bacterial and fungal infection via enhanced mucosal production of mucus and antimicrobial peptides, IL-17A and IL-17F are also involved in several autoimmune disorders (Bettelli et al., 2007; Yang et al., 2008; Ishigame et al., 2009).

Here, we demonstrate dietary GOS inclusion can maintain transcript levels of IL-17A and suppress IL-17F post colonization with *C. jejuni*. Previously we have shown *C. jejuni* challenge at 6 days triggered a transient increases in IL-17A and IL-17F in broiler chickens at 15 days (9 dpi) compared with non-colonized birds (Connerton et al., 2018), and more recently that dietary GOS increases the expression of IL-17A in juvenile birds up to 15 days (Richards et al., 2019b). In mice IL-17A expression has been proposed to benefit intestinal barrier function and IL-17F to weaken intestinal integrity, since IL-17A inhibition exacerbates induced colitis (Maxwell et al., 2015) and IL-17F suppression is protective (Tang et al., 2018). Evidence suggests the modulation of IL-17A is associated with the regulation of the tight junction formation and regulation of the mucosal barrier via activation of the ERK MAPK pathway (Awane et al., 1999; Cario et al., 2000; Kinugasa et al., 2000). *C. jejuni* colonization of the chicken intestine appears to result in the expression of both the IL-17 subtypes with opposing effects mediated at different times. The dominant response may in part explain why there are differences in the impact of *C. jejuni* colonization reported for different bacterial types on various broiler chicken breeds (Humphrey et al., 2014, 2015). An increase of IL-17A in response to dietary GOS in the presence or absence of *C. jejuni* colonization may well benefit intestinal health and contribute to the increased growth rate observed for GOS-fed birds. GOS does not prevent *C. jejuni* colonization but may also lead to performance improvements by suppression of IL-17F under circumstances when *C. jejuni* colonization has been reported to be associated with bird health and productivity (Bull et al., 2008). These current studies were conducted in clean controlled biosecure facilities such that the birds would not be subject to exposure to endemic viral, bacterial and protozoal pathogens that are frequently encountered by commercial broiler chickens. Under these circumstances the impact of prebiotic priming of intestinal innate immunity may be of greater importance in field applications.

Consistent with previous reports we observed GOS-driven changes in the cecal microbiota of chickens featuring specific operational taxonomic units identifiable as lactobacilli (Hughes et al., 2017; Azcarate-Peril et al., 2018). Colonization by *C. jejuni* did not prevent the differential increase in abundance of Otu0017 (*L. johnsoniii*) associated previously with dietary GOS (Richards et al., 2019b). At 2dpi for the 6-dc birds Otu0002 (*L. crispatus*) and Otu0017 (*L. johnsoniii*) showed increases in abundance in association with the GOS-diet. However, Otu0017 (*L. johnsoniii*) remained an abundant member of the GOS + *Campylobacter* treatment group over the course of the experiment, and in contrast the relative abundance of Otu0002 (*L. crispatus*) fell such that it exhibited significantly greater abundance at 9 and 29 dpi in the *Campylobacter* treatment group fed the control diet. These data are consistent with *L. johnsonii* outcompeting *L. crispatus* in GOS-fed birds, and consistent with the hypothesis that *L. johnsonii* contributes to the stability of the innate immune response observed in GOS-fed birds. *L. johnsonii* is an established probiotic species that has been reported to improve growth performance, intestinal development, and act as competitive exclusion agent against bacterial pathogens in broiler chickens (La Ragione et al., 2004; Wang et al., 2017).

IL-17A responses to probiotics have been reported for *ex vivo* Peyer's patch stimulated T cells derived from mice orally administered with lactic acid bacteria (*L. bulgaricus* or *Streptococcus thermophilus*). Over 7 days the stimulated T cells exhibited increases in the levels of IL-17 whilst IL-10 and Th2 IL-4 remained unchanged (Kamiya et al., 2016). In chickens transient IL-17 induction has been observed during the natural development the intestinal microbiota (Crhanova et al., 2011). In the absence of IL-22, pro-inflammatory Th17 induction did not result in intestinal damage but upon *Salmonella* Enteritidis challenge tissue damage was observed as a result of a Th17 response that features the cytokines IL-17 and IL-22.

CONCLUSION

In conclusion the data support the contention that GOS diet-induced microbiota shifts can: (1) Improve the growth rate

of broiler chickens independent of *C. jejuni* colonization. (2) Maintain ileal and cecal IL-17A transcription that can positively influence gut health in the presence of *C. jejuni*. (3) Suppress IL-17F expression arising as a consequence *C. jejuni* colonization that has the potential to impair gut integrity and health.

DATA AVAILABILITY STATEMENT

All 16S rDNA sequence data originally reported here is available at under accessions SRR10059315 to SRR10059356 in NCBI SRA study SRP133552. All other 16S rDNA sequences reported in this study are also available from NCBI SRA study SRP133552. Raw zootechnical and qPCR gene expression data is available from https://github.com/PJRichards/lafontaine_campy_gos.

ETHICS STATEMENT

Experiments involving the use of birds were subjected to approval process under National Guidelines by the United Kingdom Home Office. Work on this project was approved under United Kingdom Government Home Office Project Licensing ASPA 86. The project license has been reviewed and approved by the University Ethics Committee prior to submission to the Home Office, which includes the scrutiny of animal welfare, ethics and handling.

AUTHOR CONTRIBUTIONS

NF contributed to the study design. NF and IC conceived and designed the experiments. GF, PR, PC, PO'K, NG, NC, and IC performed the experiments. GF, PR, PC, and IC analyzed the data and wrote the manuscript. All authors approved the final manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03030/full#supplementary-material

FIGURE S1 | Rarefaction curves indicating sampling efficiency of cecal bacterial communities. Collectors curved were constructed for 16S rDNA sequences curated using mothur pipeline. Communities are presented by cohort as indicated by text in strip at the top of each panel. The colors indicate the rarefaction curves for individual bird in each cohort (key inset).

FIGURE S2 | GOS responsive OTUs in age-matched broilers. Discriminative OTUs were identified using LEfSE between 6-dc challenged birds: Treatments *Campylobacter* and GOS + *Campylobacter* **(A)**, 20-dc challenged birds: Treatments *Campylobacter* and GOS + *Campylobacter* **(B)**, and 6-dc *Campylobacter*-challenged and GOS mock-challenged control birds: Treatments *Campylobacter* and GOS **(C)**. Comparisons of OTU relative abundance were made between age-matched cohorts that were rarefied to include only OTU $\geq$ 10 reads. For clarity only OTU with $p < 0.05$ for the embedded Kruskal Wallis ANOVA test and LDA ($\log_{10}$) > 2 are reported (Mothur defaults).

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The remaining authors declare that the research was conducted in the absence of any conflict of interest.

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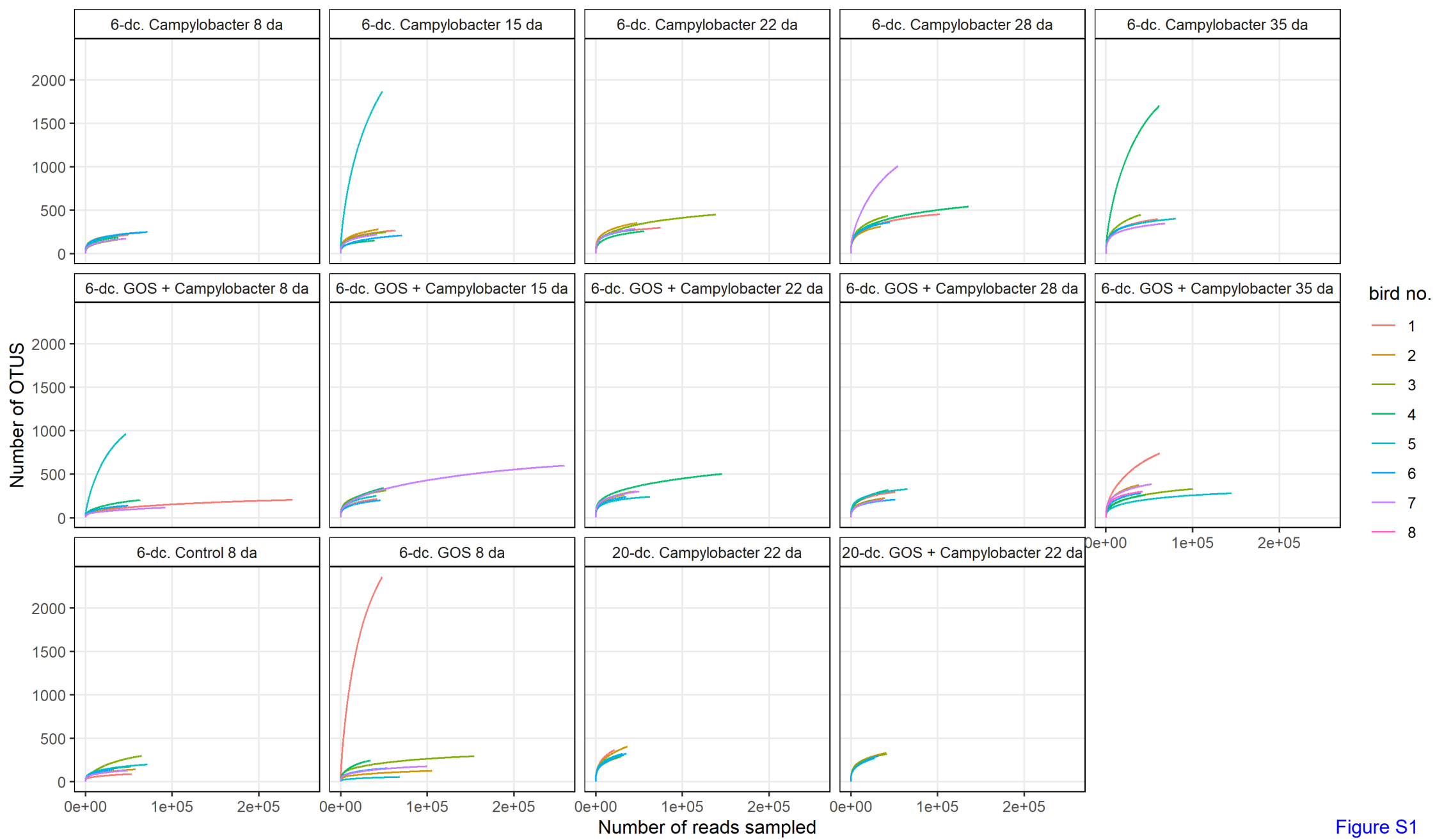

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Number of OTUS
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1500
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Number of reads sampled
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Figure S1

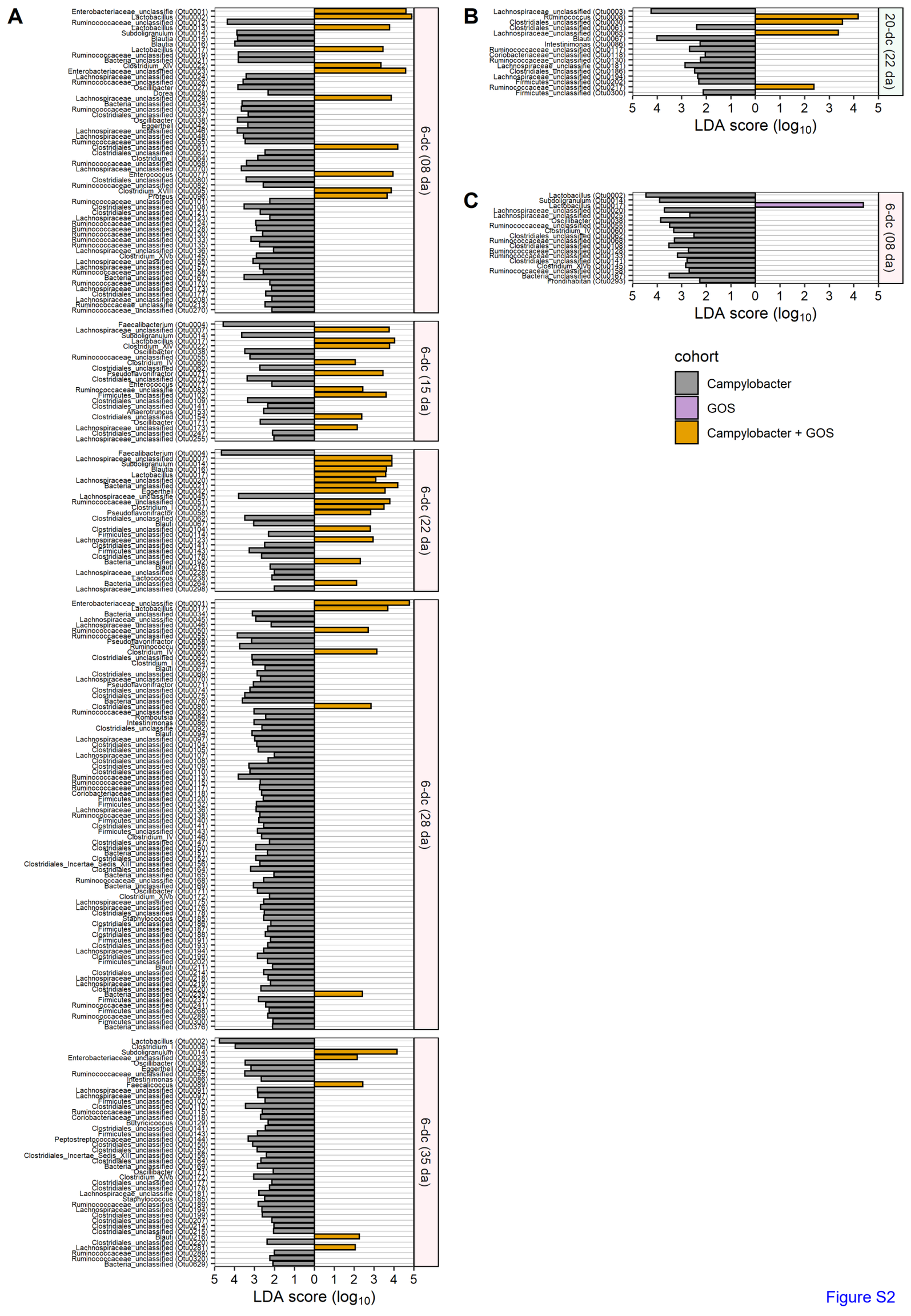

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LDA score (log10)
cohort
Campylobacter
GOS
Campylobacter + GOS

Figure S2

CHAPTER 5. CHARACTERIZING THE EFFECT OF *IN VIVO* PHAGE PREDATION OF *CAMPYLOBACTER JEJUNI* ON THE MICROBIOTA

This chapter relates to the results reported in the peer-reviewed, open access article cited below and reproduced following this section (pp. 29).

Richards, P.J., Connerton, P.L., Connerton I.F. 2019. Phage biocontrol of *Campylobacter jejuni* in chickens does not produce collateral effects on the gut microbiota. *Frontiers in Microbiology* 10. DOI: 10.3389/fmicb.2019.00476

5.1 Summary

In Richards et al. (2019) we have shown that exposing broiler chickens to a cocktail of phages reduces *C. jejuni* colonization of the gastrointestinal tract by ~2 $\log_{10}$ CFU ml^{-1}. Although we have established that the *C. jejuni* HPC5 strain used induces a mild pro-inflammatory response in the ceca (see Chapter 4) we observed no concomitant dysbiosis of the gut microbiota. We did not profile the host response to *C. jejuni* challenge.

5.2 Attribution of work

For the work presented in Richards et al. (2019), I isolated gDNA from caecal and ileum content from which the 16S rDNA amplicon libraries were generated for sequencing. Post-sequencing, I analysed the microbiota data that was generated. I also analysed the *C. jejuni* and phage counts and contributed to the manuscript. I had no role in the collection of the biological samples or the laboratory enumeration of the recovered phage or *C. jejuni*.

5.3 Phage biocontrol of pathogens

Trials performed in commercial broiler houses have shown that phage therapy can be used as an effective intervention against *C. jejuni* in broiler chickens (Kittler et al., 2013). On the basis of comparative genome analysis it has been recommended that the Epsilonproteobacteria are should be excluded from the phylum Proteobacteria and be reclassified a Campylobacterota (Waite et al., 2017). Phage with cross-species infectivity against *C. jejuni* and *Campylobacter coli* have been shown to have no activity against other Campylobacterota or against a panel of four different Enterobacteriaceae genus *in vitro* (Carrillo et al., 2005). However, prior to publication of Richards et al. (2019) it had not been established whether *Campylobacter* phage had broader activity against other bacterial species populating the chicken gut. Additionally, *C. jejuni* has a pro-inflammatory effect in

the broiler chicken cecum (Connerton et al., 2018; Reid et al., 2016) and it was unknown if rapid reduction in *C. jejuni* levels followed by adaptation of host immunity had the potential to destabilize the ecological balance in the gut resulting in dysbiosis. We did not detect any changes in microbiota composition apart from a reduction in the relative abundance of *C. jejuni*. Our work shows that exposure of the caecal microbiota to phage targeting *C. jejuni*, and *C. coli*, only reduces the levels of the target organisms and did not induce gut dysbiosis. However, we did not profile the host response to *C. jejuni* challenge.

Mixed populations of microbiota-associated phage have been linked to the stunting of children (Mirzaei et al., 2020), but there is no evidence that narrow-spectrum phage targeted at restricted taxa within the microbiota are detrimental to the host. An elegant study that ameliorated alcoholic-induced liver disease in mice by reducing cytolytic *Enterococcus faecalis* levels by ~1 $\log_{10}$ CFU g^{-1} with targeted phage therapy did not detect collateral changes in the microbiota (Duan et al., 2019). Likewise, as discussed in Richards et al. (2019), targeting pathogenic *E. coli* with phage therapy released less endotoxin that β-lactam antibiotics (Dufour et al., 2017). These findings suggested that targeted phage therapy does not cause chaotic shifts in microbiota constitution. However, it must be noted that these studies did not profile host immune parameters in the gut. Gut inflammation has been linked to microbiota composition in humans (Forbes et al., 2018), but additional confirmatory trials will be required to firmly establish that targeted phage-therapy is not harmful. If targeted phage therapy can be employed without collateral effects on the microbiota or host then this approach may be powerfully leveraged to determine effect of keystone taxa on the host:microbe:diet axis.

Richards, P.J., Connerton, P.L., Connerton I.F. 2019. Phage biocontrol of *Campylobacter jejuni* in chickens does not produce collateral effects on the gut microbiota. *Frontiers in Microbiology* 10, 476. DOI: 10.3389/fmicb.2019.00476

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Phage Biocontrol of *Campylobacter jejuni* in Chickens Does Not Produce Collateral Effects on the Gut Microbiota

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Bacteriophage biocontrol to reduce *Campylobacter jejuni* levels in chickens can reduce human exposure and disease acquired through the consumption of contaminated poultry products. Investigating changes in the chicken microbiota during phage treatment has not previously been undertaken but is crucial to understanding the system-wide effects of such treatments to establish a sustainable application. A phage cocktail containing two virulent *Campylobacter* phages was used to treat broiler chickens colonized with *C. jejuni* HPC5. *Campylobacter* counts from cecal contents were significantly reduced throughout the experimental period but were most effective 2 days post-treatment showing a reduction of 2.4 $\log_{10}$ CFU g^{-1} relative to mock-treated *Campylobacter* colonized controls. The administered phages replicated *in vivo* to establish stable populations. Bacteriophage predation of *C. jejuni* was not found to affect the microbiota structure but selectively reduced the relative abundance of *C. jejuni* without affecting other bacteria.

Keywords: bacteriophage, campylobacter, microbiota, chicken, biocontrol

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INTRODUCTION

Campylobacter ssp. cause foodborne illness worldwide (Kaakoush et al., 2015) and represent the most commonly reported zoonotic pathogens in the European Union with 246,307 confirmed cases of gastrointestinal illness in 2016 (European Food Safety Authority [EFSA], 2017). *Campylobacter jejuni* is the most common species causing human disease (83.6%), but the burden of disease caused by *Campylobacter coli* is also significant (8.5%) (European Food Safety Authority [EFSA], 2017). Both species readily colonize the poultry gut, where the impact on flock health and production parameters varies with husbandry practice and the colonizing organism (Gormley et al., 2014; Humphrey et al., 2015). Estimates of broiler chicken flock prevalence vary between nations with 0.6 to 13.1% in the Nordic countries up to 74.2–80% in other European countries (European Food Safety Authority [EFSA], 2010a). Source attribution studies have predicted that up to 80% of human illness is as a result of exposure to campylobacter arising from poultry sources (European Food Safety Authority [EFSA], 2010b). A recent source attribution study referenced at the point of exposure confirmed chicken meat as the most important source of *Campylobacter* enteric disease, with an estimated 65–69% of human campylobacteriosis cases (Ravel et al., 2017). Poultry meat contaminated with digesta during slaughter/processing therefore represents a significant risk to public health (Osimani et al., 2017).

Strict on-farm biosecurity measures to mitigate the *Campylobacter* colonization of poultry have been implemented in many countries, but these alone do not eliminate campylobacter from poultry. Additional to these efforts, intervention strategies have been developed to reduce the *Campylobacter* colonization levels of poultry, which have the potential to reduce human exposure if the reductions can be realized on poultry meat (Rosenquist et al., 2003; Newell et al., 2011). The use of *Campylobacter*-specific bacteriophages (commonly referred to as phages) to reduce the intestinal carriage of *Campylobacter* by broiler chickens is one such intervention that has shown promise in controlled trials (Loc Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009) and in broiler house applications (Kittler et al., 2013). These studies have been conducted with phage applications of one or more phage to achieve reductions in the cecal counts of *Campylobacter* colonized chickens of approximately 2 $\log_{10}$ CFU g^{-1} (Carvalho et al., 2010; Connerton et al., 2011; Hammerl et al., 2014).

The use of multiple types of phage targeting different receptors in the form of phage cocktails offers the prospect of expanding the host range of the preparation whilst limiting the potential to develop resistance to all components of the cocktail (Chan et al., 2013). This has been explored experimentally using *Campylobacter*-specific phage cocktails containing either four (Fischer et al., 2013) or two phages (Hammerl et al., 2014). In both cases phages were selected from both Group II and Group III as classified by Sails et al. (1998). The classification was originally based on total genome size and morphology but it is now known that the two groups share little DNA similarity and generally have different host specificities (Javed et al., 2014; Jäckel et al., 2017). Group II and III *Campylobacter* bacteriophages are taxonomically classified as members of the *Myoviridae* subfamily *Eucampyvirinae*, which do not possess the genes required to form stable lysogens and therefore exhibit a lytic or virulent life cycle similar to most Myoviruses (Javed et al., 2014). *Campylobacter* phage isolated using *C. jejuni* and *C. coli* hosts may show some cross-species specificity within strains of *C. jejuni* and *C. coli* species but have not been demonstrated to infect other species in the *Campylobacter* genus, for example *C. lari*, *C. upsaliensis*, *C. fetus*, *C. Sputorum,* or *C. intestinalis* (Loc Carrillo et al., 2007). Neither have they been found to infect *Helicobacter pylori*, *Arcobacter butzleri*, *Citrobacter freundii*, *Salmonella enteritica* serovar Enteritidis, *Escherichia coli,* and *Pseudomonas aeruginosa* (Loc Carrillo et al., 2007). Therefore it may be anticipated that components of a *Campylobacter* phage cocktail would retain their specificity and not give rise to dysbiosis – a detrimental microbiota constitution that often arises post broad-spectrum antibiotic therapies (Dethlefsen et al., 2008). However, a recent study has suggested that exposure of the rat gut microbiome to a cocktail of commercial phage preparations active against *Salmonella enterica*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *E. coli* results in dysbiosis with increased inflammation and gut permeability (Tetz et al., 2017). Bacteriophage mediated cell lysis has the potential to release lipopolysaccharides (endotoxin) from Gram-negative bacterial species that are potent inducers of proinflammatory cytokines in animals and in humans (Medzhitov, 2007). Exposure to a phage cocktail targeting multiple Gram-negative species may well elicit such a response, although it is reported that targeted virulent *E. coli* bacteriophages release less endotoxin than β-lactam antibiotics (Dufour et al., 2017), and that the phage tail adhesin protein Gp12 can bind free lipopolysaccharide to counteract the inflammatory effect (Miernikiewicz et al., 2016). Typically the lipopolysaccharides of Gram-negative bacteria are composed of an outer membrane anchored lipid A substituted with a core polysaccharide structure attached to a set of repeating O-chain subunits. However, *C. jejuni* synthesizes a core oligosaccharide without the repeating O-chain, which is referred to as lipooligosaccharide (Karlyshev et al., 2005).

In this study we demonstrate that *Campylobacter* phage affect the target host bacteria without provoking intestinal dysbiosis, when phages are administered to broiler chickens at therapeutic doses.

METHODOLOGY

Bacterial Strains and Growth Media

Campylobacter were isolated and enumerated by direct plating on modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) selective medium (Oxoid, Basingstoke, United Kingdom) with addition of cefoperazone and amphotericin B selective supplement (Oxoid) using standard techniques. *C. jejuni* HPC5 was isolated from the cecal content of a commercial broiler chicken in the United Kingdom (Loc Carrillo et al., 2005; NCBI accession CP032316). The universal bacteriophage host strain *C. jejuni* PT14 was used to propagate *Campylobacter* bacteriophages (Brathwaite et al., 2013; NCBI accession CP003871). *C. coli* NCTC 12668 was used to discriminate group II and III bacteriophages (Frost et al., 1999). *C. jejuni* HPC5 for the inoculation of broiler chickens and campylobacter for the production of bacterial lawns were cultured on horse blood agar (Blood agar base No 2; Oxoid) with addition of plus 5% (v/v) defibrinated horse blood, (TCS, Buckingham, United Kingdom) under microaerobic conditions (5% O_2, 5% H_2, 10% CO_2, 80% N_2, produced by the evacuation and replacement technique) at 42°C for 24 h.

Bacteriophages and Propagation

Campylobacter-specific bacteriophages CP20 and CP30A were isolated from commercial broiler chicken excreta collected in the United Kingdom in 2001. Bacteriophages were isolated by making a 10% suspension of excreta in SM buffer (50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 8 mM $MgSO_4.7H_2O$, and 0.01% gelatin; Sigma Aldrich, Gillingham, United Kingdom). This was incubated at 4°C with agitation, for 24 h followed by centrifugation at 13,000 × *g* for 5 min to remove bacteria. The resulting supernatant was filtered through a 0.2 μm filter (Minisart, Sartorius, Goettingen, Germany) to remove any remaining bacteria.

When screening for the presence or absence of bacteriophage, 10 μl filtrate aliquots were applied to bacterial lawns of *C. jejuni* PT14 prepared using the soft agar overlay method

as previously described (Connerton et al., 2004). Bacteriophage were propagated by complete plate lysis and recovered in SM buffer before filtration (0.2 μm filter) and concentration by centrifugation at 37,000 × *g*.

CP20 (NCBI nucleotide accession MK408758) corresponds to a group II *Campylobacter* bacteriophage and CP30A (NCBI nucleotide accession JX569801) a group III based on genome sizes, capsid morphologies determined by transmission electron microscopy and genomic DNA sequences (Scott et al., 2007a; Siringan et al., 2011, 2014; Javed et al., 2014; Brathwaite et al., 2015).

Experimental Birds

Commercial *Campylobacter*-free male Ross 308 broiler chicks were obtained as hatchlings (PD Hook, Oxfordshire, United Kingdom). Birds were housed in a controlled environment in individual pens under strict conditions of biosecurity. Temperatures were as outlined in the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Birds were provided with commercial broiler diets (starter, grower, and finisher) and water *ad libitum* for the duration. The birds were weighed and randomly assigned to 3 groups at 14 days of age. These were: Control group consisting of *Campylobacter*-free sentinel birds, Group Cj_phg, consisting of *Campylobacter* infected birds to be administered with phage and Group Cj, consisting of *Campylobacter* infected birds to be administered with placebo instead of phage (mock treatment). Cloacal swabs were taken on day 14 and tested for *Salmonella* by direct plating on Xylose-Lysine desoxycholoate agar (XLD) agar (Oxoid) and for *Campylobacter* by direct plating on mCCDA agar. Excreta samples, from the same day, were tested for *Campylobacter* phage (see below) and for *Salmonella* by enrichment in Rappaport-Vassiliadis soya peptone broth (Oxoid) then plating on Xylose-Lysine desoxycholoate agar (XLD) agar (Oxoid). *C. jejuni* HPC5 has previously been demonstrated to establish intestinal colonization of Ross 308 broiler chickens at 20 days of age within 48 h of oral gavage, and to maintain colonization levels without significant differences in cecal counts over 15 days (Loc Carrillo et al., 2005; Connerton et al., 2018). Four days post oral gavage was selected for phage administration to ensure intestinal colonization, and that any variation in the cecal counts would be evident over the period of the experiment. Birds from Cj and Cj_phg groups were colonized with *C. jejuni* HPC5 at 20 days of age. Each bird received 7 $\log_{10}$ CFU *C. jejuni* in 1 ml of PBS (phosphate buffered saline) by oral gavage. Phages were administered to Group Cj_phg as a single dose of $\log_{10}$ 7 PFU of CP20 and CP30A combined in 1 ml of 30% $CaCO_3$ (antacid) by oral gavage at 24 days of age. Group Cj were administered with 1 ml of 30% $CaCO_3$ as a placebo at 24 days of age. Five birds were sacrificed at 24 h intervals from 25 to 29 days of age following administration of phage or placebo. The ceca, ileum, and colon of the birds were first separated by ligature and then removed by sterile dissection. The luminal contents were collected for *Campylobacter* and bacteriophage isolation as described below and aliquots stored at −80°C for DNA extraction.

Enumeration of Campylobacters

Serial dilutions of digesta were made in maximum recovery diluent (MRD; Oxoid) and enumerated using the Miles and Misera technique on mCCDA agar with 2% (w/v) additional agar to reduce swarming. Plates were incubated under microaerobic conditions at 42°C for 48 h before typical *Campylobacter* colonies were counted.

Enumeration of Bacteriophages

Bacteriophages were recovered by making a 10% suspension of chicken digesta in SM buffer as described above. To enumerate bacteriophage CP20 and CP30A in the phage treated groups, independent lawns of *C. jejuni* HPC5 and *C. coli* NCTC 12668 were prepared. The *C. coli* strain NCTC 12668 was used as a second host because it was sensitive to CP20 but not CP30A, which allowed discrimination of the two phages administered. The CP30A titers were obtained by subtraction of the CP20 titer on *C. coli* 12668, from the total phage count on the *C. jejuni* HPC5 host, which was sensitive to both phages. Serial dilutions of intestinal contents were prepared in SM buffer and applied to these lawns as 10 μl spots in triplicate. The plates were then incubated for 24 h at 42°C under microaerobic conditions.

Acquisition of Bacteriophage Resistance

In order to establish the frequency of resistance to bacteriophages post-intervention, single colonies were lifted from the primary isolation plates onto which cecal content from all phage-treated and mock-treated control birds had been inoculated. Three colonies per cecal sample were picked for each phage-treated bird and subcultured on horse blood agar plates. Bacterial lawns were prepared from successful subcultures and CP20 and CP30A phage were applied at a range of dilutions from 1 to 3 $\log_{10}$ PFU to establish if resistance had been acquired with respect to the efficiency of plating.

DNA Isolation

DNA was isolated from both ileal and cecal content using the Mobio PowerSoil kit (now QIAGEN Ltd., Manchester, United Kingdom). The method used is as described in the Human Microbiome Project SOP for processing of stool Specimens (see Manual of Procedures for Human Microbiome v12, section 7.7 onward[1].

Microbiome Analysis

The V4 regions of the bacterial 16S rRNA genes were PCR amplified using the primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGAC TACHVGGGTWTCTAAT 3′) (Caporaso et al., 2011). Amplicons were then sequenced on the Illumina MiSeq platform using 2 × 250 bp cycles. The 16S rRNA gene sequences were quality filtered and clustered into operational taxonomic units (OTUs) in Mothur (Schloss et al., 2009) using the Schloss lab. MiSeq SOP[2] (Kozich et al., 2013). Batch files of Mothur commands used

[1]http://hmpdacc.org/resources/tools_protocols.php

[2]https://www.mothur.org/wiki/MiSeq_SOP, (accessed 2018-10-05).

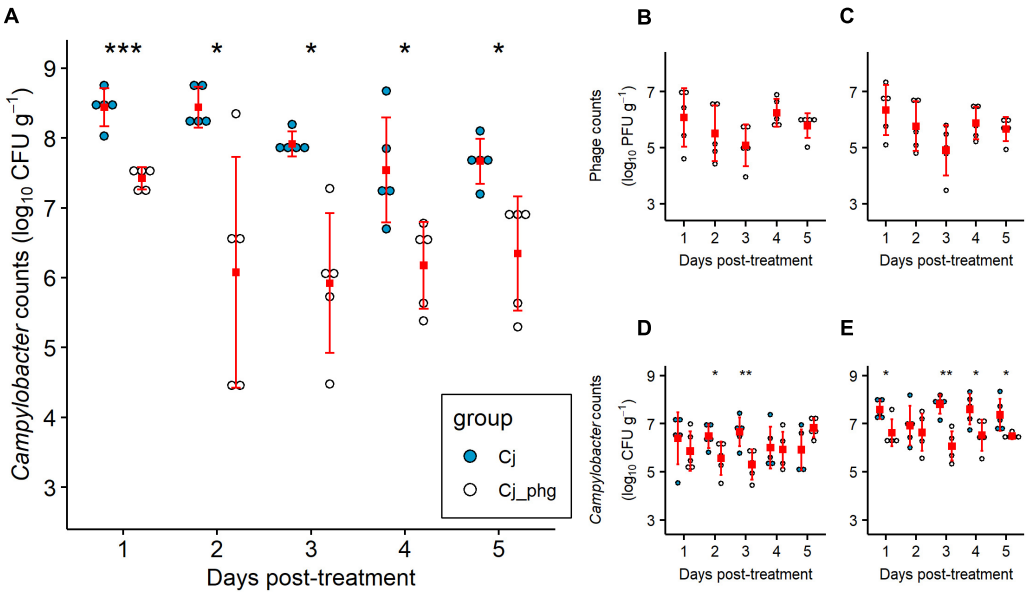


FIGURE 1 | *Campylobacter jejuni* levels are reduced by phage predation post-treatment. Independently housed Ross 308 broilers were given 7 $\log_{10}$ CFU *C. jejuni* by oral gavage at 20 days-old. At 24 days-old birds were orally administered a mixture of two phage at 7 $\log_{10}$ PFU each or a placebo of carrier alone. Five chickens from each group were sacrificed on each day from 25 to 29 days-old, from which *C. jejuni* were enumerated from the intestinal contents of the ceca **(A)**, ileum **(D)**, and colon **(E)**. Titers of phage CP20 **(B)** and CP30A **(C)** were determined from cecal content. Filled squares indicate means. Error bars indicate standard deviation. Asterix indicate statistical significance: *, $p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

in this study are available at: https://github.com/PJRichards. Post-processing rarefaction curves were plotted to assess sampling effort (**Supplementary Figures S1–S3**). Based on these observations the Day 1 (1dpt) ileum communities and two further ileum communities (Group Cj: Day 2 replicate 2; Group Cj_phg: day 4 replicate 4) were judged as having insufficient depth, and were therefore excluded from the analysis. No template controls were included in the analysis.

Raw sequence data are deposited in the NCBI database within the Bioproject PRJNA506577 under the SRA study SRP170194.

Ethics Statement

All experimental animal work was performed in accordance with United Kingdom and EU law. This study was approved by the Local Ethics Committee of the University of Nottingham and performed under Home Office license.

Statistical Analysis

The article was written in R 3.5.1 (R Core Team, 2018) and Rmarkdown 1.10.8 (Allaire et al., 2018; Xie et al., 2018) using Rstudio 1.1.456 (RStudio Team, 2015). R code used to make the figures/tables presented here are available at: https://github.com/PJRichards/Richards_phage_microbiota. **Figure 3** was drawn in R using code adapted from Torondel et al. (2016) and kindly made available at: http://userweb.eng.gla.ac.uk/umer.ijaz. OTUs discriminatory between communities were identified using LEfSE (Segata et al., 2011). Commonality of 16S rDNA sequences of OTUs discriminative of phage-treated (Group Cj_phg) and untreated birds (Group Cj) with other trials performed in our laboratory were determined using stand-alone ncbi-blast-2.7.1+ (Zhang et al., 2000)[3].

RESULTS

Dual *Campylobacter* Bacteriophage Treatment of Reduces Levels of *Campylobacter jejuni* Colonization

Campylobacter were enumerated using standard culture methods from intestinal luminal contents collected from the ileum, ceca, and colon of all birds. High levels of *Campylobacter* were recovered from all digesta collected from *C. jejuni* colonized birds (Group Cj) throughout the 5-day period of the experiment (mean $\log_{10}$ CFU g^{-1}: ileum 6.289, ceca 8.000, colon 7.452 for $n = 25$). A control group comprising of a cohort of non-colonized sentinel birds confirmed the effectiveness of the biosecurity measures adopted as they remained free of *Campylobacter* and phage contamination.

Co-administration of a single phage dose containing CP20 (7 $\log_{10}$ PFU) and CP30A (7 $\log_{10}$ PFU) 4 days after *Campylobacter* exposure at 24 days of age, significantly reduced *C. jejuni* numbers in the ceca of phage-treated birds (Group Cj_phg) compared to mock-treated birds throughout the period of the experiment ($p \leq 0.032$; **Figure 1A**). The phages were most effective 2 days post-treatment (dpt), resulting in a reduction in *C. jejuni* numbers of 2.365 $\log_{10}$ CFU g^{-1} in Group Cj_phg compared to *C. jejuni* colonized controls in Group Cj.

[3]https://ftp.ncbi.nlm.nih.gov/blast/executables/LATEST/

After this time, the numbers of *C. jejuni* in Group Cj_phg birds increased but remained significantly lower than the levels observed from mock-treated birds (Group Cj) by 1.321 $\log_{10}$ CFU g^{-1} after 5 days.

Phage treatment was most effective in the cecum, the organ that represents the greatest biomass of digesta and reservoir of *C. jejuni*. However, treatment was also effective in reducing *C. jejuni* in the ileum (p = 0.044 and 0.008 at 2 and 3 dpt, respectively) and the colon at all days excluding 2 dpt ($p \leq 0.041$; **Figure 1E**). The reductions in *Campylobacter* counts were not as great as those observed for the ceca, with a maximum reduction in the ileum of 1.359 $\log_{10}$ CFU g^{-1} at 3 dpt and a maximum reduction in the colon of 1.740 $\log_{10}$ CFU g^{-1} also at 3 dpt (**Figures 1D,E**). Bacteriophages were enumerated over the 5 day trial period and were detected in the cecal contents of all 5 birds treated in Group Cj_phg from 24 h after administration (**Figures 1B,C**). The CP20 phage titre recovered from cecal contents of the treated birds remained stable over time (mean 5.738 $\log_{10}$ PFU g^{-1}; *SD* 0.460; **Figure 1B**), confirming that the phage were replicating *in vivo*. Similarly, the mean CP30A titre was 5.708 $\log_{10}$ PFU g^{-1} (*SD* 0.517; **Figure 1C**) confirming the two phages co-exist without competitive exclusion. The levels of phages recovered from ileum and colon contents also remained stable over the course of the experiment (**Supplementary Figure S4**).

Bacteriophage Resistance Post Treatment

The overall levels of phage resistance (isolates resistant to one or both phage) in *C. jejuni* HPC5 isolates in Group Cj_phg was approximately 10% (n = 7/67) of the strains recovered post-treatment. Of these, three (4.5%) were resistant to both CP20 and CP30A phages whilst one (1.5 %) was resistant to CP20 but not CP30A and three (4.5%) were resistant to CP30A only. No phage resistance was detected in the *C. jejuni* recovered from birds that had not received phage (n = 32).

Bacteriophage Predation of *Campylobacter jejuni* Does Not Affect Microbiota Structure

The α-diversity (inverse Simpson index) of the cecal or ileal microbiota of bacteriophage-treated birds (Group Cj_phg) were not significantly different to those from mock-treated birds (Group Cj) ($p \geq 0.095$ and $\geq$0.841, respectively; **Figures 2A,C**). There was no difference in the richness (Chao) of the cecal

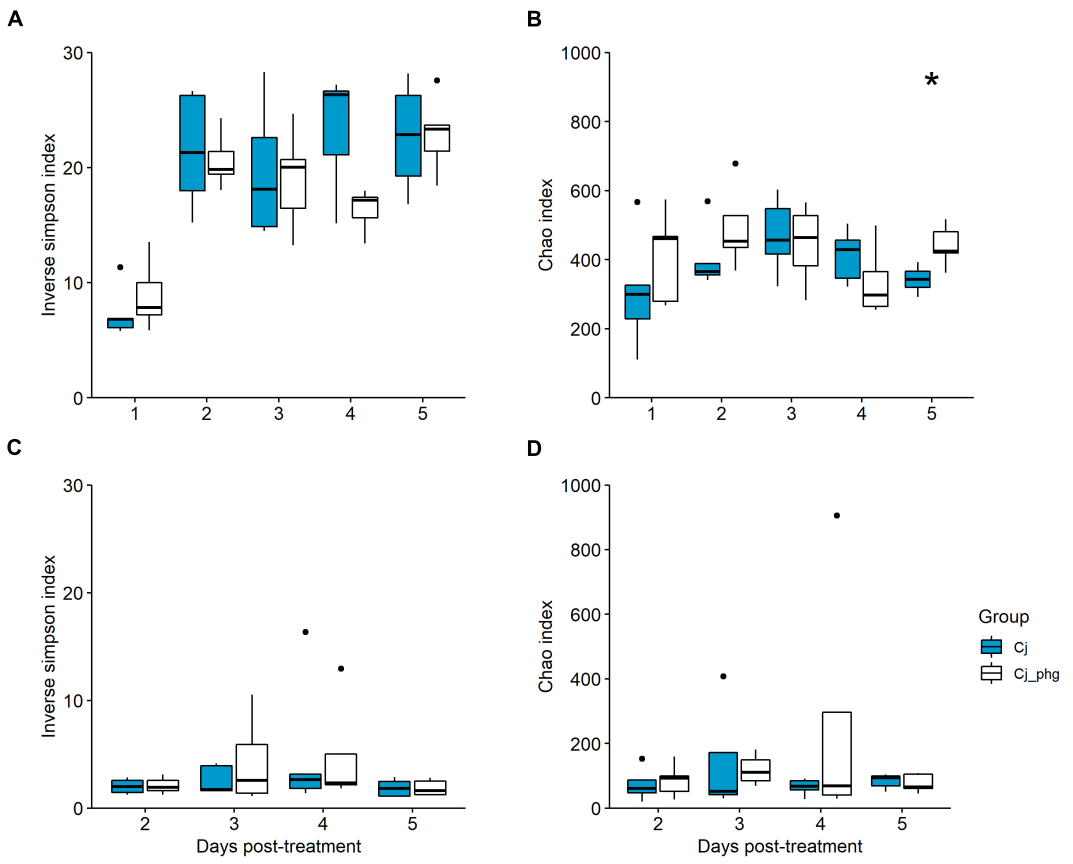


FIGURE 2 | Bacterial diversity within the gastrointestinal tract is not affected by phage treatment. Box-and-whisker plot describing bacterial 16S rDNA gene content surveys of cecal lumen contents from phage-treated (Cj_phg) or mock-untreated (Cj) groups in terms of α-diversity (inverse Simpson's index; **(A)** and richness (Chao index; **(B)**. Corresponding α-diversity and richness for ileal lumen contents are shown in panels **(C,D)**, respectively. Asterix indicate statistical significance: *$p \leq 0.05$.

microbiota between 1 and 4 dpt ($p \geq 0.151$) or the ileum microbiota at any time ($p \geq 0.548$; **Figures 2B,D**). The Chao-richness of the cecal microbiota of bacteriophage-treated birds (Group Cj_phg) was significantly different from mock-treated birds (Group Cj) by 5 dpt ($p = 0.032$; **Figure 2B**).

Exposure to *Campylobacter*-Phage Selectively Reduces Proportions of *C. jejuni* Without Affecting the Wider Microbiota

Throughout the experiment the predominant bacterial phylum present in the ileal lumen was the Firmicutes with a median relative abundance (RA) of 83.091% (**Supplementary Figure S5A**). There were no differences in relative abundance between age-matched bacteriophage-treated (Group Cj_phg) and mock-treated birds (Group Cj) in this phylum ($p \geq 0.063$, Wilcoxon test; **Supplementary Figure S5A**). The next most abundant phylum are the Proteobacteria where a difference at 4 dpt was observed ($p = 0.016$), but not on any other day ($p \geq 0.730$, Wilcoxon test; **Supplementary Figure S5A**). Phyla-level composition of the cecal microbiotas of both groups were likewise dominated by Firmicutes (median 90.620% RA), and to a much lesser extent Proteobacteria (4.747% RA) (**Supplementary Figure S5B**). However, there was no difference in the relative abundance of Firmicutes or Proteobacteria between phage-treated (Group Cj_phg) and mock-treated birds (Group Cj) at any time post-treatment ($p \geq 0.178$, *t*-test; and $p \geq 0.151$, Wilcoxon test; respectively).

At the OTU level, phage treatment did not affect the β-diversity (Bray Curtis distance) between communities of ileal lumen bacteria from age-matched phage-treated (Group Cj_phg) and mock-treated birds (Group Cj) at any time point ($p = 0.106$; AMOVA; **Supplementary Figure S6A**). The 11 most abundant OTUs in the ileum lumen are shown in **Figure 3A**. After the OTUs were filtered to include only those ≥1% of total reads, the only discriminative OTU between phage-treated and mock-treated birds identified using LEfSE (Segata et al., 2011) was OTU0013 [*Campylobacter* (100)] at 3 dpt ($p = 0.009$).

The 11 most abundant OTUs in the cecal lumen are shown in **Figure 3B**. For populations of cecal bacteria the β-diversity was not different at 1 to 4 dpt ($p \geq 0.088$, **Supplementary Figure S6B**). However, bacterial populations could be distinguished at 5 dpt ($p = 0.021$). At 5 dpt OTU0009 [Clostridiales_unclassified (100)] was the sole discriminative OTU between the treatments ($p = 0.027$; LEfSE; **Supplementary Figure S7D**), which was present in significantly greater proportions in the mock-treated birds (Group Cj) relative to phage-treated birds (Group Cj_phg) (mean % RA = 1.211 and 6.668, respectively) (**Figure 3A**). Previous work from our laboratory identified an OTU with 100% DNA sequence

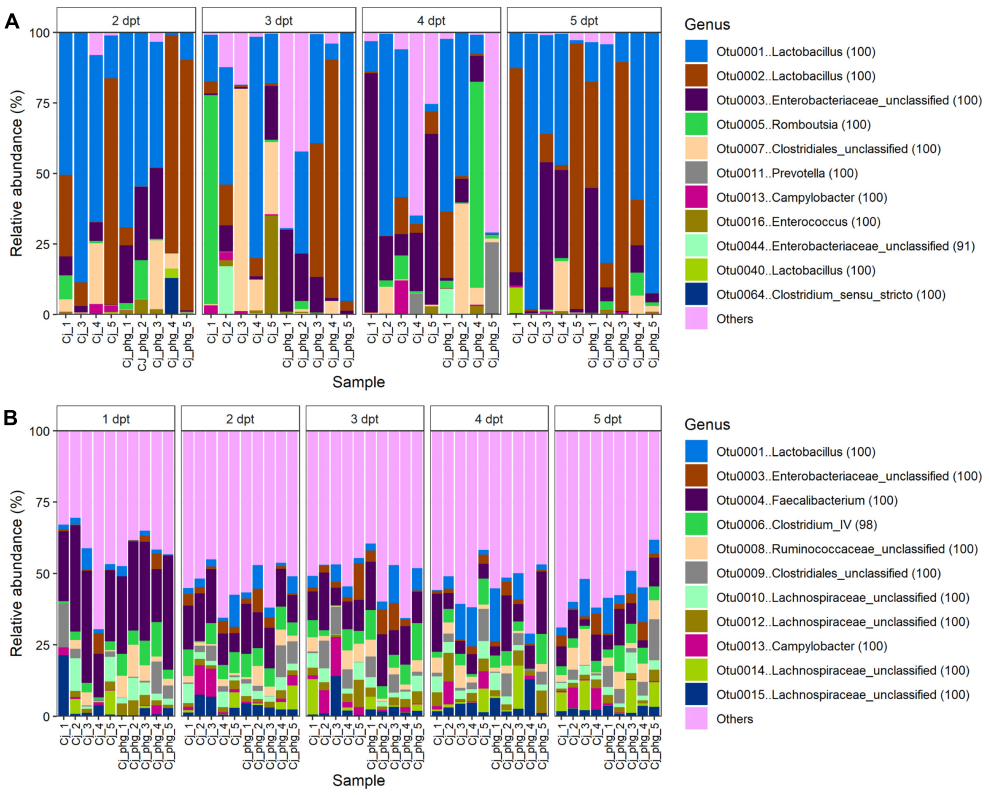


FIGURE 3 | Bacteriophage treatment reproducibly only affects *C. jejuni* proportions. Stacked bar charts showing bacterial community compositions from the ileum **(A)** and ceca **(B)** of all birds. For clarity only the 11 most abundant OTUs are described with all other OTUs summarized as "other."

identity to OTU0009 as being associated with *Campylobacter*-colonized birds [see OTU0073 in Connerton et al. (2018)]. Discriminative analysis of the cecal communities associated a greater abundance of *Camplylobacter* (OTU0013) with the mock-treated birds (Group Cj) at 3 and 4 dpi ($p < 0.047$; **Supplementary Figures S7B,C,** respectively). A positive association was observed between phage-treated (Cj_phg) birds at 1 dpt and OTUs 0006 and 0021, representing Clostridium IV (98) and Clostridiales_unclassified (100), respectively ($p = 0.047$; **Supplementary Figure S7A** and **Figure 3B**).

At 5 dpt the bacterial communities of the ceca or ileum of *Campylobacter*-free sentinel control birds could not be distinguished from mock-treated *Campylobacter*-colonized birds (Group Cj, $p > 0.05$; **Supplementary Figures S6A,B**), but cecal populations of bacteria in the phage-treated birds (Group Cj_phg) could be distinguished from non-colonized control birds ($p = 0.014$). Correspondingly, the OTUs that are associated with phage-treated treated birds (Group Cj_phg) are OTU0006 Clostridium IV (98) and OTU0009 Clostridiales_unclassified (100), which were also identified as discriminating phage-treated treated birds from mock-treated birds at 1 and 5 dpt, respectively (**Supplementary Figure S8B**). Although there was no difference in the β-diversity of non-colonized and mock-treated colonized birds in these cohorts, as expected OTU0013, representing *Campylobacter* (100), showed association with colonized birds, but conversely OTU0031, *Bifidobacterium* (100), showed association with the non-colonized control birds (**Supplementary Figure S8A**). This observation is also consistent with our previous study which demonstrates the differential association of an OTU with 100% DNA sequence identity with non-colonized birds compared to those 2 days post-colonization with *C. jejuni* at 20 days of age (NCBI database SRA study SRP133552; Connerton et al., 2018).

Interestingly, the low diversity of the ileal bacterial communities, relative to the cecal microbiota (see **Figures 2A,B**), is revealed in the observation that the top 11 most abundant OTUs across all ileal communities constitute 90.754% of reads, whereas the top 11 most abundant OTUs across all cecal communities account for a lower quotient of 48.670% reads (**Figures 3A,B**). We note that ileal samples showed some similarity to no template controls at phyla level (**Supplementary Table 1**), and that there was notable correspondence in the γ-proteobacteria [*Escherichia*/*Shigella* (100)] among the top 11 most abundant OTUs from ileal and cecal samples and the most abundant kitome/contaminant OTUs (**Figures 3A,B**; **Supplementary Table 2**).

DISCUSSION

The results described here provide further evidence of the efficacy of phage treatment to reduce the *Campylobacter* colonization of chickens. It has been widely suggested that an advantage of phage biocontrol over antibiotic use or other broad-spectrum types of therapy against pathogens that inhabit the intestinal tract, is the specificity of the bacteriophage selected for a particular host (Sulakvelidze and Barrow, 2004; Sulakvelidze and Kutter, 2004). This specificity is presumed to avoid the possibility of causing dysbiosis but recently this assumption has been challenged using a multi-phage treatment to affect collateral changes in the composition of the microbiota of rats with decreases in the abundance of *Blautia*, *Catenibacterium*, *Lactobacillus,* and *Faecalibacterium* species, and increases in the abundance of *Butyrivibrio*, *Oscillospira,* and *Ruminococcus* (Tetz et al., 2017). This is in contrast to directed studies using simulated gut microbial consortia (duodenum and ileum) containing a specific *Escherichia coli* as a bacteriophage target, where the impact of phage therapy was compared with ciprofloxacin treatment (Cieplak et al., 2018). Bacteriophage and antibiotic therapies were equally as effective in reducing the target *Escherichia coli* population by 2 to 3 $\log_{10}$ CFU ml^{-1} but notably the bacteriophage treatment had no measurable impact on non-target bacteria.

Although *Campylobacter* phages selected for the biocontrol of campylobacter in chickens appear to be confined to replication in *C. jejuni* and *C. coli* as hosts (Loc Carrillo et al., 2007), this discrimination had not previously been verified from the intestinal microbiota of *Campylobacter* colonized chickens. Frequency and abundance estimates of campylobacters and phages recovered from the ceca of commercial broiler chickens support the contention that production birds are often exposed to phage, and that phage presence coincides with a reduction in the mean *Campylobacter* cecal counts by approximately 1.8 $\log_{10}$ CFU g^{-1} (Atterbury et al., 2005). However, this study also demonstrated that phage which replicate on *Campylobacter* could be recovered in the absence of culture detectable *Campylobacter* host bacteria. The study left an open question as to whether the presence of phage under certain circumstances can drive *Campylobacter* populations below the culture detection limit (<2 $\log_{10}$ CFU g^{-1}) or that phage infecting campylobacters could also replicate on alternative host bacteria present in the microbiota of chickens. Further motivation for the current study was to establish whether phage therapy under these circumstances constitutes a minimal targeted intervention that utilizes biocontrol agents that are not detrimental to the intestinal microbiota of farmed chickens, and to which consumers are already exposed.

Phage therapy of *C. jejuni* colonized chickens produced significant reductions in intestinal *C. jejuni* counts compared to mock-treated controls over 5 days. However, the introduction of the phages did not affect the structures of the cecal or ileal microbiotas of the birds based on calculations of α-diversity (inverse Simpson index). The richness (Chao) of the microbiota remained similarly indistinguishable until 4 dpt. At the phyla level no difference in the abundance of the major components were observed for the cecal microbiotas representing the greatest biomass, and at only one time point was any difference observed post-phage treatment for the ileal microbiotas (4 dpt). Analysis of differences in the relative abundance between phage-treated and mock-treated ileal and cecal community OTUs highlights significant differences in OTU0013 that represents the phage therapy target, *C. jejuni*.

Additional to this, the cecal community member OTU0009 (Clostridiales_unclassified) showed significantly greater proportions in the mock-treated birds relative to phage-treated birds, as had previously been identified on the basis of the association of a DNA sequence identical OTU with *Campylobacter*-colonized birds (Connerton et al., 2018). The consistent association could be indicative of a key reliance for the corresponding clostridial organism(s) on high levels of *Campylobacter* colonization of the chicken gut. These observations further the idea that campylobacters can act as a hydrogen sink to improve the growth and competitive standing of specific clostridia (Kaakoush et al., 2015). The *Bifidobacterium* OTU0031 showed association with non-colonized sentinel birds, which is in contrast to 16S rRNA qPCR data reported by Thibodeau et al. (2015) that showed an increase in the molecular detection of *Bifidobacterium* sp. upon *C. jejuni* colonization. The authors noted that *Bifidobacterium* had previously been reported to hinder *C. jejuni* colonization (Ding et al., 2005; Santini et al., 2010), and that subtle effects may occur during *C. jejuni* colonization of chickens.

We observed the emergence of phage resistant *C. jejuni* post-phage treatment in this study as reported previously (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Fischer et al., 2013; Hammerl et al., 2014). We did not recover any phage resistant *C. jejuni* from the non-phage treated chickens despite the propensity of *C. jejuni* to undergo phase variation in genes leading to phage resistance (Aidley et al., 2017), and suggesting the observed phage escape mutation frequency of 10% in this experiment was a consequence of selection due to phage predation. The observed phage resistance frequency is within those reported previously of 1–14% (Loc Carrillo et al., 2005; El-Shibiny et al., 2009; Carvalho et al., 2010; Fischer et al., 2013; Hammerl et al., 2014). The phage treatment of chickens to reduce *Campylobacter* colonization has been demonstrated to be most effective over a 2–3 day period post-treatment (Loc Carrillo et al., 2005; El-Shibiny et al., 2009), since thereafter *Campylobacter* populations begin to recover. The continued impact of *Campylobacter* phage predation on the wider intestinal microbiota could not be assessed within the treatment timeframe examined in the current experiment (Connerton et al., 2018). However, when the time to slaughter after phage therapy was extended, the cecal *Campylobacter* levels were not reported to achieve the levels observed in non-treated controls (Fischer et al., 2013). This is likely due to reduced competitive fitness of the resistant types as described previously (Loc Carrillo et al., 2005; Scott et al., 2007a,b), and supports the supposition of Wagenaar et al. (2005) that the release of virulent *Campylobacter* phages into the environment would not constitute any greater risk.

Bacteriophage CP20 is a group II phage based on genome size and DNA sequence similarities. Group II phage are generally flagellotropic that require the host to be motile with a functional flagellar (Coward et al., 2006; Scott et al., 2007a,b; Baldvinsson et al., 2014; Lis and Connerton, 2016; Liang and Connerton, 2018). CP30A is a group III phage that in common with this phage group exhibits dependence on capsular polysaccharide structures (Sørensen et al., 2011; Lis and Connerton, 2016). The post-phage treatment *C. jejuni* isolates we identified as resistant to both phage classes will be of interest to examine with respect to their ability recolonize chickens and the nature of the mutation. Although we note they remain a minority population within the chicken gut even in the presence of bacteriophage controlling the wild type *C. jejuni* populations. These bacteria are likely at a competitive disadvantage to the wild type.

We have rigorously examined the microbiota of *Campylobacter* colonized chickens treated with either phages or a placebo to provide strong evidence for the lack of any collateral effect on the gut microbiome.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI, NCBI Bioproject PRJNA506577 under the SRA study SRP170194.

AUTHOR CONTRIBUTIONS

IC designed the experiments. PR, PC, and IC executed the experiments, analyzed the data, and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00476/full#supplementary-material

FIGURE S1 | Rarefaction curves indicating coverage of *Campylobacter*-colonized (experimental) cecal bacterial communities. 16S rDNA bacterial communities from: **(A)** 1 day post-treatment (dpt); **(B)** 2 dpt; **(C)** 3 dpt; **(D)** 4 dpt; **(E)** 5 dpt. Cohort: **(i)** Group Cj **(ii)** Group Cj_phg.

FIGURE S2 | Rarefaction curves indicating coverage of *Campylobacter*-free non-colonized (control) cecal bacterial communities. 16S rDNA bacterial communities from *Campylobacter*-free non-colonized control birds at 29 days-old, equivalent to 5 days post-treatment.

FIGURE S3 | Rarefaction curves indicating coverage of ileal bacterial communities. 16S rDNA bacterial community from: **(A)** 2 day post-treatment (dpt); **(B)** 3 dpt; **(C)** 4 dpt; **(D)** 5 dpt. The non-phage treated cohort (Group Cj) are shown in panels Ai to Di, and the phage treated cohort (Group Cj_phg) in panels Aii to Dii.

FIGURE S4 | *Campylobacter* phage titers in the ileum and colon post-treatment. Phage tiers were determined for CP20 **(A)** and CP30A phage **(B)** from ileal content and colon content **(C)**, CP20; **(D)**, CP30A). Filled squares indicate mean. Error bars indicate standard deviation.

FIGURE S5 | Stacked barchart showing phyla-level microbiota composition for ileal and cecal communities. OTUs were filtered so that only Phyla present in more than one ileal or cecal community are included in the analysis. Figures in parenthesis in the key indicate bootstrap values for that taxonomic assignment generated in Mothur. All taxa shown in the key were detected in the ileum lumen including those of low abundance **(A)**. However, note that only Actinobacteria, Bacteria_unclassified, Bacteroidetes, Firmicutes, and Proteobacteria phyla were detected in cecal communities **(B)**.

FIGURE S6 | Relationship between communties of gut bacteria from phage-treated and mock-treated birds. PCoA plot of Bray-Curtis distance between **A**) ileal (R^2 = 0.41; **A,B**) cecal communities (R^2 = 0.75; **B**).

FIGURE S7 | Cecal bacterial taxa responsive to *Campylobacter*-phage treatment. OTUs were filtered to only include those representing at least 1% of the total reads before all communities were randomly subsampled an equal depth for unbiased comparison. Discriminatory OTUs were then described between age-matched groups using LEfSE. LDA refers to Linear Discriminant Analysis. 1 dpt **(A)**, 3 dpt **(B)**, 4 dpt **(C)**, 5 dpt **(D)**. There were no differential OTUs at 2 dp.

FIGURE S8 | Gut bacterial taxa responsive to *Campylobacter*-colonization at 5 days post-treatment. OTUs were filtered as described for **Supplementary Figure S7** and discriminatory OTUs identified using LEfSE. LDA refers to Linear Discriminant Analysis. Control-Group Cj comparison **(A)**, Control-Group Cj_phg comparison **(B)**.

TABLE S1 | Description of phyla associated with no template controls. Due to potential for low biomass and dominant contaminant profile from ileum samples the kitome microbiota profile is included. Table text in parenthesis indicates Mothur taxonomy bootstrap values. Samples: kitome for all samples, excluding 1 dpt (kit Ai and kit Aii); kitome for 1 dpt (kit B); sequencing negative control for all samples, excluding 1 dpt (seq Ai and seq Aii); sequencing negative control for 1 dpt (seq B).

TABLE S2 | Description of top 10 OTU associated with no template controls. Table text in parenthesis indicates Mothur taxonomy bootstrap values.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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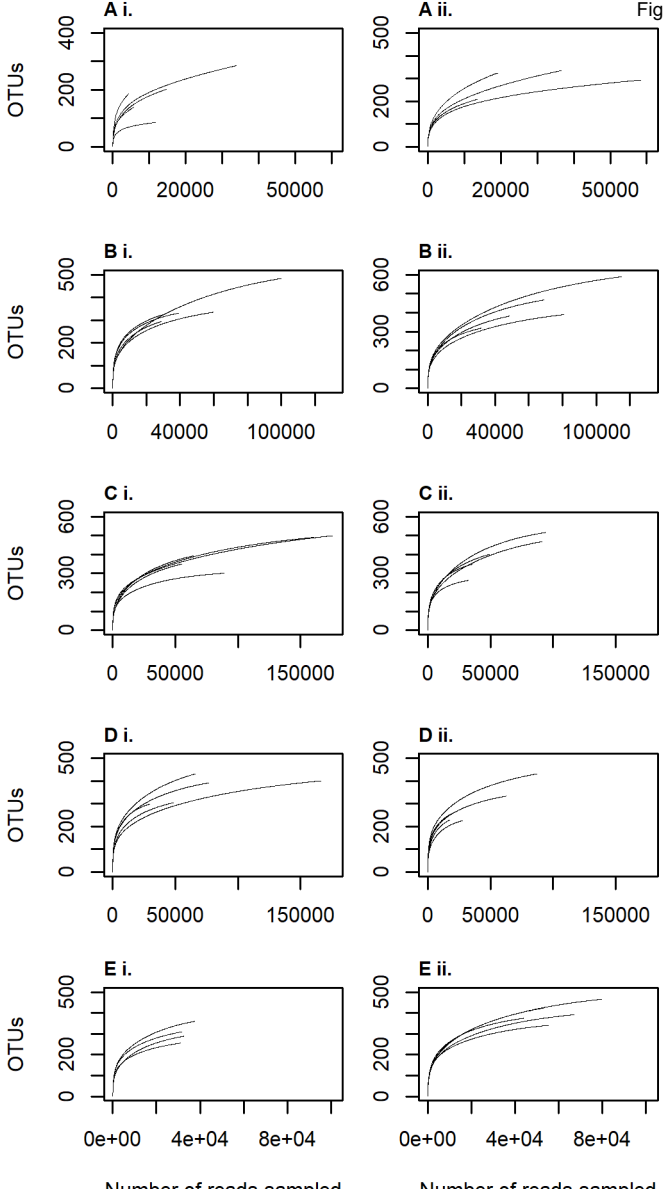

A i.
A ii.
B i.
B ii.
C i.
C ii.
D i.
D ii.
E i.
E ii.
OTUs
0
200
400
500
600
300
0
20000
50000
40000
100000
150000
0e+00
4e+04
8e+04
Number of reads sampled

Figure S2

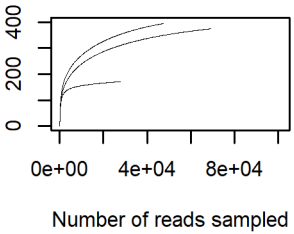
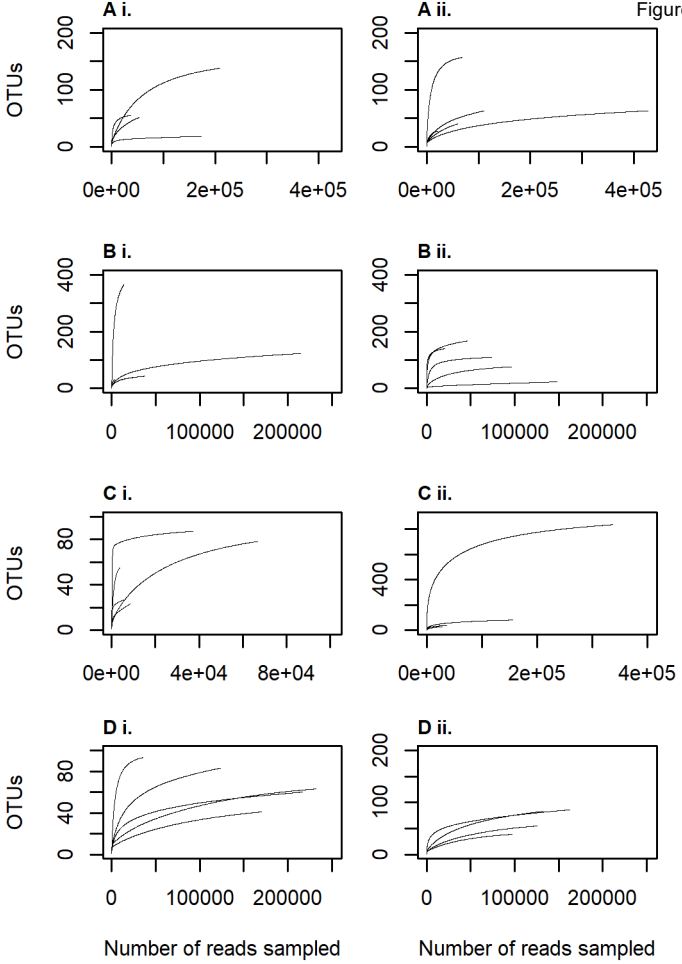
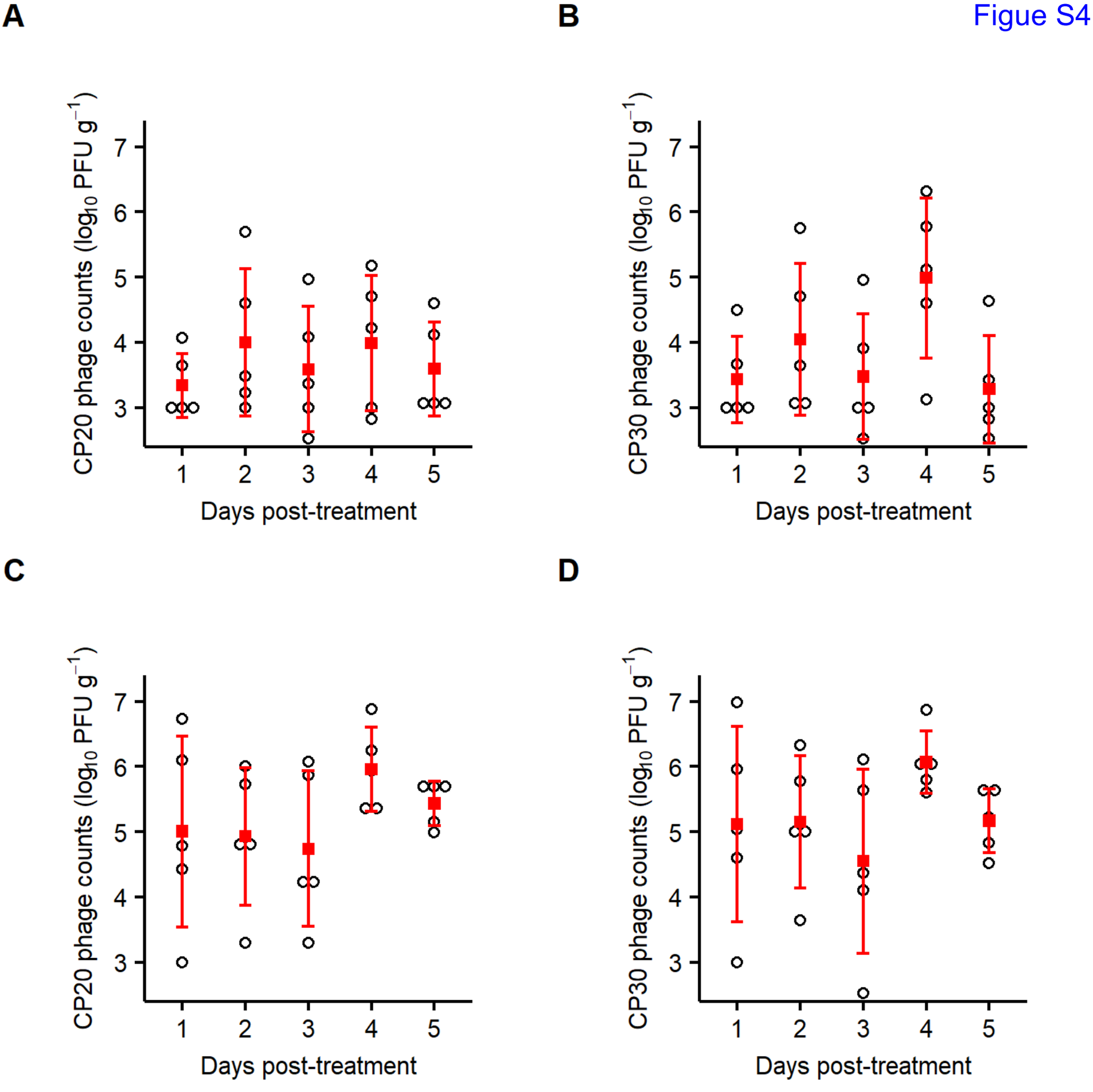
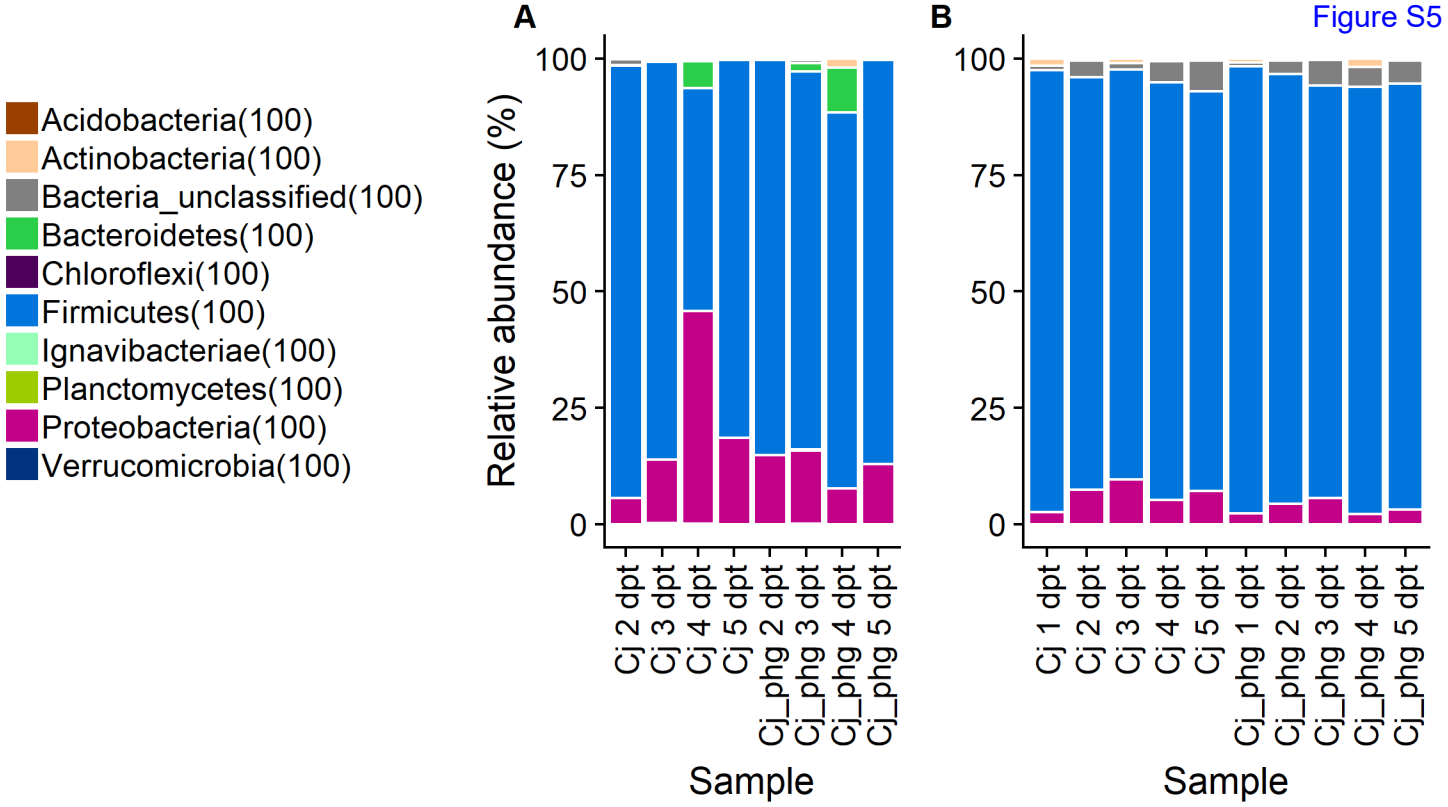


Figure S3


A i.
A ii.
B i.
B ii.
C i.
C ii.
D i.
D ii.
OTUs
Number of reads sampled
0
100
200
400
80
40
0e+00
2e+05
4e+05
8e+04
4e+04
100000
200000

Figue S4


A
B
C
D
CP20 phage counts (log_{10} PFU g^{-1})
CP30 phage counts (log_{10} PFU g^{-1})
Days post-treatment
1
2
3
4
5
3
4
5
6
7


Acidobacteria(100)
Actinobacteria(100)
Bacteria_unclassified(100)
Bacteroidetes(100)
Chloroflexi(100)
Firmicutes(100)
Ignavibacteriae(100)
Planctomycetes(100)
Proteobacteria(100)
Verrucomicrobia(100)
A
B
Relative abundance (%)
100
75
50
25
0
Cj 2 dpt
Cj 3 dpt
Cj 4 dpt
Cj 5 dpt
Cj_phg 2 dpt
Cj_phg 3 dpt
Cj_phg 4 dpt
Cj_phg 5 dpt
Cj 1 dpt
Cj_phg 1 dpt
Sample
Sample

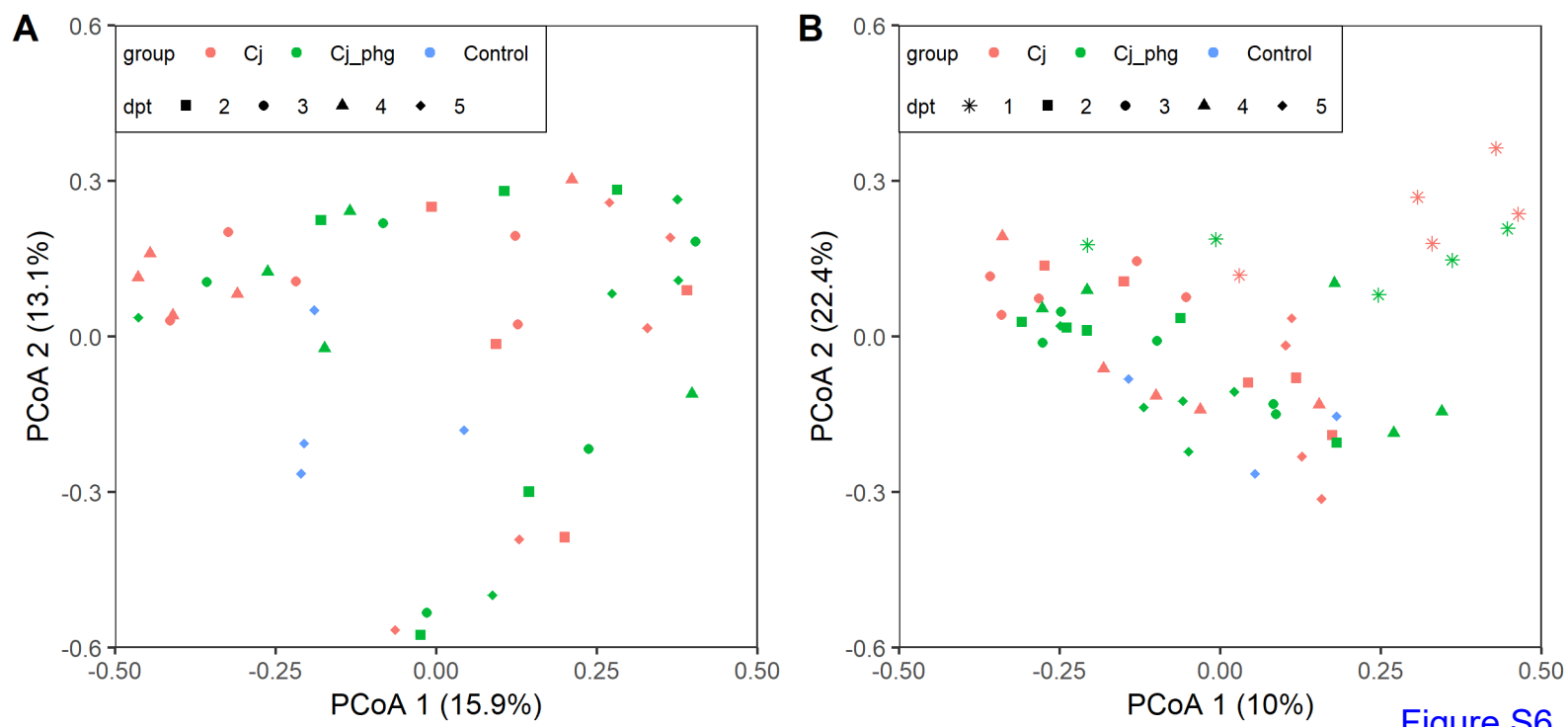

A
group Cj Cj_phg Control
dpt 2 3 4 5
PCoA 2 (13.1%)
PCoA 1 (15.9%)
0.6
0.3
0.0
-0.3
-0.6
-0.50
-0.25
0.00
0.25
0.50
B
group Cj Cj_phg Control
dpt 1 2 3 4 5
PCoA 2 (22.4%)
PCoA 1 (10%)

Figure S6

Figure S7

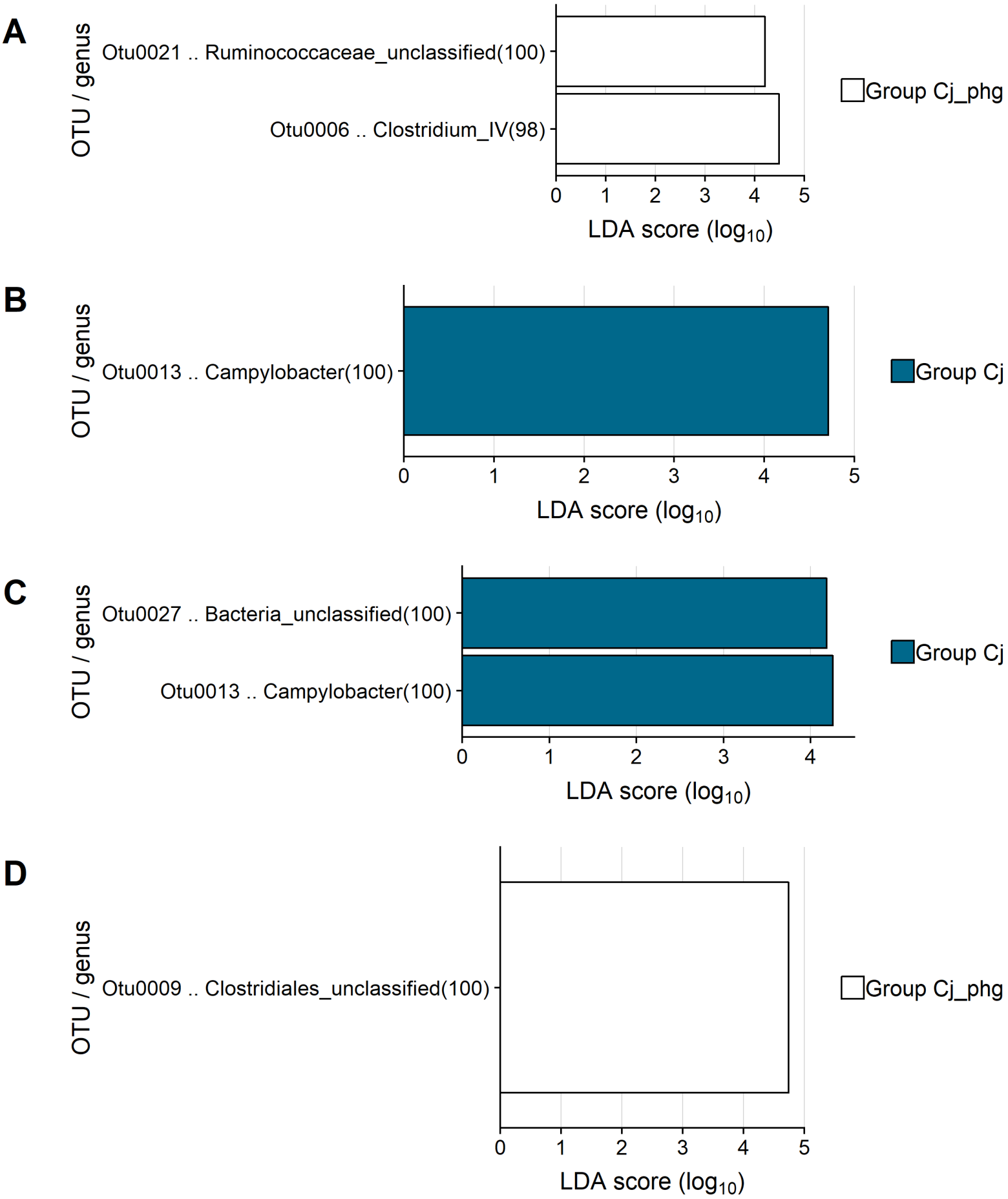
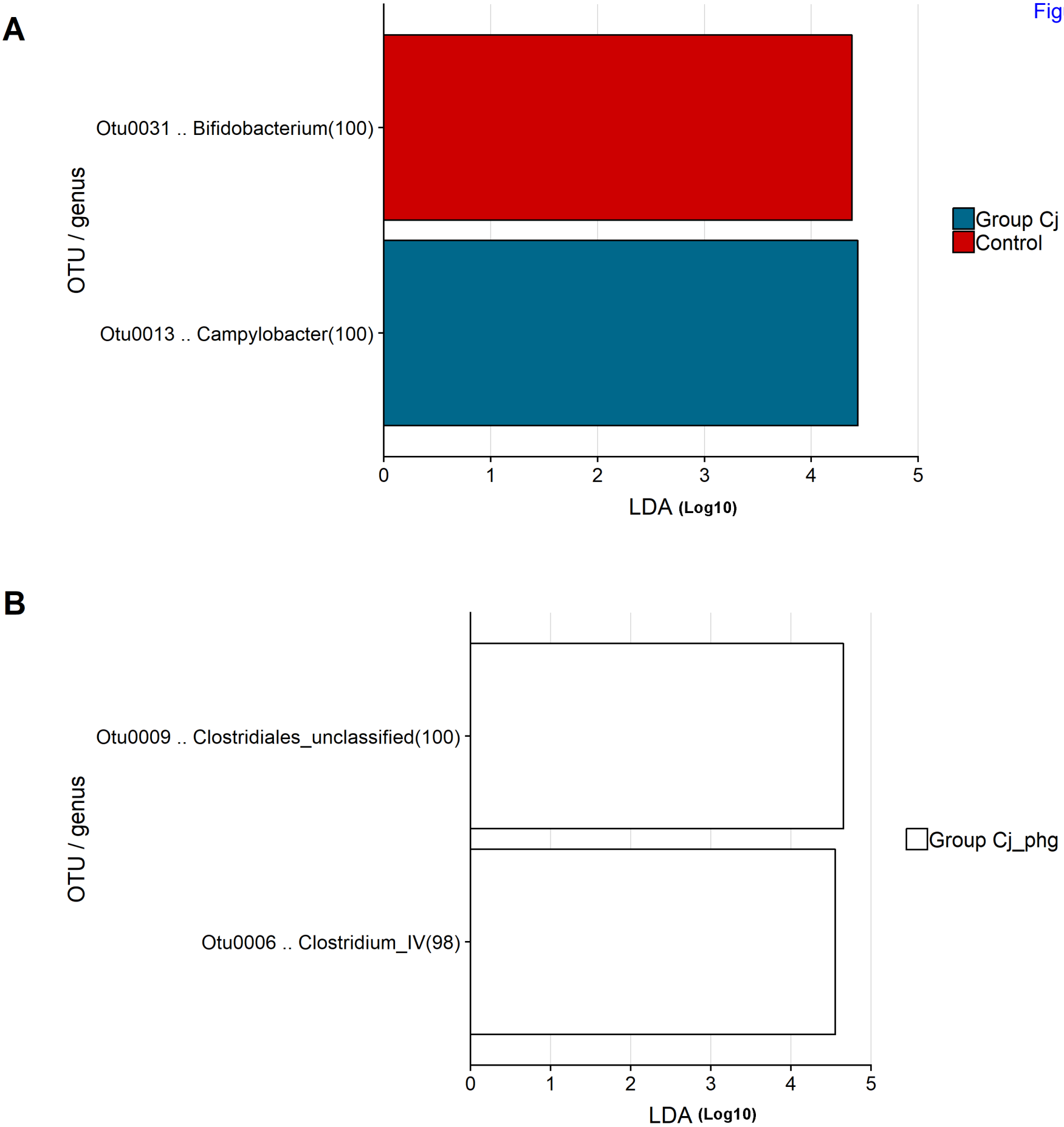

A
OTU / genus
Otu0021 .. Ruminococcaceae_unclassified(100)
Otu0006 .. Clostridium_IV(98)
0 1 2 3 4 5
LDA score (log10)
Group Cj_phg
B
OTU / genus
Otu0013 .. Campylobacter(100)
0 1 2 3 4 5
LDA score (log10)
Group Cj
C
OTU / genus
Otu0027 .. Bacteria_unclassified(100)
Otu0013 .. Campylobacter(100)
0 1 2 3 4
LDA score (log10)
Group Cj
D
OTU / genus
Otu0009 .. Clostridiales_unclassified(100)
0 1 2 3 4 5
LDA score (log10)
Group Cj_phg

Figure S8


A
Otu0031 .. Bifidobacterium(100)
Otu0013 .. Campylobacter(100)
OTU / genus
0
1
2
3
4
5
LDA (Log10)
Group Cj
Control
B
Otu0009 .. Clostridiales_unclassified(100)
Otu0006 .. Clostridium_IV(98)
OTU / genus
0
1
2
3
4
5
LDA (Log10)
Group Cj_phg

Phylum	kit Ai	kit Aii	kit B	seq Ai	seq Aii	seq B
Actinobacteria(100)	0.3895451	2.1676301	0.00000	2.7673681	1.369863	0.000000
Bacteria_unclassified(100)	0.3895451	0.3853565	0.00000	0.3603344	0.000000	7.088608
Bacteroidetes(100)	0.6534305	5.9730250	0.00000	11.8694148	2.739726	18.227848
Cloacimonetes(100)	0.0000000	0.0000000	0.00000	0.0000000	0.000000	1.518987
Firmicutes(100)	6.4526263	34.6339114	23.63636	21.4759297	86.301370	4.810127
Planctomycetes(100)	0.0000000	0.0000000	0.00000	0.0000000	0.000000	8.101266
Proteobacteria(100)	92.1148530	56.8400771	76.36364	63.5269530	9.589041	53.924051
Thermotogae(100)	0.0000000	0.0000000	0.00000	0.0000000	0.000000	3.797468
Verrucomicrobia(100)	0.0000000	0.0000000	0.00000	0.0000000	0.000000	2.531646

[Table S1]

OTU	Phylum	Class	Order	Family	Genus	Kit Ai	kit Aii	kit B	seq Ai	seq Aii	seq B
Otu0001	Firmicutes(100)	Bacilli(100)	Lactobacillales(100)	Lactobacillaceae(100)	Lactobacillus(100)	0.307866298	2.36030829	0.4545455	0.021620063	0.000000	0.00000
Otu0002	Firmicutes(100)	Bacilli(100)	Lactobacillales(100)	Lactobacillaceae(100)	Lactobacillus(100)	0.069112842	3.37186898	0.0000000	1.340443932	0.000000	0.00000
Otu0003	Proteobacteria(100)	Gammaproteobacteria(100)	Enterobacteriales(100)	Enterobacteriaceae(100)	Enterobacteriaceae_unclassified(100)	91.122141242	15.51059730	76.3636364	56.839146728	9.589041	53.92405
Otu0004	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Ruminococcaceae(100)	Faecalibacterium(100)	1.897461674	11.41618497	10.9090909	0.007206688	0.000000	0.00000
Otu0006	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Ruminococcaceae(100)	Clostridium_IV(98)	0.006282986	1.83044316	1.3636364	0.014413376	0.000000	0.00000
Otu0007	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Clostridiales_unclassified(100)	Clostridiales_unclassified(100)	0.012565971	0.04816956	0.0000000	0.000000000	0.000000	0.00000
Otu0008	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Ruminococcaceae(100)	Ruminococcaceae_unclassified(100)	0.000000000	0.86705202	0.4545455	0.007206688	0.000000	0.00000
Otu0009	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Clostridiales_unclassified(100)	Clostridiales_unclassified(100)	0.483789897	0.04816956	0.0000000	0.000000000	0.000000	0.00000
Otu0010	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Lachnospiraceae(100)	Lachnospiraceae_unclassified(100)	0.012565971	0.52986513	0.0000000	0.007206688	0.000000	0.00000
Otu0011	Bacteroidetes(100)	Bacteroidia(100)	Bacteroidales(100)	Prevotellaceae(100)	Prevotella(100)	0.006282986	0.00000000	0.0000000	4.151052176	2.739726	0.00000
Otu0012	Firmicutes(100)	Clostridia(100)	Clostridiales(100)	Lachnospiraceae(100)	Lachnospiraceae_unclassified(100)	0.961296808	0.43352601	0.0000000	0.000000000	0.000000	0.00000

[Table S2]

CHAPTER 6. DISCUSSION

6.1 Summary

Chicken meat is an important source of dietary protein and, unless alternative sources become available, heightened production will be required to meet a global protein demand that is predicted to grow by 50% by 2050 (Westhoek et al., 2011). The major obstacle to meeting this target is that many of the cost-effective approaches that were successfully employed by the poultry industry to massively improve the productivity of broiler chickens in the past are no longer available. Selective breeding programs to improve the genetics of fast-growing broiler chickens are approaching their limit (Tallentire et al., 2018) and AGPs have been outlawed or spurned by consumers. In addition, new feed components are being sought (Truong et al., 2019) due to the price pressure exerted on feed components such as corn, wheat and soy by competition with both human food and biofuel markets. New approaches, that are both ethical and sustainable, are urgently needed to improve the growth performance of hygienically-safe broiler chickens.

My PhD was part of a larger effort to establish a research program to better understand gastrointestinal health in chickens within the Division of Microbiology, Brewing and Biotechnology at the University of Nottingham. The experimental work reported in the articles that make up this thesis were the outputs of this effort and use a systems biology approach to describe the effect of GOS or *C. jejuni* challenge on both gut microbes and the host. The analysis I undertook of the gut microbiota described was performed using 16S rDNA profiling and was used to determine gross shifts in the bacterial community and pinpoint GOS or *C. jejuni*-responsive taxa for further analysis. Measurements of the host response, by survey of the gut architecture and profiling of cytokine/chemokine expression, and isolation of synbiotic organisms was performed by my co-workers.

The articles that make up this thesis describe how application of prebiotic GOS, a by-product of the dairy industry, to the broiler chicken gut microbiota affects zootechnical performance. This work also reports the results of using the same experimental strategy to describe host:microbe interactions between the broiler chicken and *C. jejuni*, a major zoonotic pathogen.

6.2 Prebiotic GOS improves zootechnical performance of male Ross 308 broiler chickens sustained on wheat-soymeal feed

Two of the articles that make up this thesis report the potential of prebiotic GOS to improve broiler performance and gut health. Consumption of GOS has been reported to have physiological effects in humans by alleviating the symptoms of lactose-intolerance subjects (Azcarate-Peril et al., 2017) or by increasing fasting glucose levels (Liu et al., 2017). Neither of these studies profiled the host response in the gastrointestinal tract, but both reported that GOS was associated with increased proportions of *Bifidobacterium* spp. (Azcarate-Peril et al., 2017; Liu et al., 2017). In our analysis although the *Bifidobacterium* genus was detected, albeit at low relative abundance, it did not respond to GOS (data not shown). Azcarate-Peril et al. (2017) also reported that the *Lactobacillus* genus positively responded to GOS, but Liu et al. (2017) did not. In a study conducted on broiler chickens, Jung et al. (2008) reported that GOS did not improve the growth performance and did affect the levels of faecal *Lactobacillus*. However, the same study did report that probiotic GOS increased the levels of faecal *Bifidobacterium* spp. (Jung et al., 2008). It is unclear whether the difference between the observations of Jung et al. (2008) and those we report are attributable to the different breed of birds used or differences in feed composition. It is also possible that colonization of chickens with GOS-responsive *Bifidobacterium* spp. in early life could account to the findings reported by Jung et al. (2008). The stochastic basis of bacterial succession in the chicken gut was outlined previously (section 1.6). In Richards et al. (2020) we observed that prebiotic GOS and *L. johnsonii* were associated with greater body weight at slaughter (see Chapter 3) and we provided further evidence for the relationship by deliberately exposing chickens to *L. johnsonii* DC22.2 in early-life. However, previous reports of distributions of gut bacteria that are correlated with growth performance have struggled to identify bacterial taxa common to birds with good performance across independent studies (Stanley et al., 2013, 2016). This is also likely to be the case with the correlation between *L. johnsonii* DC22.2 and performance reported in Richards et al. (2020). The microbiota data reported in Richards et al. (2020) was collected from unchallenged birds that were part of the same trial as the chickens challenged with *C. jejuni* at 6 days-old and reported in Connerton et al. (2018) and Lafontaine et al. (2020). It is therefore of note that the caecal microbiota of chickens challenged with *C. jejuni* at 20 days-old and sustained on GOS-supplemented or control diets (Lafontaine et al., 2020), which was performed independently some months later, exhibited differences in *Lactobacillus* spp. with similar OTUs.

However, inter-trial variation was not unexpected and our experiments were not designed to exclude it. The broiler chicken breed was controlled as only male Ross 308 broiler chickens were used and sourced from the same hatchery. Animal husbandry was controlled as birds were sustained on the same control and GOS feed and housed in the same conditions under ACDP2 biosecurity. However, no effort was made to standardise the gastrointestinal microbiota between trials. As any application of these studies would require similar functional responses from a range of husbandry practices in commercial operations we rationalised that the gut microbiota of the chickens would be more representative of on-farm practice than of a highly controlled, experimental model. Given that microbial development of the caeca is often the result of stochastic exposure to colonizing microbiota (Rychlik, 2020) the early development of the gut microbiota was a source of uncontrolled variation. I am unaware of any studies that describe how the gut microbiota of chickens produced by commercial hatcheries vary over time. However, zootechnical performance of GOS-fed birds exceeded that of birds on a control diet in several trials; and our findings suggest that when there was no difference between the body weights of GOS-fed and control birds it was because both achieved optimal growth. This suggests that the same microbiota bioprocesses may underpin the improved performance, but that they may not be performed by the same bacterial taxa. Smits et al. (2016) have shown that feeding 'humanized' mice, ie. germ-free mice colonized with a human donor microbiota, prebiotic fructooligosaccharide changes the composition of the faecal microbiota, but that this does not predict community functionality as determined by metabolomic profiling.

6.3 Interactions between *Campylobacter jenuni* and the caecal microbiota

We have shown that *C. jejuni* colonization of broiler chickens, at either 6 or 20 days old, is accompanied by minor changes in the microbiota and low level increases in the expression of pro-inflammatory cytokine (Connerton et al., 2018). Both of these observations have been reported independently elsewhere (Reid et al., 2016; Thibodeau et al., 2015), but to my knowledge this is the first time they have been recorded together in the same birds. In Richards et al. (2019) we have shown that reduction in the levels of *C. jejuni* by phage-therapy also causes only minor changes in the gut microbiome. Taken together these findings indicate that the low-level inflammation that accompanies *C. jejuni* infection of the caeca is probably not co-opted by *C. jejuni* to effectively colonize the chicken gut at high-levels, as has been reported for *Salmonella* (Litvak et al., 2019). However, the gut microbiota does affect *C. jejuni* colonization as germ-free chickens are colonized with

higher levels of the *C. jejuni* than conventionally-colonized birds (Han et al., 2017), and preparations of anaerobic bacteria from mature birds have been shown to reduce, but not exclude, subsequent *C. jejuni* colonization (Alrubaye et al., 2019; Mead et al., 1996). Although the specific bacterial taxa shown to be protective against *C. jejuni* colonization are predominantly lactic acid bacteria (Cean et al., 2015; Ghareeb et al., 2012; Mañes-Lázaro et al., 2017) the fundamental nature of the interaction between *C. jejuni* and the competitor organisms remains unclear. Further studies will be necessary to determine whether exclusion of *C. jejuni* is best achieve through competition for resources, as in the case of *E. coli* exclusion of *Salmonella* from chickens (Barrow and Tucker, 1986; Litvak et al., 2019), through the production of molecules inimical to *Campylobacter*, or modulation of the host-immune response or by a combination of each of these phenomena (Mohan, 2015).

Our work has shown than *C. jejuni* HPC5 increases the expression of IL-17A and IL-17F in broiler chickens in early-life (Connerton et al., 2018) and that prebiotic GOS increases caecal expression of IL-17A in early-life in the absence of *C. jejuni*. As discussed in Lafontaine et al. (2020), on the basis of studies of mice it has been proposed that IL-17A expression benefits intestinal barrier function, whereas IL-17F weakens intestinal integrity (Maxwell et al., 2015; Tang et al., 2018). In Lafontaine et al. (2020) we report that prebiotic GOS increased IL-17A expression and suppressed IL-17F expressed following colonization with *C. jejuni*, but that the levels of colonization were unaffected. This is in keeping with earlier observations that *C. jejuni* does not co-opt the host pro-inflammatory to colonize the caeca so a dampening the pro-inflammatory response is unlikely to prove effective in reducing colonization. This work does provide a foundation for the rational formulation of chicken feed with ingredients that can beneficially modulate the host:microbe:diet axis.

6.4 Effect of *Campylobacter* phage therapy on the gastrointestinal microbiome

In Richards et al. (2019) we report that oral gavage with a cocktail of two taxonomically distinct *Campylobacter* phages reduced the numbers of *C. jejuni* in the gastrointestinal tract of pre-colonized Ross 308 broiler chickens. The reduction in *C. jejuni* levels was not accompanied by detectable changes in the gastrointestinal microbiota, an observation in keeping with another report of targeted phage-therapy against *E. faecalis* in mice (Duan et al., 2019). Advances in understanding of the role of the gut microbiome on systemic health have been mirrored by a growing interest in the importance of intestinal bacteriophages to human health, with a growing body of evidence of a direct immunological response to phage in the gut of mammalian models (reviewed in Sausset et al., 2020). Gogokhia et al.

(2019) have shown that mice have a IFN-γ and TLR9 mediated response to *E. coli*-targeted phage belonging to the order *Caudovirales*. Richards et al. (2019) did not report the immunological effects of exposure of the gastrointestinal tract to the *Campylobacter* phage CP20 and CP30A that also belong to the order *Caudovirales*. Although phage intervention against *Campylobacter* spp. in chickens has not been associated with any reported detrimental effects (Chinivasagam et al., 2020; Connerton et al., 2011; Fischer et al., 2013; Loc Carrillo et al., 2005; Wagenaar et al., 2005). This is perhaps not surprising as these phage classes are frequently encountered by the chicken intestine from environmental sources (El-Shibiny et al., 2005; Loc Carrillo et al., 2007; Owens et al., 2013). Prior to wider adoption of these approaches by industry it will be valuable to investigate whether exposure to *Campylobacter* phage promotes gastrointestinal inflammation, particularly with regard to exacerbation of inflammation when birds are colonized with more invasive *C. jejuni* strains (Humphrey et al., 2014; Humphrey et al., 2015).

6.5 Future work

Host immune response to *C. jejuni* is dependent on the chicken breed (Reid et al., 2016) and *C. jejuni* strain (Chaloner et al., 2014). Host immunity is also primed by feed formulation, likely mediated through microbial activity (Richards et al., 2020). The findings reported in this thesis are specific to the experimental design and biological models. To fully appraise the applicability of the findings presented here it will be necessary to repeat the experiments with alternative models and parameters, thereby properly assessing the impact of feed composition, chicken breed and *C. jejuni* strain. For example, to exploit these findings it is important to determine whether the immune responses we reported for fast-growing broilers are similar in slow-growing broiler chickens or birds sustained on corn-soymeal feed. It will also be important to profile the effect of GOS on the crop microbiota as this has proposed as a key site of *Lactobacillus* activity (Tannock, 2004; Walter, 2008). The effect of GOS prebiotic on zootechnical performance also needs to be expanded as we have focussed on growth performance criteria and have not observed other criteria such as carcass deposition.

To better understand the fundamental bioprocesses that underpin the GOS-mediated growth performance it will be necessary to understand microbiota functionality by describing the caecal microbiome (Sergeant et al., 2014) or the metabolome of either the caecel lumen or the host (Wilmanski et al., 2019). To better validate the 16S rDNA profiling data it will be necessary to use alternative primers, such as the widely used 27F - 338R pair

(Chung et al., 2016) or an alternative platform such as PacBio, which can sequence the complete length of the 16S rRNA gene (Callahan et al., 2019).

For the microbiota results reported here we have made the raw DNA sequence data available through freely-accessible data repositories and published the programmatic code for sequence curation and figures on github (https://github.com/PJRichards). Future work will more fully embrace the principles of open science (Allen and Mehler, 2019; Schloss, 2018) and will publish all raw data and corresponding programmatic code. To make reproduction easier analytical packages will also be bundled together and made available through a container such as Docker (https://docker.com/).

6.6 Conclusion

The novel findings presented here are of interest to the poultry and poultry feed industries as they are the first step in determining a mode of action for GOS and potentially other prebiotic oligosaccharides. Further work will be required to fully elucidate the bacterial and host biochemical pathways responsible for improved growth performance, this will require wider metagenomic and metabolomic approaches with established methods of determining the levels of energy that the chicken is able to harvest from feed.

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