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

Philip J. Richards, Geraldine M. Flaujac Lafontaine, Phillippa L. Connerton, Lu Liang, Karishma Asiani, Neville M. Fish, Ian F. Connerton

Division of Microbiology, Brewing and Biotechnology, School of Biosciences, University of Nottingham, Loughborough, Leicestershire, United Kingdom

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
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, dendritic 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 symbiotic 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,
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 35da in trial 2 (GOS-fed birds, 2,584g; control birds, 1,838g; P = 0.001 [Fig. 1C]) and trial 3 (GOS-fed birds, 2,501g; control birds, 2,291g; 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 ≥ 0.295; Shannon diversity, P ≥ 0.187 [Fig. S1A and B]). Community richness (Chao) was not significantly different throughout the trial (P ≥ 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,
identified as *Lactobacillus* spp., were discriminatory of the different diets in the early rearing period up to 15 da (OTU0006, \( P < 0.01 \); OTU0010, \( P < 0.022 \)). 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 (\( P = 0.001 \)).
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).

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/H11001 GOS in the absence of monosaccharides. Cultures were incubated for 72 h under anaerobic conditions at 37°C in basal medium with either DP2/H11001 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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**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             | 06610a                        | 07265                         | Lactose permease                | 41        |
| lacA             | 06605                         | 07260                         | β-Galactosidase                 | 41        |
| lacL/M           | 06620                         | 07275                         | β-Galactosidase                 | 34        |
| lacE/F           | 06625                         | 07280                         | PTS lactose transporter         | 34        |
| lacG             | 04955                         | 06095                         | Phospho-β-galactosidase         | 34        |
| mucBP            | 08660a                        | 08750                         | Adhesion                        | 28        |
| fbpA             | 09625                         | 08180                         |                                      |           |
| cbsA/B           | 00840                         | 08695–08755                   | Adhesion                        | 28        |
| apf1/2           | 07620                         | 08695                        | S-layer                          | 32        |
| epsA-E           | 05690–05730                   | 01350                         | Aggregation factor              | 31        |
| Bacteriocin leader | 02750                        | 09005                         | Exopolysaccharide               | 29        |
| hlv              | 00185                         | 07800                         | Bacteriocin                     | 28        |
| Bacteriocin leader | 00290                        | 10185                         | Helveticin J                    | 28        |
| bsh              | 00415                         | 08515                         | Bile salt hydrolase             | 30        |

*a* Internal stop codons present.
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 \(\text{FC} = 83\); corresponding probability \(P_{\text{adj}} = 0.002\)) at 8 da (Fig. 4A; Table 3) and downregulation of IL-10 (FC = 0.02; \(P_{\text{adj}} = 0.002\)), while IL-17F remained unchanged.

![FIG 3](http://msystems.asm.org/) 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 with basal medium plus DP2+ GOS or glucose as a carbon source.

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

| Diet or parameter and age (days) | Villus length (\(\mu\)m) | Crypt depth (\(\mu\)m) | 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            |
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
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 transcriptswere 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 \textit{L. johnsonii} (OTU0010).

**Abundance of \textit{L. johnsonii} in the cecal lumen positively correlates with bird growth performance.** Correlations between abundances of the \textit{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 \textit{L. johnsonii} genome copy number.

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**TABLE 3 Modulation of intestinal cytokine and chemokine responses to dietary GOS in broiler chickens**

| Cytokine or chemokine | Ileum | Cecum |
|-----------------------|-------|-------|
|                       | 8 da  | 15 da | 22 da | 35 da | 8 da  | 15 da | 22 da | 35 da |
| IFN-γ                 | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-1β                 | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-4                  | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-10                 | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-6                  | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| ChCXCLi-1             | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| ChCXCLi-2             | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-17A                | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |
| IL-17F                | FC    | FC    | FC    | FC    | FC    | FC    | FC    | FC    |

|               | 8 da  | 15 da | 22 da | 35 da |
|---------------|-------|-------|-------|-------|
| FC            | 0.5   | 0.6   | 0.2   | 0.1   |
| Padj          | 0.8   | 0.7   | 0.9   | 0.9   |
|               | 0.5   | 0.6   | 0.7   | 0.8   |
| Padj          | 0.8   | 0.9   | 0.9   | 0.9   |
|               | 0.8   | 0.9   | 0.8   | 0.8   |
| Padj          | 0.8   | 0.8   | 0.1   | 0.1   |
|               | 0.8   | 0.8   | 0.8   | 0.8   |
| Padj          | 0.8   | 0.8   | 0.8   | 0.8   |

**Corresponding probabilities (Padj) were calculated from log10-transformed 2^{-ΔΔCt} values using nonparametric Wilcoxon rank sum tests adjusted with Benjamini-Hochberg false-discovery rate correction.**

**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^{-ΔΔCt} was determined as indicated in the legend to Fig. 4, and the log10-transformed expression values were plotted against each other (n = 7).
determined by quantitative PCR (qPCR) from the cecal microbiota (Pearson’s $r = 0.876$; $P = 0.001$ [Fig. 6A]). A significant negative correlation between $L. \text{ crispatus}$ genome copy number and mass was also observed (Pearson’s $r = -0.763$; $P = 0.001$ [data not shown]). The abundances of $L. \text{ crispatus}$ and $L. \text{ johnsonii}$ 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 $L. \text{ johnsonii}$ genome copy number (Pearson’s $r = 0.502$; $P = 0.003$). These results together strongly suggest that $L. \text{ johnsonii}$ 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 $L. \text{ crispatus}$ DC21.1 or $L. \text{ johnsonii}$ 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 $L. \text{ crispatus}$/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 $L. \text{ johnsonii}$ corresponded with disparate differences in the weights of mature birds at 35 da. The mean body weight of the birds with low cecal $L. \text{ johnsonii}$ abundance (administered with competitive $L. \text{ crispatus}$) fed on the control diet was $1.86 \pm 0.16$ kg, compared with $2.83 \pm 0.11$ kg for the birds with high cecal $L. \text{ johnsonii}$ abundance ($P < 0.001$). Figure 7B and C show the respective correlations between body mass and $L. \text{ johnsonii}$ genome copy number from the cecal microbiota of birds administered either $L. \text{ johnsonii}$ DC22.2 (Pearson’s $r = 0.353$; $P = 0.038$) or $L. \text{ crispatus}$ DC21.1 (Pearson’s $r = 0.504$; $P < 0.001$). These data provide further evidence for the positive relationship between the cecal abundance of $L. \text{ johnsonii}$ and growth performance. The impact of the early exogenous introduction of $L. \text{ crispatus}$ DC21.1 was to reduce the abundance of $L. \text{ johnsonii}$, 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 $L. \text{ johnsonii}$ and improved body masses at 35 da, the birds in the $L. \text{ crispatus}$ treatment groups did not develop a comparable
relative abundance of \textit{L. johnsonii} or achieve the weight at 35 days observed for mock-treated birds (Fig. 7C). Collectively, these data indicate \textit{L. crispatus} is a competitor of \textit{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.

**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: \textit{Bacteroides}, \textit{Enterobacteriaceae}, \textit{Clostridium}, \textit{Ruminococcus}, \textit{Faecalibacterium}, and \textit{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 \textit{Lactobacillus} genus in the cecal microbiota of juvenile birds on control and GOS diets were observed. \textit{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.
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 lacEF. Converse to this, _L. johnsonii_ DC22.2 has retained lacS and lacA but has lost the lacEF 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. pasteurii_ 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...
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 (Tβ-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
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. Chranova 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, δ-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-oligosaccharide, 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...
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 CO2 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. Ideal 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 pen 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 log10 CFU in 0.1 ml of MRD (Oxoid, Basingstoke, UK) were administered to chicks by cloacal gavage. Control groups were mock administrated 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 DNA was 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 3H2O, 2 g of diammonium hydrogen citrate, 0.2 g of magnesium sulfate 7H2O and 0.05 g of manganese sulfate 4H2O (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 log10 CFU/ml (OD600 of approximately 1.5). The suspension was diluted 1 in 100 into the growth medium. The
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 target genes was determined using the 2−ΔΔCT method, where the CT is the PCR cycle threshold and 2−ΔΔCT corresponds to the difference between the CT values of the cyto/chemokine gene and the reference housekeeping genes to give normalized transcript levels, and ΔΔCT is the difference between transcript levels of birds on the GOS and control diets (87). The means of triplicate Ct values were used for analysis, where target gene Ct 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/PRichards/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.
Competitive indices of the *Lactobacillus* species present in the ceca were calculated as the ratio of the *L. crispatus* (OTU006) 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/PJR Richards/GOS_broiler/tree/master/zootechnical.

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/PJR 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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