Effect of prebiotic oligosaccharides on gut health and performance of chickens

By

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ABSTRACT

Interest in prebiotics and their potential application for human and animal health is flourishing. Here, the effects of galacto-oligosaccharide (GOS) on the performance and health of chickens, with the potential for human applications was evaluated. Following a review of the poultry meat industry from economic and animal welfare standpoints, the outcomes of GOS feedstuff on the gut health and immune function of broiler chickens were evaluated. First, the effects of in-feed inclusion of GOS on broiler chicken performance and intestinal immune status were assessed in the absence of intestinal challenge. The GOS diet was shown to modulate the juvenile gut microbiome and innate immunity to increase weight gain and reduce the cumulative feed conversion ratio. GOS-associated activation of mucosal Th17 immune response at a young age was accompanied with a shift in the microbiota composition promoting one member of autochthonous *Lactobacillus spp* at the expense of another. The cecal abundance of immuno-modulatory *L. johnsonii* was shown to increase on the GOS diet and positively correlate with bird growth weight at 35 days of age. The impact of GOS was further assessed upon bacterial challenge with the foodborne pathogen *Campylobacter jejuni. C. jejuni* challenge effects on birds maintained on a normal diet were first characterised. The study identified age-dependent differences in the kinetics of cecal colonisation, microbiome compositional shifts, and Th 17 induced intestinal immune responses. Upon *C. jejuni* challenge, broilers maintained on a GOS diet continued to exhibit the growth advantage. Despite successful cecal colonisation by *C. jejuni*, GOS selectively induced microbiota shifts associated with Th17 induction. Due to limited evidence of the direct impact of dietary fibres on the intestinal barrier, the effects of GOS and fructo-oligosaccharides (FOS) on the transcriptome of polarised

human colonic epithelial cells were evaluated. Both oligosaccharides improved epithelial tight junctions as indicated by increases in trans-epithelial resistance. However, the effects of FOS on the transcriptome were reduced when compared to GOS. Our strategy produced a comprehensive curated gene expression database that will permit further work to link gene expression signatures of cultured cells to their mode of action, thus potentially facilitating product choices in human or animal intervention studies.

FOREWORD

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ABBREVIATIONS

AGPs	antimicrobial growth promoters
APC:	antigen-presenting cell(s)
CT:	cecal tonsils
d.a.:	day of age
DC:	dendritic cell(s)
DEFRA:	Department for Environment, Food and Rural Affairs
ED:	embryonic day
EU:	European Union
ExPEC:	extra-intestinal pathogenic *E. coli*
FAO:	Food and Agriculture Organization
FCR:	feed conversion ratio
FOS:	fructo-oligosaccharides
GALT:	gut-associated lymphoid tissues
GOS:	galacto-oligosaccharides
HMOs:	human milk oligosaccharides
IEL:	intraepithelial lymphocytes
IFN:	interferon
Ig:	immunoglobulin
kg:	kilogram
LAB:	lactic acid bacteria
LP:	lamina propria
mAb:	monoclonal antibody
MALT:	mucosa-associated lymphoid tissues
MHC:	major histocompatibility complex
MoDC:	monocyte-derived dendritic cell
NK:	natural killer
OECD:	Organisation for Economic Co-operation and Development
p.i.:	post-infection
PBMC:	peripheral blood mononuclear cell(s)
PP:	Peyer's patch
qPCR:	quantitative polymerase chain reaction
SCFA:	short chain fatty acid
SL:	sialyllactose
TCR:	T-cell receptor
TNF:	tumour necrosis factor
UK:	United-Kingdom

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The effect of the timing of exposure to *Campylobacter jejuni* on the microbiome and inflammatory responses of broiler chickens. (2017). Geraldine M. Lafontaine, Phillippa L. Connerton, Philip J. Richards, Peter M. O'Kane, Nacheervan Ghaffar, Nicola J. Cummings, Darren L. Smith, Neville M. Fish and Ian F. Connerton; 19th *Campylobacter*, *Helicobacter* and Related Organisms Conference (CHRO2017), 10-14th of September 2017, Nantes, France

The effect of the timing of exposure to *Campylobacter jejuni* on the microbiome and inflammatory responses of broiler chickens. (2017). Geraldine M. Lafontaine, Phillippa L. Connerton, Philip J. Richards, Peter M. O'Kane, Nacheervan Ghaffar, Nicola J. Cummings, Darren L. Smith, Neville M. Fish and Ian F. Connerton; SfAM Annual Applied Microbiology Conference, 3-6 July 2017, Gateshead, UK

CERTIFICATIONS

Animals (Scientific Procedures) Act 1986 Personal Licence. Species: Birds, Mice, Rabbits, Rats. Delivered on the 14th of October 2016 by Animals in Science Regulation Unit, Home Office Science, London (UK)

Animal PIL A EU modules EU 3.1/3.2, EU 4, EU 5, EU 7, EU 8 from Directive 2010/63/EU. Species: Pigs. Delivered on the 25th of August 2016 by Scottish Accreditation Board (SAB).

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1 Chapter 1. Introduction

In the first part of this introduction, the current economic context and challenges to poultry meat production are considered. In a second part, the state of knowledge on broiler chicken performance, gut health and the use of in-feed GOS and FOS are reviewed.

1.1 Economic context of poultry production

Global meat consumption levels in the developed world are already high, nevertheless as meat has become more affordable demand continues to increase (OECD/FAO 2019). Worldwide, in 2017 a person consumed on average around 14 kilograms of poultry meat, making it the most consumed meat *per capita* followed by pig meat (Figure 1).

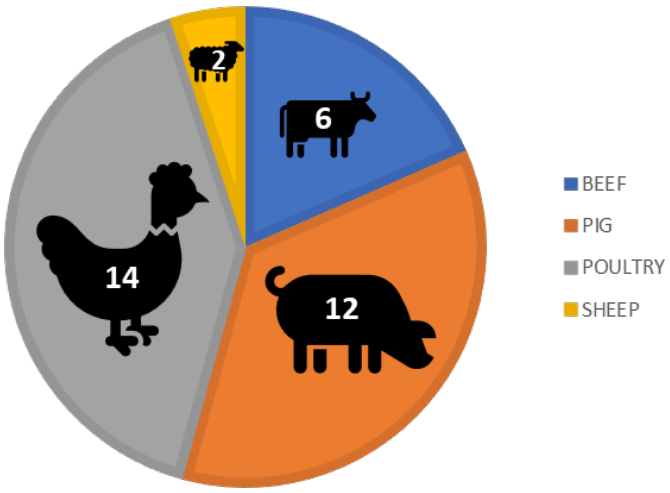

Figure 1. World meat consumption

Meat consumed per person (Kg/capita) in 2017. Sources: (OECD/FAO 2019).

Global production of poultry meat has increased over the last 50 years more than 12-fold (Ritchie and Roser 2020) with the current European market standing at 2 billion Euros import-export (EU Commission 2020), thus emphasizing the economic power of the poultry industry across borders. Going forward, the poultry sector is expected to continue to grow with the demand for eggs and meat driven by growing populations, rising incomes and urbanisation (Mottet and Tempio 2017). Poultry cover a wide range of domesticated birds such as chickens, ducks, turkeys, guinea fowl, geese, quail, pigeons, ostriches and pheasants, nonetheless chickens are by far the leading species raised for meat consumption worldwide (FAO 2016; Scanes 2007).

Broiler chicken meat represents the most consumed meat product in the UK with an average of 20 million broilers slaughtered per week (DEFRA 2020). In a growing and challenging food supply market, animal performance and feed conversion efficiency of healthy fast-growing birds are paramount elements to the profitability of the poultry industry. Antimicrobials have been widely used in commercial farms to treat or prevent occurrences of disease and to enhance animal performance as growth promoters (AGPs) of high-density flocks. It is also becoming ever clearer that food safety, food quality and animal welfare are essential considerations to the consumer. Public and regulatory concerns have been mounting regarding the development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota (Castanon 2007). Consequently, the European Union (EU) has banned the inclusion of AGPs in livestock feed since 2006. Although antimicrobials are still globally widely administered, there have been reductions in their therapeutic use in poultry production, which have led to an increase in intestinal health problems (Van Immerseel et al. 2017). To palliate the undesirable effects of antimicrobial reduction, several strategies such as dietary prebiotics, probiotics, phytobiotics, yeast and beneficial enzymes feed additives have been evaluated in poultry

production (Sethiya 2016; Thacker 2013; Erickson and Hubbard 2000; Gadde et al. 2017). Worldwide, intense research is in progress to assess the beneficial effects of prebiotics aimed to improve human health. Dietary prebiotic applications are also targeting farm animal health and performance whilst reducing the need for antibiotic applications. Prebiotics are non-digestible feed ingredients that can be metabolized by specific members of the intestinal microbiota to provide health benefits to the host. In this context Dairy Crest Ltd, a functional ingredients manufacturer, sought to evaluate the effects of galacto- and fructo-oligosaccharide prebiotics on the performance and health of monogastric farm animals, with the potential for human applications. Therefore, the aim of this project was (1) to assess the prebiotic effects (microbiota-dependent) of galacto-oligosaccharides on the performance and gut health of meat-producing broiler chickens and (2) to measure the direct effects (microbiota-independent) of galacto-oligosaccharides and fructo-oligosaccharides on human intestinal epithelial cells.

1.2 Prebiotics

Prebiotics have been initially described in 1995 by Gibson and Roberfroid as "non-digestible oligosaccharide food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon" (Gibson and Roberfroid 1995). Two decades later, the concept of saccharides acting in the colon has been extended to substances selectively utilized by host micro-organisms conferring a health benefit (probiotics) (Gibson et al., 2017). Some members of the chicken natural microbiota have previously been characterized and associated with growth performance outcomes (Torok, Hughes, et al. 2011; Stanley et al. 2016). Furthermore, antimicrobial in-feed addition demonstrated a profound effect on juvenile birds microbiome composition with a reduced relative abundance of Lachnospiraceae, *Lactobacillus johnsonii*,

Ruminococcaceae, Clostridiales and Oxalobacteraceae to the potential benefit of other community members such as *Lactobacillus crispatus*, *Lactobacillus reuteri* or unclassified Clostridiales and Enterobacteriaceae (Schokker et al. 2017; Torok, Allison, et al. 2011). These findings support the development of alternatives to current antimicrobial agents such as probiotics (Sethiya 2016; Gadde et al. 2017). Besides the need for improved understanding of beneficial bacteria mode of action, characterising their nutritional requirements to become established members of the microbiota appears paramount.

Oligosaccharides are important natural components of food products that escape hydrolysis by salivary and intestinal digestive enzymes. They are neither digestible nor absorbed in the stomach or small intestine, and so act as indigestible soluble fibres capable to reach the large intestine, where beneficial colonic bacteria break them down to absorbable nutrients through fermentation processes. Ingestion of prebiotic food can have a large impact on the microbiota composition, the level of fermentation products such as short chain fatty acids (SCFAs) produced, the intestinal barrier integrity and the host immune status (Kraehenbuhl and Neutra 1992). For instance, fermentation and by-products of oligosaccharides, such as SCFAs, have been shown to promote beneficial effect on intestinal mucosa (reviewed by Koh *et al.*, 2016). The ability of prebiotics to improve gut fitness through manipulation of probiotic microbiota composition and host gut immune function are widely assessed in human and animal health thus it is considered as a viable alternative to some chemical feed additives and antibiotics (Schley and Field 2002; Macfarlane, Macfarlane, and Cummings 2006). This implies the effect of a prebiotic to be essentially indirect because it selectively feeds a limited number of microorganisms from the microbiota, however more information remains to be unveiled regarding unfermented oligosaccharides present in the intestinal lumen that could potentially act through direct or 'non-prebiotic' effect on the intestinal epithelium should they reach the mucosa.

1.2.1 Human milk oligosaccharides

Studies addressing the potentials of several oligosaccharides in human and animal health are surging. In terms of human health, milk oligosaccharides (HMOs) are the subject of intense research to characterise their chemical structures and functions supporting the health of new-born infants. One mode of action proposed for breast-fed infants increased protection from intestinal burdens is through anti-adhesive and anti-proliferation properties of HMOs against viral, protozoan parasite or bacterial pathogens (reviewed by Bode, 2015; Morozov et al. 2018). The action of HMOs has been suggested to occur by creating selectivity toward certain members of the microbiota. For example, Garrido *et al.* demonstrated *in vitro* that within *Bifidobacterium* species HMOs utilization and gene expression were different between *B. infantis* and *B. bifidum* species (Garrido et al. 2015), implying a possible selective effect based on oligosaccharide utilisation capacity. Moreover, mounting evidence suggest HMOs association with immunomodulatory properties and effects on epithelial barrier function support the contention that HMOs increase the maturation of the intestinal mucosa (He et al. 2014). Holscher *et al.* demonstrated the effects on differentiation, digestive function, and epithelial barrier function of cell monolayers were dependent upon the chemical structure of the HMO in question (Holscher, Davis, and Tappenden 2014). Foetal lymphocyte maturation properties were further assessed using umbilical cord blood. Eiwegger *at al.* were able to demonstrate different fractions of HMOs could activate T cells and promote cytokine release (Eiwegger et al. 2004). Interestingly, the authors uncovered further evidence of the aptitude of oligosaccharides to promote epithelial transport through Caco-2 cell monolayers (Eiwegger et al. 2010). It is apparent human milk is a complex and rich prebiotic medium with multifaceted beneficial properties driven by underlying

molecular mechanisms that are relatively unknown. In an attempt to mimic the beneficial effects of HMOs, galacto-oligosaccharides and fructo-oligosaccharides are often included in infants milk formula, thus leading intense research on these key components.

1.2.2 Galacto- and fructo- oligosaccharides

Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are fermentable carbohydrates that can be produced from whey, thus valorising cheese's most significant waste (Geiger et al. 2016). GOS synthesis through β-galactosidase-catalysed trans-glycosylation of lactose creates molecules of differing lengths and linkage types. Degrees of polymerization (DP) usually range from 2 to 8 monomeric units with β(1→3), β(1→4) and β(1→6) linkages between galactose units coupled to a terminal glucose (Van Leeuwen et al. 2016).

Fructo-oligosaccharides

Fructo-oligosaccharides (FOS) are commonly produced from inulin, a natural food ingredient, by acid- or enzyme-catalysed hydrolysis (Martins et al. 2019) resulting in fructan oligomers of 1 to 7 units (DP 1-7) sometimes with a terminal glucose (Niness 1999; Martins et al. 2019).

Oligosaccharides such as GOS can promote the growth of beneficial autochthonous bacterial families, including *Bifidobacteria*, *Bacteroides*, and *Lactobacillaceae* in humans and animals (Jung et al. 2008; Hughes et al. 2017; Tian et al. 2019; Van Bueren et al. 2017). When comparing GOS to FOS the effects on cultures of healthy infant fecal samples *in vitro*, GOS was found to promote the greatest bifidogenic effect as revealed by outgrowth of *Bifidobacterium* (*B. longum*) at the expense of *E. coli* abundance, which was coupled with increases in acetic acid concentration

and pH reduction (Stiverson et al. 2014). The authors concluded that GOS may increase intestinal Bifidobacteria and benefit infant health. Further details comparing HMO sialyllactose (SL) and GOS revealed distinct modulation of the microbiota composition and the production of short chain fatty acids (SCFAs) such as acetate, lactate and propionate. In a study evaluating infant and adult microflora composition using *in vitro* fecal cultures, Perdijk *et al*. showed that SL induced propionate-associated growth of *Bacteroides* whereas GOS promoted *Bifidobacterium* linked with a rise in lactate, while lactobacilli remained unaffected (Perdijk et al. 2019). Furthermore, in the same study the authors demonstrated SL and GOS could directly influence epithelial barrier function by promoting cell differentiation and the re-epithelialization phase of the wound repair process in an epithelial cell monolayer model.

Immunomodulatory properties

Immunomodulatory properties in humans have been suggested for GOS and FOS. This was demonstrated on naïve T cells co-stimulated with monocyte-derived dendritic cells (MoDC) exposed to FOS, GOS and lactic acid bacteria (LAB) *Bifidobacterium breve* and *Lactobacillus rhamnosus* (Lehmann et al. 2015). Lehmann *et al*. showed a GOS-FOS mixture could upregulate the number of functional suppressive $Foxp3^+$ T cells and induce release of IL-10, an anti-inflammatory cytokine, by MoDC stimulation in the absence of IL-12 secretion, thus the same effect was observed upon exposure of naïve T cells and MoDC to LAB. Moreover, the anti-inflammatory response of LAB was found enhanced when the GOS-FOS mixture was introduced implying a synergetic effect of oligosaccharides mixture and probiotic LAB. In light of these findings the authors suggested oligosaccharides promoted the induction of regulatory T cells directly and through probiotic interaction.

Microbiota maintenance

To comprehend the underlying mechanisms by which oligosaccharides can support the growth of autochthonous bacteria, the effect of GOS on the metabolism of *Bacteroides thetaiotaomicron.* was evaluated (Martens, Chiang, and Gordon 2008; Van Bueren et al. 2017). As a prominent member of the human microbiota, *B. thetaiotaomicron* is known for its ability to "forage" on host mucin by modulating the expression of genes involved in targeting host glycans, and mucin O-glycan specifically. It has been established that the "foraging" aptitude enables maintenance and intestinal persistence of the bacterium, thus implying a pivotal role in the maintenance of the gut community structure (Martens, Chiang, and Gordon 2008). In a recent study, Van Bueren *et al.* were able to identify an endo-β (1 → 4)-galactanase responsible for GOS catabolism by *B. thetaiotaomicron* (Van Bueren et al. 2017). The authors further demonstrated GOS ability to modulate bacterial gene expression by inducing a subset of polysaccharide utilization loci directed at O-glycan and host glycan. Strikingly, the authors were able to associate different GOS DP fractions with their direct physiological effects on the bacterium (Van Bueren et al. 2017). It is conceivable that the development and maintenance of autochthonous members of the microbiota within their niche may be one of the ways oligosaccharides support the host protection against pathogen adherence and colonisation. Hence, microbiota-rich organs such as gut, lung and skin can be beneficially affected by prebiotic molecules.

Chicken productivity

An average of 90 million commercial broiler chicks were placed each month in farms in the UK (DEFRA 2020). Cost effective production of chicken meat relies on achieving healthy good performing birds. Selective breeding and feed optimization have resulted in considerable increases in the growth rate and feed efficiency of broiler chickens since the 1940s

(Zuidhof et al. 2014). However, it is generally recognized that increases in performance are slowing as these approaches are reaching their biological limit (Tallentire, Leinonen, and Kyriazakis 2018). Over a typical rearing cycle of 35 to 42 days, the average liveweight of broiler chickens at the point of slaughter reaches 2.2 kg (Aviagen 2019). Live weight and feed intake are key performance indicators in poultry production with huge economic and environmental impact. The feed conversion ratio (FCR) is a measure of the amount of feed required (in kilogrammes) to produce one kilogramme of body weight. Consequently, feed efficiency increases relate to a reduction in the quantity of feed consumed per kg of meat produced. Therefore feed optimization has the promise to reduce the environmental impact and greenhouse gas intensity resulting from poultry production (Ritchie and Roser 2020; Zuidhof et al. 2014). Prebiotic and probiotic in-feed additives have shown promising weight increases in chickens usually coupled with modulation of the gut microbiota (Silva et al. 2010; Song et al. 2014; Van Immerseel et al. 2017; Rubio 2019; Jung et al. 2008). However, carbohydrate-related changes in the gut microbiota have not always translated into improved broiler performance (Geier et al. 2009). Growth performance is directly linked with the intestinal capacity to absorb nutrients. Therefore, the effect of any feed additive on the intestinal epithelium is likely to be of significance for the rate of growth. It is accepted that ileal microscopic features are related to its absorptive function, and to specific and non-specific defence systems that scan and respond to pathogens or hazardous components. Intestinal morphology has become a tool for assessing nutritional effects on the intestine (Lilburn and Loeffler 2015; Wang and Peng 2008). In chickens and piglets, probiotics and prebiotic GOS have been reported to improve ileal architecture (Silva *et al.*, 2010; Song *et al.*, 2014; Varasteh *et al.*, 2015; Alizadeh *et al.*, 2016), whilst heat stress in chickens was found to be detrimental (Geyra et al. 2014).

Animal welfare and health are crucial elements to the profitability of the poultry industry. Long distance shipping of chicks from the hatchery is commonplace, and high flock densities on farm represent stressful situations with health challenges for newly hatched chicks and fast-growing juvenile birds. Animal welfare is currently protected by EU legislation with specific set of rules to safeguard chickens kept on farm for meat production (Directive 2007/43). In the UK, animal welfare legislation has been implemented through a Code of Practice made under the Animal Welfare Act 2006 (Act of Parliament 2006; DEFRA 2018). For example, there are specific rules for environmentally controlled loose-housing systems that address maximum stocking density, litter quality and access, feed, ventilation performance, and lighting intensity and pattern. Legislation also applies during transport, setting rules such as maximum journey time without feed and water and the space allowance based on the liveweight of the bird (European Commission 1998; DEFRA 2018). Moreover, a "from farm to fork" food safety concept has been developed to address modern consumers expectations in delivering high-quality products compliant with high standards of production and animal welfare throughout the farming process (Oakley et al. 2013). Nevertheless, high density farming and the coprophagic behaviour of birds favour the spread and expansion of unwelcome organisms from the environment.

Chicken welfare

Broilers are frequently confronted with respiratory diseases, enteric infections by coccidia (Quiroz-Castañeda and Dantán-González 2015), pathogenic bacteria such as *Escherichia coli* (Kemmett et al. 2014), *Clostridium*, *Salmonella*, and *Campylobacter* (Humphrey et al. 2014), some of which are endemic but are still responsible for growth depression or death. In some instances, poultry products have been suggested to serve as a source of human pathogens. For example, avian pathogenic *Escherichia coli* has been previously linked with extra-intestinal

pathogenic *E. coli* (ExPEC) responsible for sepsis and urinary tract infections in humans (Manges and Johnson 2012), although the transmission process remains to be elucidated. However, the most notable human pathogens are foodborne zoonotic bacteria of the genera *Salmonella* and *Campylobacter*.

Salmonella enterica is responsible for foodborne salmonellosis, a major concern worldwide. Broiler meat alongside layer egg and egg-products account for the majority of food-borne salmonellosis outbreaks in the EU. In 2016, salmonellosis cases mounted to 96,039 reported cases with *Salmonella* Enteritidis and *S.* Typhimurium representing the most frequently acquired serovars (Koutsoumanis et al. 2019). Under EU Directive (2003/99/EC) broiler flock testing for *S.* Enteritidis and *S.* Typhimurium is required and reported to EFSA (Koutsoumanis et al. 2019). Commercial farms contaminated with multiple *Salmonella* serovars are common and often asymptomatic. *Salmonella* is typically introduced in flocks from the environment, feed, or from infected chicks that may have acquired *Salmonella* within the egg from the parent breeder flock or from cross-contamination in the hatchery (Pande et al. 2016). Although several vaccines are in use, variable levels of efficacity are driving the search for additional methods of control. An evaluation of FOS showed the prebiotic didn't reduce *Salmonella* cecal load for chicken challenged with *Salmonella* Enteritidis (Fukata et al. 1999). The same observation was made for pigs (Naughton et al., 2001). Lately, the use of GOS, in conjunction with an attenuated mutant of *Salmonella enterica* serovar Typhimurium, have shown promising results in reducing the level of cecal colonisation, which was attributed to microbiota composition shift (Azcarate-Peril et al. 2018). However, GOS alone did not significantly reduce the level of Salmonella cecal colonisation despite evident microbiota compositional changes (Hughes et al. 2017).

Campylobacter

In terms of food safety, Campylobacteriosis is at the forefront of the most frequently reported human zoonotic disease with 246,158 confirmed cases of gastrointestinal illness in the EU in 2017 (EFSA 2018). *Campylobacter* cause acute human bacterial food-borne enteritis, fatal in some cases (Adak, Long, and O'Brien 2002), and have been associated with severe complications such as Guillain-Barré syndrome (Takahashi et al. 2005) and Inflammatory Bowel Syndrome (Neal, Barker, and Spiller 2002; Spiller and Lam 2012). The most common species associated with human disease and found widely amongst broiler farms is *Campylobacter jejuni* (84.4%), but *C. coli* also represents a significant disease burden (9.2%) leading to broiler flock testing and reporting to EFSA (Sahin et al., 2015; EFSA, 2018; Skarp et al., 2016). Poultry meat is frequently contaminated with intestinal content harbouring high levels of *Campylobacter* cells during slaughter and carcass processing, which constitutes the main risk to public health (Osimani et al. 2017). *Campylobacter* are flagellate spiral-shaped Gram-negative microaerophilic thermophilic bacteria, with an optimum growth temperature of 42°C, although they are capable to grow at lower temperatures (reviewed by Silva et al., 2011; Public Health England, 2018) . Their main reservoirs are warm blooded-animals such as poultry, pigs, and cattle (Newell et al. 2011; Sahin et al. 2015). *C. jejuni* are extremely prevalent in poultry production. Although some authors consider *Campylobacter* as a commensal of the intestinal microbiota of chickens (Wilson et al. 2008), others suggest *C. jejuni* can induce clinical symptoms through observation of natural and experimental infections (Ruiz-Palacios, Escamilla, and Torres 1981). Often *Campylobacter*-positive farms have been associated with low performance and high level of transmission to poultry meat following processing (Guerin et al. 2010; Bull et al. 2008; Newell et al. 2011; Humphrey et al. 2014). Factors leading to the introduction of *Campylobacter* in bird flocks remain under investigation. In commercial flocks it has been observed that *C. jejuni* is absent in chicks less than 2

weeks of age, also called the lag-phase, suggesting they may have intrinsic resistance to *Campylobacter* colonization possibly due to the presence of specific maternal antibodies (Han, Pielsticker, et al. 2016; Sahin et al. 2003). Nevertheless, growing evidence suggests farms become *Campylobacter* positive upon human intervention during the rearing period at thinning and at depopulation stages (Lawes et al. 2012; Hald, Wedderkopp, and Madsen 2000). Thinning is a routine practice involving the removal of a proportion of birds (approximately 20–30 %) for processing at around 35 days of age, allowing the remaining birds to develop to a larger weight for processing at around 42 days. Production without thinning and diligent biosecurity interventions have shown to significantly reduce the odds of a broiler house being diagnosed as *Campylobacter* positive (Hald, Wedderkopp, and Madsen 2000; Higham et al. 2018). Nonetheless, complementary interventions are required to enhance *Campylobacter* control at farm level (Newell et al. 2011). Consequently, much attention has focused on reducing both the incidence of *Campylobacter* in poultry flocks and growth performance improvement. Little is known about the local immune response and systemic humoral immune responses which would distinguish whether *C. jejuni* may or may not be a commensal bacterium of chickens (Humphrey et al. 2014). *Campylobacter* positive poor flock performance has been observed (Bull et al. 2008), but not in every case (Gormley et al. 2014), implying variation due to chicken breed and *Campylobacter* species. Besides reduction in body weight, *C. jejuni* has been associated with reduced intestinal barrier integrity and alteration of small intestine architecture implying a reduction in the intestinal barrier function (Awad, Molnár, et al. 2015; Awad, Smorodchenko, et al. 2015). Analysis of the chicken gut microbiome composition during *C. jejuni* colonization identified microbiota shifts indicating a window of opportunity for *Campylobacter* colonisation, thus calling for intervention strategies within the first 3 weeks of the rearing cycle (Ijaz et al. 2018). It has been speculated that prebiotics diets may modify the cecal microbiota and gut health of chickens therefore affect the

prevalence of *C. jejuni* in older chickens' gastrointestinal tract (Han, Willer, et al. 2016).

1.3 Chicken intestinal immunity

In addition to fulfilling the function of nutrient absorption, the chicken gut is the largest immunological organ and the first protective mechanism to shield against exogenous pathogens that can colonise and/or enter host cells and tissues (Choct 2009). The intestine is a complex organ requiring microbiota tolerogenic functions and efficient responses to undesirable microbes or products and forms the basis for the prevention of translocation to the venal system. The intestinal mucosa constitutes a physical and immunological protective barrier for the integrity of the gastro-intestinal tract. The intestinal lumen harbours trillions of micro-organisms, thus supporting digestion processes and protective intestinal functions, which in return are under surveillance of the mucosal immunity system (Kraehenbuhl and Neutra 1992). On commercial poultry farms newly hatched chicks face life threatening enteric infections as a result of immature digestive and immune system along with poor feed intake (Klasing 2007). Thus, any intervention that affects the health of the gut will undoubtedly influence the animal as a whole and consequently alter its nutrient uptake and performance. Commensal bacteria are necessary for the development and maintenance of a healthy immune system. Indeed, the host relies on the commensal microbes for many basic physiological and metabolic functions, as well as immune functions (Ivanov and Honda 2012). Decades of studies in germ-free animals have established the importance of microbiota for proper host immune function and its role in maintaining intestinal homeostasis (Macpherson and Harris 2004). Today, gut health is a major topic for research since maintenance and enhancement are essential for welfare and peak productivity of animals when antibiotics are not allowed in feed (Choct

2009). The search for suitable pre- and pro-biotics that stimulate immune responses is mostly driving current research of the avian intestinal immune system (Yurong et al. 2005; Haghighi et al. 2008; Janardhana et al. 2009). Compared to mammals, birds' immune systems are rather less well understood, partly due to dissimilarities to other systems and the lack of reliable commercially available antigens and antibodies to identify the cells involved in immune functions. Differing from the lymphoid system of mammals that are shaped in lymph nodes, the avian lymphoid system is organised through diffuse secondary mucosa-associated lymphoid tissue (MALT), which are the main sites of antigen-specific activation of mucosal B and T cells.

1.3.1 Chicken gut associated lymphoid tissues (GALT)

Chickens have developed local mucosa associated lymphoid tissues such as nasal (NALT), conjunctival (CALT), bronchial (BALT) and gut (GALT) (reviewed by Lillehoj and Trout, 1996; Liebler-Tenorio and Pabst, 2006). GALTs are present as aggregations of lymphoid cells or organized in lymphoid follicles and tonsils, depending on the location (Fig.2). Except for the bursa of Fabricius, which is a primary lymphoid organ, all chicken MALT lymphoid tissues are secondary lymphoid organs (reviewed by Liebler-Tenorio and Pabst, 2006). In fowl, the primary lymphoid organs include the embryonic yolk sac, the lympho-epithelial organs (thymus and bursa of Fabricius), and the bone marrow.

The bursa of Fabricius

The bursa of Fabricius is both a primary lymphoid organ, essential to provide an environment for stem cells to divide and mature into B cells and generation of antibody diversity for the normal development of the humoral immune system for B cell development, and a secondary

lymphoid tissue responsible for immune response initiation. Through its connection to the cloacal lumen, the bursa of Fabricius is a major avenue through which environmental antigens such as indigenous bacteria and intestinal contents stimulate the immune system post-hatch (Ekino et al. 1985; Ratcliffe 2006). Historically, B cells have been described originally in chickens by Glick *et al.* in the 50's (1956) as "antibody-producing cells residing in the bursa of Fabricius", a follicular structure located dorsally to the cloaca (Glick, Chang, and Jaap 1956). The avian bursa is described as the equivalent to mammals' bone marrow where, *in ovo,* bloodborne stem cells migrate from embryonic spleen through the blood stream between day 8 (ED8) and 14 (ED14) of embryonic development, for proliferation and maturation. Using the B cell surface antigen Bu-1 and the antigen Ov as a marker for T cells, Houssaint *et al.* had demonstrated that early segregation of the B and T cell lineages occurred prior to the colonization of the bursa, thus the authors suggested B cell precursors were committed prior to their entry into primary lymphoid organs (Houssaint, Mansikka, and Vainio 1991; Ratcliffe 2006). During embryonic migration, Laparidou *et al.* elegantly confirmed *in vitro* and *in vivo* that chemotaxis between the embryonic B cell receptor CXCR4 and the bursal chemokine CXCL12 led to the cells chemo-attraction toward the bursa (Laparidou et al. 2020).

GALT secondary lymphoid system

The GALTs secondary (or peripheral) lymphoid system, which contains immune cells such as T, B, and other lymphoid cells, controls mature naive lymphocytes maintenance and immune response initiation. GALTs consist of lymphoid cells located in the intestine mucosal lining and in specialised structures (Peyers patches, cecal tonsils, Meckel's diverticulum; Fig. 2). The lymphoid tissues are frequently covered by a lympho-epithelium infiltrated by lymphoid cells containing microfold cells (M cells) specialized in antigen sampling and transport to the underlying lymphoid

tissue (reviewed by Casteleyn et al., 2010; Balic et al., 2019; Jeurissen et al., 1999).

The intestinal mucosa (the closest layer to the lumen) consist of an epithelium, lamina propria (LP), and muscularis mucosae (Fig 3). The muscularis mucosae is a thin muscular wall. The intestinal epithelium, comparable to mammals, consists of columnar enterocytes (absorptive cells), goblet cells (mucus secreting cells), Paneth cells, M cells and intra-epithelial lymphocytes (IEL) dispersed along the intestinal epithelium. IEL represent a diverse population of lymphocytes comprising mainly T cells, B cells and to a lesser extent, non-T and non-B cells. The LP occupies the cores of villi, envelops crypts, and includes thin blood vessels and smooth muscle that may extend into villi and host numerous immune system cell types including plasma cells, effector and memory lymphocytes, macrophages and granulocytes. LP leukocytes are relatively enriched with immunoglobulin-producing B cells (Arnaud-Battandier, Clinton Lawrence, and Blaese 1980; Göbel et al. 1994).

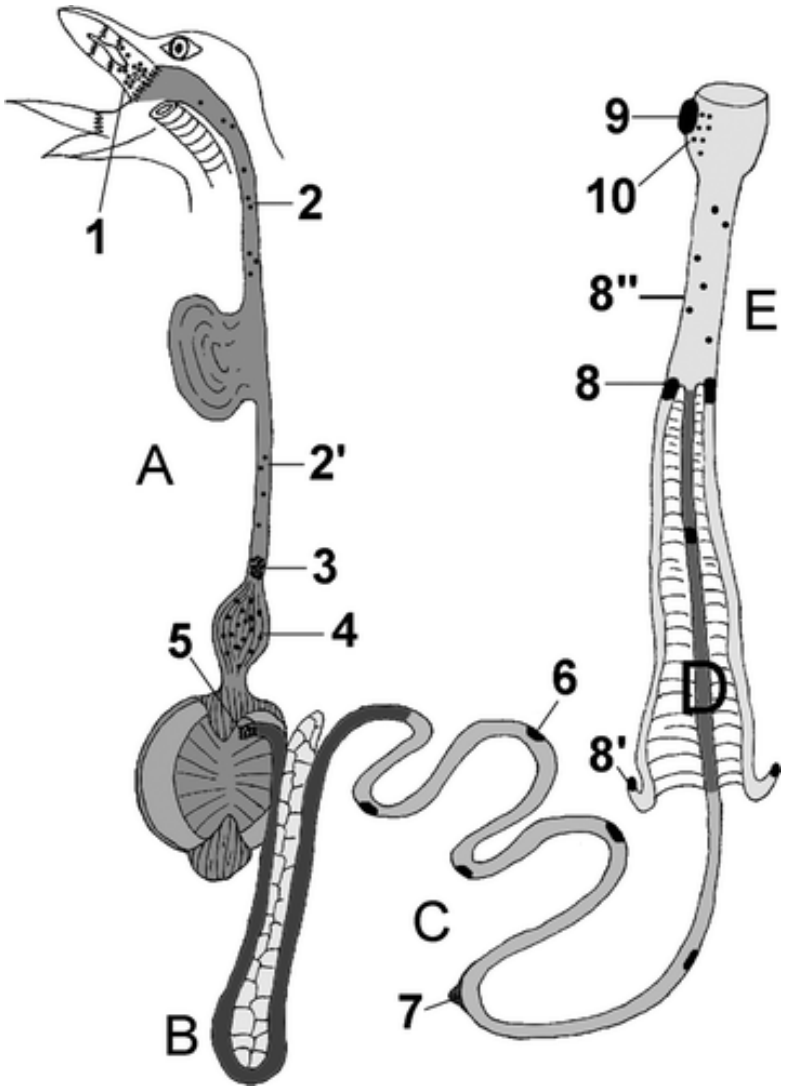

Figure 2. Chicken intestinal tract GALT representation

*Schematic drawing of the chicken intestinal tract indicating the locations of GALT: 1, pharyngeal tonsil; 2 and 2', lymphoid tissue in the cervical and thoracic parts of the oesophagus, respectively; 3, oesophageal tonsil; 4, lymphoid tissue in the proventriculus; 5, pyloric tonsil; 6, Peyer's patch; 7, vitelline diverticulum (Meckel); 8, caecal tonsils; 8', lymphoid tissue in the apical wall of the caecum; 8'', lymphoid tissue in the rectum; 9, cloacal bursa (Fabricius); 10, lymphoid tissue in the proctodeum. Segment **A**, oesophagus with ingluvies, proventriculus and ventriculus; segment **B**, duodenal loop with pancreas; segment **C**, jejunum; segment **D**, ileum; segment **E**, caeca, rectum and cloaca. Figure reproduced from Casteleyn* et al. *(2010) with publisher's permission.*

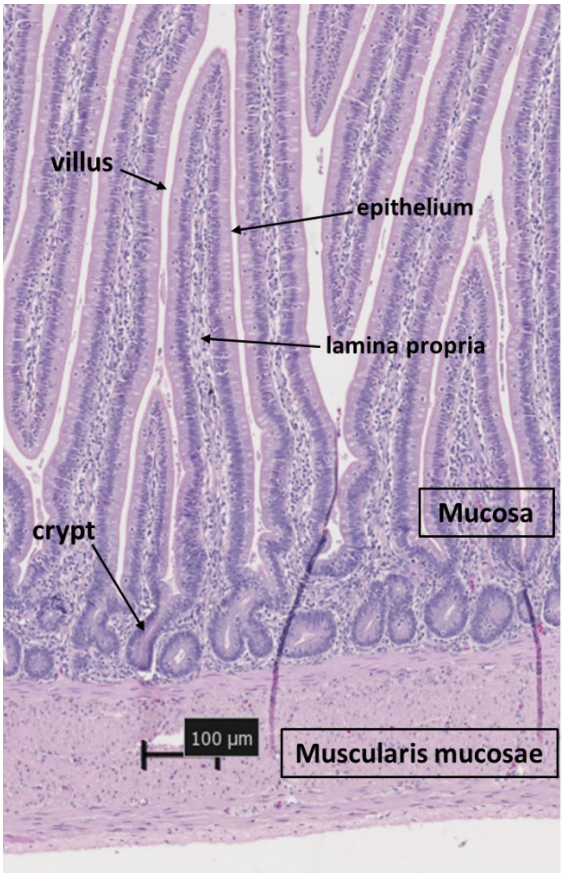

Figure 3. Intestinal mucosa architecture

Histological section taken from chicken ileum, showing the intestinal mucosa stained with modified haematoxylin and eosin (H&E).

In mammals, T lymphocytes are composed of two functionally distinct subpopulations distinguishable by their surface phenotypes. Cytotoxic T lymphocytes ($CD8^+$) recognize foreign antigens in the context of MHC class I molecules, whereas helper T cells ($CD4^+$) recognize antigens in association with MHC class II molecules. Initially, chicken T cells were divided into three receptor subgroups: T cells associated with CD4 receptor, T cells associated with CD8 co-receptor and T cells associated with CD3 co-receptor linked with γδ T cells, recognized by T cell receptor 1 (TCR1) monoclonal antibody (mAb), and with αβ T cells, identified by TCR2 and TCR3 mAb (reviewed by Lillehoj and Trout, 1996).

In chickens, identification of mucosal immune system cells, their functions and their effectors constitute work in progress. Knowledge on cytotoxic $CD8^+$ T cells has expanded greatly owing to intensive research on viral infections such as infectious bronchitis virus (IBV) and Marek's disease to establish new vaccines (reviewed by Erf, 2004). However, cell characterisation and functions of other IEL such as $CD4^+$ T helper cells (Th) requires more research.

Lillehoj *et al*. (1992) showed both $TCR1^+$ and $TCR2^+$ cells co-expressed the CD8 antigen and were present in the intraepithelium and lamina propria in proportions that were age-dependent, reaching adult levels at around 4 to 5 weeks post-hatch (Lillehoj and Chung 1992). There has been increased interest in the selective homing of TCR $\gamma\delta^+$ cells to the intestinal epithelium in mice and in chickens (Janeway et al. 1988; Bucy et al. 1988). The major population of LP γδ T cells being $CD8^+$, it is also suggested γδ T cells predominate in the LP with only few αβ T cells. (Lillehoj and Chung 1992; Liebler-Tenorio and Pabst 2006). Upon environmental stimulation, a variety of immune system cell types produce effectors such as cytokines to activate and regulate the immune response. The type of cytokines produced, and the corresponding effects vary depending on the cell type, surface receptor and function thus leading to the activation or downregulation of the cellular activity. Chicken cytokines have been classified according to their properties and cell function similarities to mammals, however, overlap between these categories occur (reviewed by Wigley and Kaiser, 2003). Cytokines IL-1β, IL-6 and IL-8 have been classed as "pro-inflammatory" (or macrophage activation related); cytokines IFN-γ, IL-2 and IL-18 are associated with T helper cells Th1 that mostly lead to activation of macrophages and development of a cell-mediated immune response; Th2 cells that are important for clearing extracellular organisms produce IL-4, IL-5 and IL- 10 and Th3 cells mainly produce transforming growth factor-β (TGF-β) in response to antigen (reviewed by Wigley and Kaiser, 2003). Since this initial classification, the development of genomic

molecular methods such as qPCR have enabled new findings. However current availability of reliable commercial antibodies to avian cytokines is still 'embryonic'. A vast majority of recent new findings have been unveiled through studies evaluating pathogen challenges and /or dietary interventions. This is the case for newly described IL-17.

1.3.2 Th17 immune response

Initially described in mammals, IL-17-producing $CD4^+$ T lymphocytes (Th17 cells) were described more than a decade ago as potent inducer of autoimmune tissue injury (Harrington et al. 2005; Park et al. 2005). Cytokines produced by Th17 cells including IL-17A, IL-17F and IL-22, prompt neutrophil recruitment and production of antimicrobial peptides by intestinal epithelial cells (IECs). Th17 cells have been described as important host protection mediators against pathogen infection and while involved in several immune disorders, they are crucial for maturation of immune response at early age (Ivanov et al. 2009; Atarashi et al. 2015). In contrast, $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells that produce IL-10 counterpoise Th17 functions, which suggests under healthy conditions Th17 cells and Tregs should co-exist in a well-regulated balance (Bettelli et al. 2008).

Recently, using molecular tools Reid *et al.* showed *C. jejuni* challenge of chickens induced a Th17 response and regulation of IL-10 as implied by up-regulation of IL-17A and IL-6 (Reid et al. 2016). Next, Walliser et *al.* generated mAb able to detect specific chicken IL-17A and IL-17F (Walliser and Göbel 2017). The authors demonstrated IL-17A could be produced by a wide range of immune cells described as splenic $TCR1^+$ γδ T cells, $CD3^+$ T cells, $CD4^+$ γδ T cells and $CD8^+$ (although at lower frequency), implying the presence of different sources of chicken IL-17A with various targets of the cytokine (Walliser and Göbel 2018). These findings suggest chicken IEL may be more pluripotent than initially thought.

Th17 immune response in mammals

It is now well established that mutualistic commensals with immunomodulatory effects (autobionts) affect the development and function of various immune cell populations such as regulatory T-cells (Treg), Th17 cells, IgA-secreting plasma cells, natural killer cells (NK), macrophages, dendritic cells (DCs), innate lymphoid cells (ILCs) (Honda and Littman 2016; Ivanov et al. 2009). To date, little is known about the mechanisms by which autobionts exert their immunomodulatory effects, partly due to the difficulty in culturing these organisms *ex-vivo* and the relative lack of genetic tools to study their genome function. For example, segmented filamentous bacteria (SFB), described as immunomodulatory commensals, have been investigated and the potential cellular and molecular mechanisms involved in their interaction with the host that leads to a healthy steady immune state has been hypothesized. SFB-monocolonised mice have been generated, and using these studies have shown that SFB have major immunomodulatory effects. In addition to a Th17 cell induction effect, SFB colonisation induces production of secretory IgA, serum amyloid A protein (SAA), regenerating islet-derived protein 3 (Reg3) and nitric oxide synthase 2 (Nos2), and activates IELs (Ivanov et al. 2009; Atarashi et al. 2015; Schnupf et al. 2015).

It has been shown the development of $CD4^+$ cell subsets was differentially regulated by certain members of the intestinal microbiota, including *Bacteroides fragilis*, Clostridia species and SFB (Ivanov et al. 2008; Round and Mazmanian 2009; Atarashi et al. 2011). Identification of individual examples of such immunomodulatory commensals and understanding their mechanisms of interaction with the host are key to designing therapeutic strategies to reverse intestinal dysbiosis and recover immunological homeostasis. Conventional probiotics such as *Bifidobacterium* and *Lactobacillus* are the subject of much attention (reviewed by Liévin-Le Moal and Servin, 2014). Several studies have shown *in-vitro* cytokine responses of human peripheral blood mononuclear cells

(PBMC) and DC to lactobacilli can be strikingly different depending on both the species and the strains (Foligne et al. 2007; Christensen, Frokiaer, and Pestka 2002). This suggests that multiple factors can influence the immune phenotype. For example, a comparison of three patented probiotic LAB, *Lactobacillus plantarum* NCIMB8826, *L. rhamnosus* GG (LGG) and *L. paracasei* B21060 *in vitro* and in mice demonstrated different immunological properties. For instance, LGG, and L. *plantarum* exhibited immunostimulatory functions similar to those of pathogenic *Salmonella* Typhimurium aggravating colitis in mice, whereas *L. paracasei* was a poor inducer of cytokines, thus implying *Lactobacillus* strains contrast in their ability to activate DCs and to drive the polarization of T cells.(Mileti et al. 2009). Other strains such as *L. acidophilus* NCFM and *L. johnsonii* NCC 533 have been described as intestinal health promoting microbes, or probiotics (Claesson, Van Sinderen, and O'Toole 2007). Consequently, manipulation of the gut probiotic population through prebiotic foodstuff exhibiting beneficial effects on human and animal health has the potential to support health and improve the sustainability of food production.

2 Chapter 2. Methodological Approaches

This chapter describes supplementary information associated with the methodologies described within the papers presented in Chapters 3, 4 and 5.

2.1 Animal studies

All animal studies complied with Directive 2010/63/EU, implemented in the UK under the 1986 Animals (Scientific Procedures) Act (ASPA), and were performed in accordance with the University of Nottingham codes of practice. To perform chicken procedures, accreditation from the UK Home Office was gained and a Personal Licence to carry out regulated procedures on living animals under ASPA was granted.

2.1.1 Birds

Work was carried out in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Data arising from this programme was published and maintained in accordance with ARRIVE and sponsor guidelines. These measures were adopted to ensure that the data could be fully evaluated, utilised with key information available on how the study was designed, conducted and analysed to prevent duplication. All metadata are available with 16S rRNA community data or are associated with DNA sequencing data in public databases.

The 3Rs principles were applied to these studies.

Replacement: There is currently no other way than to use chickens to assess the impact of dietary prebiotic intervention on an endemic intestinal zoonotic pathogen, and to be able to relate gut microbial communities to complex gut architecture and innate immune responses.

Reduction: Optimum group sizes for each experiment were calculated using power calculations, and where possible small groups were used to validate protocols and provide data as a basis to plan larger experiments. For challenge experiments a well characterised *Campylobacter* strain was selected with a reproducible ability to colonise the guts of chickens that enabled meaningful differences to be determined.

Refinement: Broiler chicken meat is the most popular source of meat and therefore must be the animal of choice to assess dietary prebiotic impacts with respect to the intestinal carriage of the bacterial foodborne pathogens. When possible, chickens were group housed to minimise stress of social animals and provided environmental enrichments to promote natural behaviour (for example, strings, reflective surfaces, and deep litter wood shavings in which to scratch and bath). Birds were monitored at least twice a day for any signs of ill health or unusual behaviour.

2.1.2 Experimental design

In respect with 3Rs principles (Festing and Altman 2013), relevant sample sizes were calculated for all animal trials. Power analysis calculation considered a significance level of 5% (p= 0.05) of one-sided *t*-test at 80% power. The calculation formula can be found online at: http://datashare.is.ed.ac.uk/handle/10283/1996 (Brown 2016).

Colonisation studies with indigenous bacteria are difficult but measurements of *Lactobacillus crispatus* from the intestinal contents of broiler chickens indicated a difference of 0.7 $\log_{10}$ count per g of cecal content as a significant difference between gavaged (doses 6-8 $\log_{10}$ CFU)

and carrier treated control birds based on qPCR of the cecal microbiota with species specific primers against a standard curve produced from genomic DNAs extracted from dilutions of axenic cultures. Using the spreadsheet for sample size calculation previously described (Brown 2016) these data indicate a minimum group size of 9 was required for a power of 80%.

Broiler chickens were challenged with *C. jejuni* HPC5 at either an early stage of development (6 days old) or late stage (20 days old). The mean caecal counts for *C. jejuni* HPC5 in broiler chickens at 35 days of age has previously been determined as 7.2 $\log_{10}$ CFU/g with a standard deviation of ±0.3 ($\log_{10}$ CFU/g of intestinal contents). Based on a measurable difference ≥1 $\log_{10}$ CFU/g the sample size for *C. jejuni* HPC5 colonization experiments can be calculated at 7 per independent experimental group to give 80% power.

For cage placement, 6 and 20 days old chickens were wing-tagged with number identification for random assignment to cages. During sampling for *Campylobacter* challenge trials (Connerton *et al.*, 2018; Flaujac Lafontaine *et al.*, 2020), samples from "uninfected" room were collected before "infected" room was sampled to avoid cross-contamination between rooms. Since chickens are coprophagic animals, individual caging of the birds allowed the bird to represent the experimental unit (n= bird). When birds were kept in pens (Richards et al. 2020), the number of pens was determined by the sample size (n) for each treatment group (n= pen). The cohort size in pens matched the number of sampling times with an extra bird for the last time point. Since the animal studies were completed, new EU legislation requiring larger enclosures for individually caged birds were implemented in the UK. Hence, from a welfare standpoint, considering that birds are natural flock animals that require "social" proximity, it is conceivable pen experimental designs will be chosen rather than caging for future work.

2.1.3 Histology

Tissue architecture was assessed from ileal sections (Connerton *et al.*, 2018; Flaujac Lafontaine *et al.*, 2020; Richards *et al.*, 2020). Sections were stained with modified haematoxylin and eosin (H&E) to assess pathological signs, measure villus height and crypt depth (figure 4) and enumerate heterophiles infiltration in villus epithelium (figure 5). Goblet cells were identified (figure 4) and tallied from sections obtained from the same blocks that were stained with periodic acid-Schiff (PAS). A minimum of 10 well-orientated villi were measured per biopsy section for three to four biopsies per treatment group, each biopsy represented a bird ileal section. Following blind assessment of the biopsies architecture and enumeration, data were reconciled with sampling day and treatment group for statistical analysis.

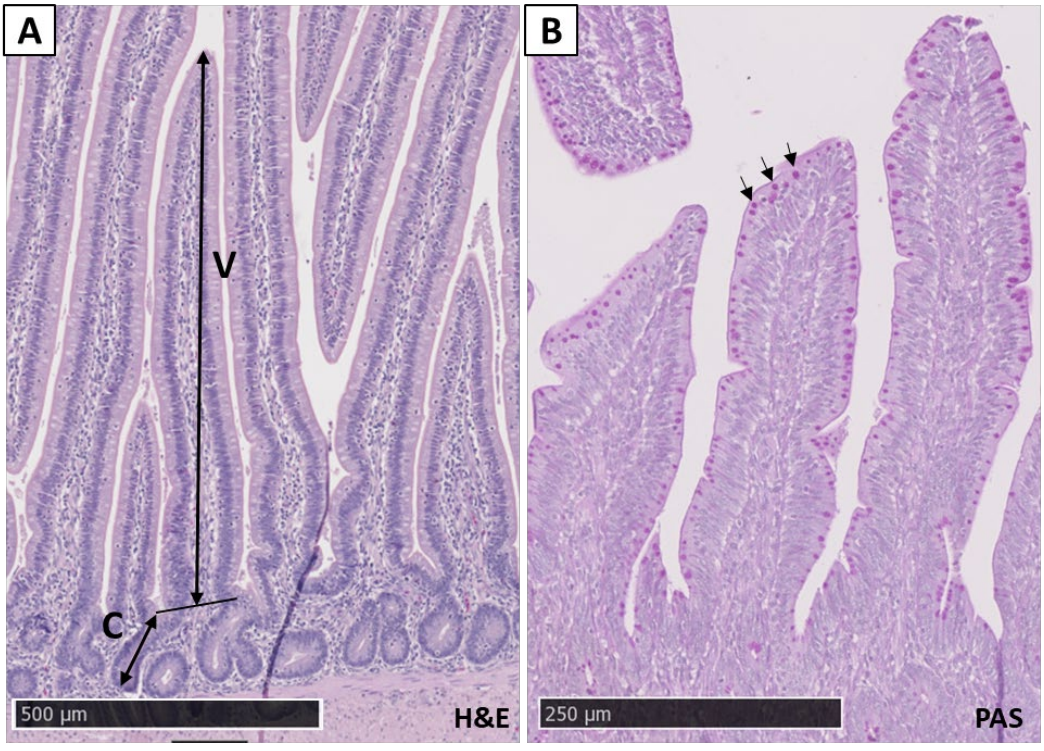

Figure 4. Ileal architecture assessment

Ileum tissue section stained with H & E (A) or PAS (B), microscopic architecture was assessed from sections scanned digitally using NanoZoomer digital pathology image program (Hamamatsu, Welwyn

Garden City, UK). (A), villus length (V), and crypt depth (C) measurements represented by long arrows; (B): goblet cells stained in purple (short arrows).

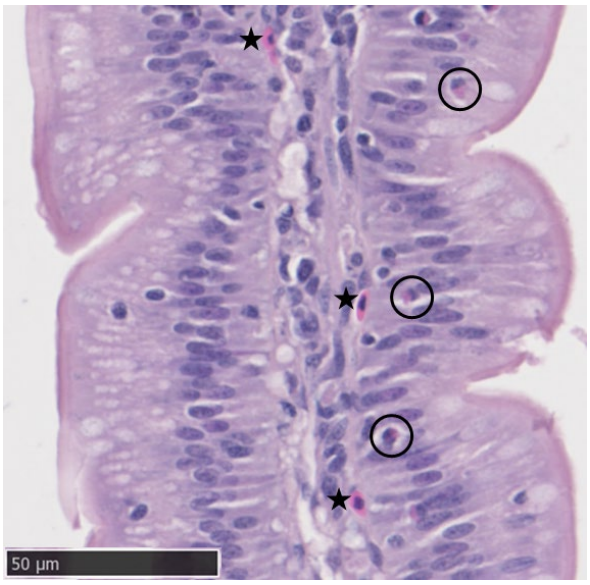

Figure 5. Heterophiles epithelial infiltration

Magnified ileum section stained with H & E showing heterophiles infiltrated in the villus epithelium (black circles) and erythrocytes in the lamina propria (black stars).

2.1.4 Metagenomic analysis

Metagenomics were assessed for alpha--and beta-diversity. Here, alpha-diversity characterised the species richness (OTU count) and species diversity (Shannon index) within each sample. Operational taxonomic unit (OTU) count analysis assessed the number of distinguishable taxa present in the sample, whilst Shannon index measured abundance level to determine how evenly these different species are distributed. Evaluation of the beta-diversity was determined between treatment groups as the difference in taxonomic abundance profiles of microbial communities. The profiles differences in microbial abundances based on abundance or

read count data (Bray–Curtis dissimilarity); in microbial composition based on presence or absence of species (Jaccard distance); or in sequence distances (UniFrac phylogenetic tree). Bray-Curtis dissimilarity and Jaccard distance are conveyed as numbers between 0 and 1, where "0" means both samples are identical and "1" means both samples are different.

2.1.5 Cytokines and chemokines gene expression

Choice of methodology

The method applied for gene expression analysis from chicken ileal and cecal tissue is described in the next two chapters (Connerton *et al.*, 2018; Flaujac Lafontaine *et al.*, 2020; Richards *et al.*, 2020), the method workflow diagram is presented in Figure 6. Chicken cytokines and chemokines expression were evaluated by two-step real-time quantitative PCR (RT-qPCR) of RNA transcripts. Real time qPCR monitors the accumulation of a fluorescent DNA product from a PCR reaction in real time as the reaction progresses, thus allowing to quantify the amount of DNA in the sample at the start of the reaction. In a two-step RT-qPCR reaction, RNA fragments are first transcribed into cDNA molecules to serve as template to the real time qPCR. The development of RT-qPCR methodologies has rendered RNA quantification simpler, faster, and more sensitive than methods such as northern blots. Alternative methods for cytokine and chemokine expression exploration such as ELISA and immunofluorescence assays employ antibodies targeting proteins. Importantly, antibody-based assays target precise protein targets that are species-specific. Commercial availability of antibodies targeting chicken cytokines and chemokines is still limited and costly. In this context, gene expression assays by RT-qPCR on chicken tissue samples can facilitate the exploration of a wide variety of targets for large number of samples at affordable cost.

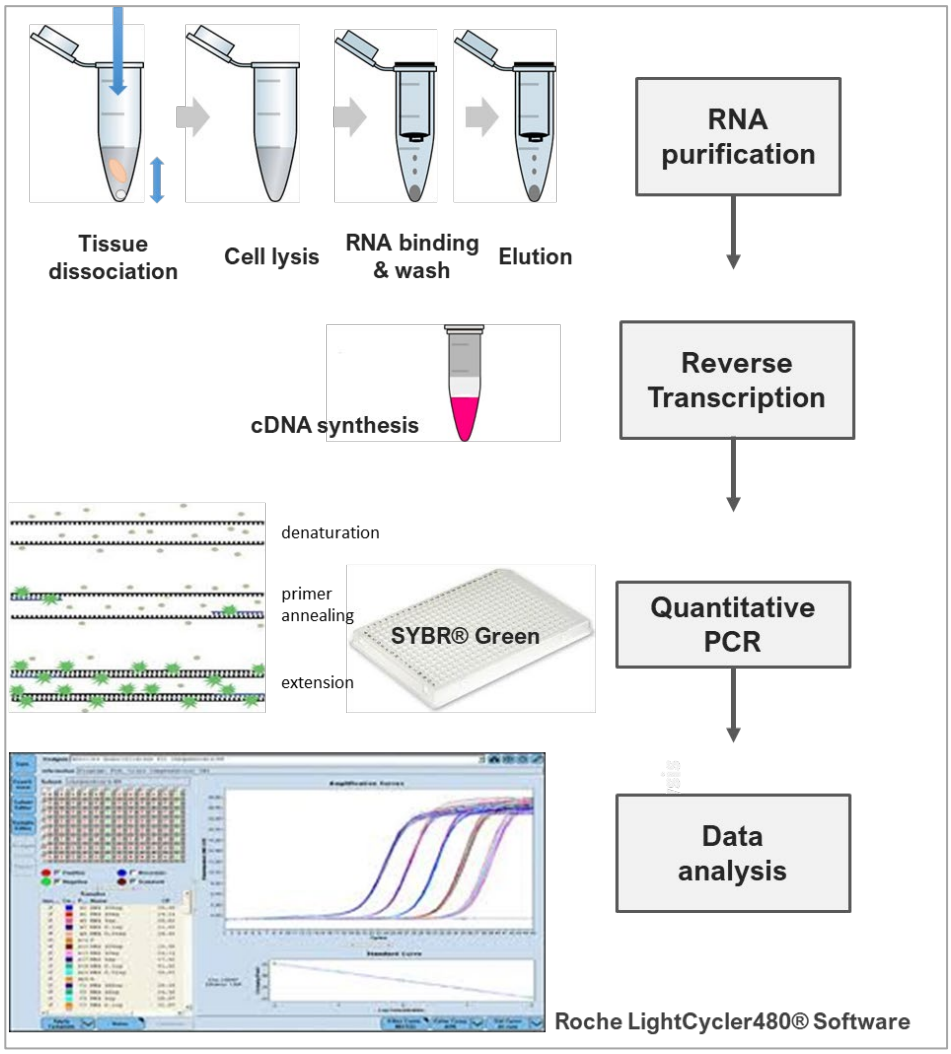

Figure 6. Chicken intestinal tissue gene expression analysis workflow

Statistical analysis

Normal distribution of continuous data was assessed in Genstat software (Genstat 14th edition, VNS international, UK). Sets of data following a normal distribution were computed for analysis of variance (ANOVA), when normal distribution was not validated, data sets were analysed using Kruskal Wallis non-parametric test. A level of $p < 0.05$ was considered significant.

2.2 In vitro Caco-2 monolayers study

This section develops the methodological approach developed for the Caco-2 cell monolayers experiments detailed in Chapter 5.

2.2.1 Experimental design

The experiment design was established on randomised blocks of three biological replicates for each treatment group (n= 3). Three different passage numbers of Caco-2 cells (biological replicates) were independently exposed to each oligosaccharides (OS) GOS or FOS (2% v/v) and their matching mock treatments (mock). Each biological replicate was randomly assigned to one of three Transwell® plate layouts to control for plate effect (Appendix. 1). An internal control maintained in complete DMEM media within each plate monitored normal cell growth during treatment exposure.

2.2.2 Cell growth monitoring

Monolayer confluence expansion was monitored by measuring their trans-epithelial electrical resistance (TEER) every two days, at the time media was replenished, growth chart is presented in Appendix 2. At the time of exposure, cells confluence was established with TEER exceeding 500 Ωcm^2, which was in line with reports from Akbari *et al.* (Akbari *et al.*, 2017).

2.2.3 Gene expression analysis

The method applied for gene expression analysis is summarised in the method workflow (Figure 7).

Choice of methodology

To investigate the entire transcriptome of cell monolayers, RNA-seq technology was chosen to determine the absolute expression level of genes. Microarrays are high throughput alternative methods to detect gene expression, however probes are usually limited to known transcripts thus, bias in level of transcript detection can vary with the probe performance and nonspecific or cross-hybridization. In contrast, RNA-seq is not limited to known transcripts and is a dynamic methodology capable to detect the expression of low abundance transcripts that can be used to resolve the structure of transcripts or find new genes. Protein coding mRNA was purified from total RNA using poly(A) mRNA magnetic enrichment (NEBNext® Poly(A) mRNA Magnetic Isolation Module; E7490; New England Biolabs (UK) Ltd). Briefly, The NEBNext Poly(A)+ RNA magnetic isolation technology is based on oligo d(T) coupled to paramagnetic beads used as a solid support for the direct binding of poly(A) RNA, thus enabling intact mRNA elution in small volumes for library preparation. Indexed strand-specific mRNA libraries were prepared for a 2×75 bases paired-end sequencing run. Stranded RNA-seq is a recommended approach for mRNA-seq studies to evaluate the relative abundance of transcripts , especially where gene overlapping occurs (Zhao et al. 2015). Indexing libraries enabled sequencing of the pooled samples in the same run, thus controlling potential between-flow cell bias.

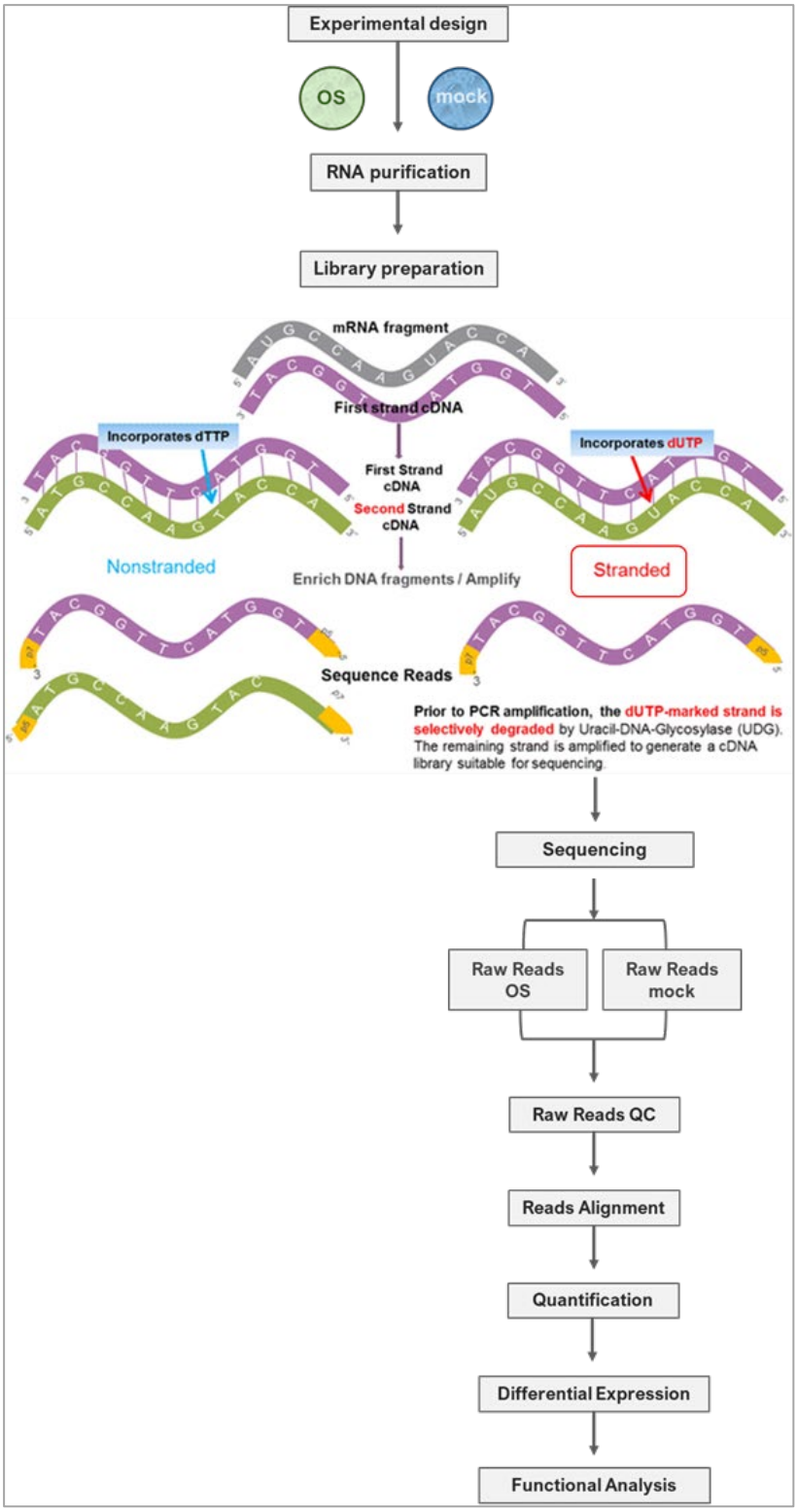

Figure 7. Caco-2 cell monolayers gene expression analysis workflow

Stranded RNA-seq cDNA libraries were prepared with a Illumina Ultra directional mRNA library prep kit (workflow adapted from Zhao et al., 2015).

Validation of expression analysis

Accuracy of relative gene expression for differentially expressed genes computed by RNA-seq was assessed by RT-qPCR. As described in Chapter 5, the methodology steps for transcripts quantification were comparable to those described for chicken gene expression analysis (Figure 6). RT-qPCR cDNA templates were generated from the same total RNA samples as those processed for sequencing.

RT-qPCR primer design

Sets of primers targeting differentially expressed genes were designed using NCBI Primer Blast designing tool, accessible from the following weblink:

http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Sets of primers were selected according to the following criteria:

- Amplicon size: 70 to 200 bp
- G-C content of 50%
- Optimum Tm of 63 °C (min. 60 °C, max. 65 °C)
- Primer must span an exon-exon junction
- Minimum of 7 bases must anneal to the template at 5' side and 4 bases at 3' side of the exon-exon junction.
- Primer must have at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end.
- Ignore targets that have 6 or more mismatches to the primer.
- Max target size: 4000 bp
- Allow primer to amplify mRNA splice variants

Normalization of RT-qPCR was achieved with GAPDH, PUM1 and ACTB reference genes, in line with Liu *et al*. assessment in human breast cancer cell lines (Liu et al. 2015).

RT-qPCR primer validation

Primers sets quality was validated for their specificity when samples melting curve inspection exhibited a single peak with Tm > 78 °C and amplicon size matched the expected PCR product size as established by the primer designing tool. Pooled RT-qPCR products were analysed by electrophoresis using Agilent TapeStation 2200 system, electrophoregrams were computed with TapeStation Analysis software (version: 2.2.24.9522). Primers proven suitable were used for the validation of computed RNA-seq gene expression analysis; exemplary primers set validation are presented in Appendix 3.

STUDY RESULTS

Next Chapters 3, 4 and 5 present study results through four published papers. My contributions to the studies were as follow:

Chapter 3. Effect of GOS on broiler chicken performance and gut health.

My contribution to the study involved assistance with experimental chickens husbandry, handling and cloacal gavage; collection of intestinal tissues and luminal contents; processing of tissue biopsies for RNA purification and performing qPCRs, data analysis and validation; histological assessment of villus and crypts measurements of ileal biopsies with data analysis; co-writing the manuscript.

Chapter 4. Performance and gut health of broiler chicken challenged with *Campylobacter jejuni*.

- The effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens.

My contribution to the study involved the collection of chickens intestinal tissues and luminal contents; processing of tissue biopsies for RNA purification and performing qPCRs, data analysis and validation; histological assessment of heterophils counts and villus-crypts measurements of ileal biopsies with data analysis; co-writing the manuscript.

- The effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens fed a GOS diet

My contribution to the study involved the collection of chickens intestinal tissues and luminal contents; processing of tissue biopsies for RNA purification and performing qPCRs, data analysis and validation; histological assessment of ileal biopsies with data analysis; leading and co-writing the manuscript.

Chapter 5. *In vitro* evaluation of prebiotics GOS and FOS on human colonic Caco-2 cells

My contribution to the study involved the experimental design and investigation, the data analysis, validation and curation, the co-administration of the project, leading and co-writing the manuscript.

I confirm that the information given is true, complete and accurate,

Geraldine M Lafontaine

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26/02/2020

To whom it may concern

Dear Sir/Madam

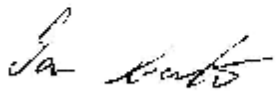
Ref: Geraldine Marina Lafontaine - 14270033

I am the primary PhD supervisor of Geraldine Marina Lafontaine and this letter is to confirm her contribution to the open access manuscripts presented in her thesis submitted 31st March 2020.

Geraldine has contributed to the research team working on immune responses documented in the papers 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 her thesis was developed by her in the course of her research and represents an original research portfolio as defined by the University of Nottingham.

Yours sincerely,



Professor Ian F. Connerton,
Head of Division of Microbiology, Brewing and Biotechnology

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3 Chapter 3. Effect of GOS on broiler chicken performance and gut health

To assess the potential of GOS application in farms as an alternative to AGPs, the first study describes their effects on unchallenged broiler chickens' performance, intestinal immunity and on the intestinal mucosa-resident microbiota interplay. In this paper, findings demonstrated that broiler chickens sustained on a GOS diet exhibited improved performance. Increased market-ready weight and up-regulation of mucosal pro-inflammatory cytokine IL-17A were concomitant with reduced anti-inflammatory IL-10. On GOS diet, the microbiota of the juvenile birds ceca shifted towards increased relative abundance of autochthonous *Lactobacillus* spp. This was one of the first studies to correlate bird performance to *L. johnsonii* abundance, thus to demonstrate GOS in-feed additive-associated cecal microbiota composition shifts were linked with the modulation and possible priming of the gut immune status in juvenile birds.

The following paper was peer-reviewed and accepted for publication by American Society for Microbiology mSystems® journal in January 2020 and is accessible online via the following weblink: https://msystems.asm.org/node/8186.full and through the following portable document format:


mSystems

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

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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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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,

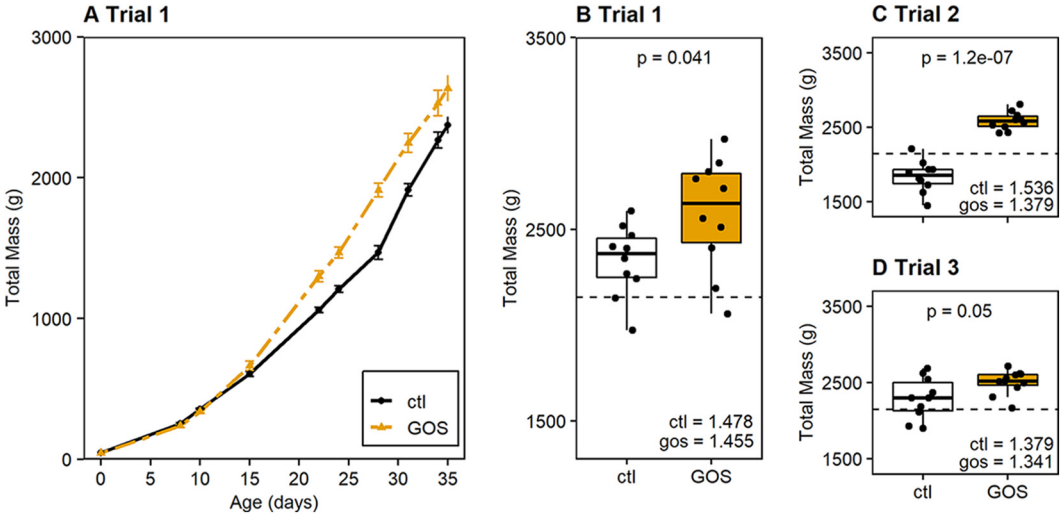

FIG 1 Galacto-oligosaccharide diet improves the growth performance of broiler chickens. (A) GOS diet trial 1 comparing median body weight of birds fed the GOS diet with that of birds fed the control (ctl) diet. Data presented are for the mass observations made for the 10 birds from each cohort that remained 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,

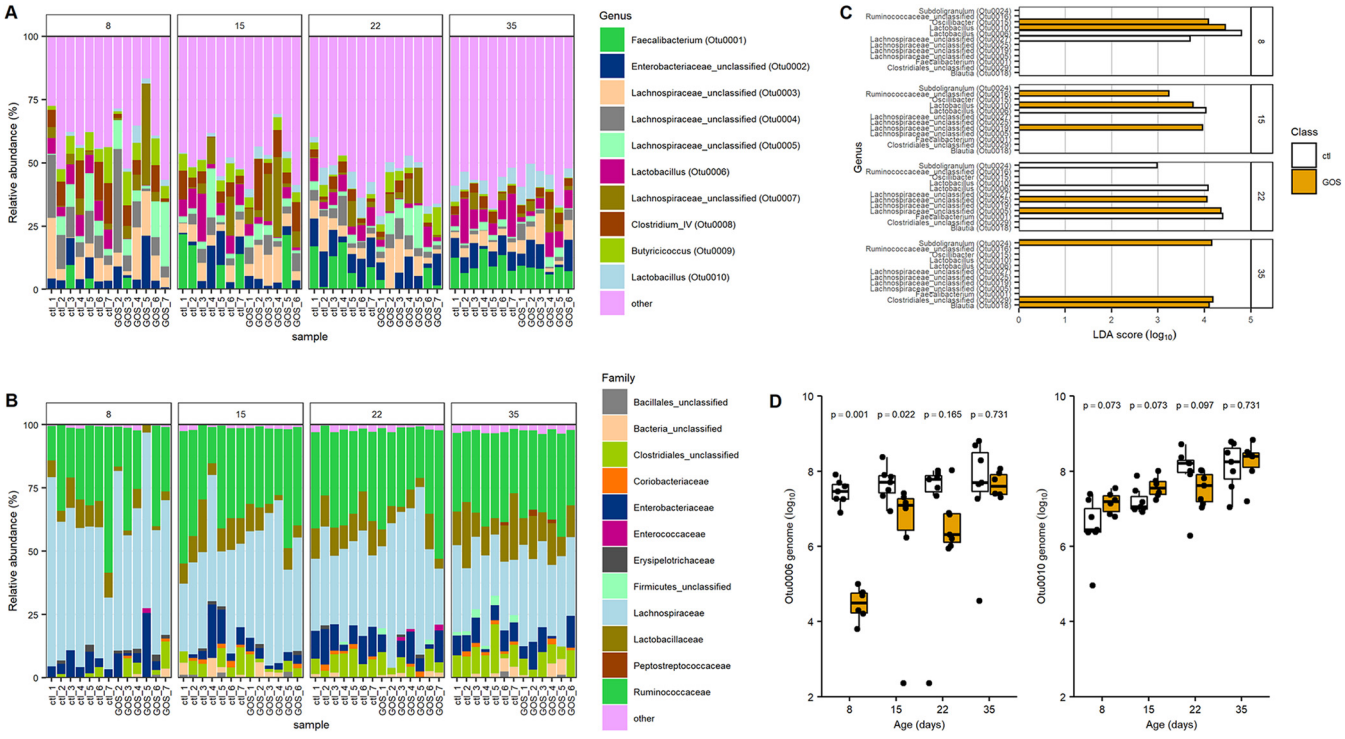

FIG 2 Dietary GOS shifts the abundance of specific taxa. Panels A and B show stacked bar charts indicating the relative abundance to OTU (A) and family (B) levels of the cecal microbiota of birds fed GOS and control diets. In three cases, the sequence data sets did not meet pre- or postprocessing quality 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 ($P = 0.001$).

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

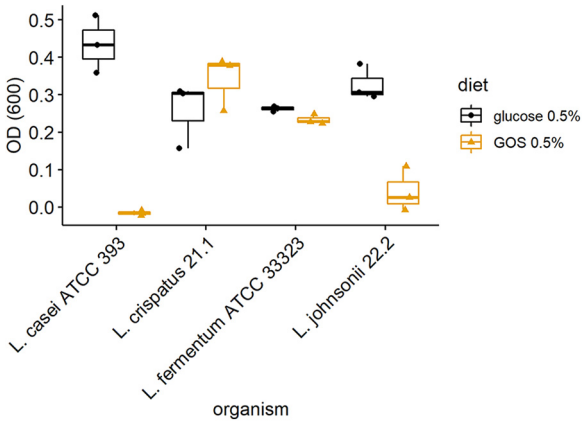

FIG 3 *In vitro* growth of lactobacilli on galacto-oligosaccharides. Utilization of DP2+ GOS by *L. crispatus* DC21.1 and *L. johnsonii* DC22.2 isolates compared with that by *Lactobacillus* type strains is shown. Anaerobic growth was recorded from OD_{600} measurements after incubation with basal medium 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/mm²
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

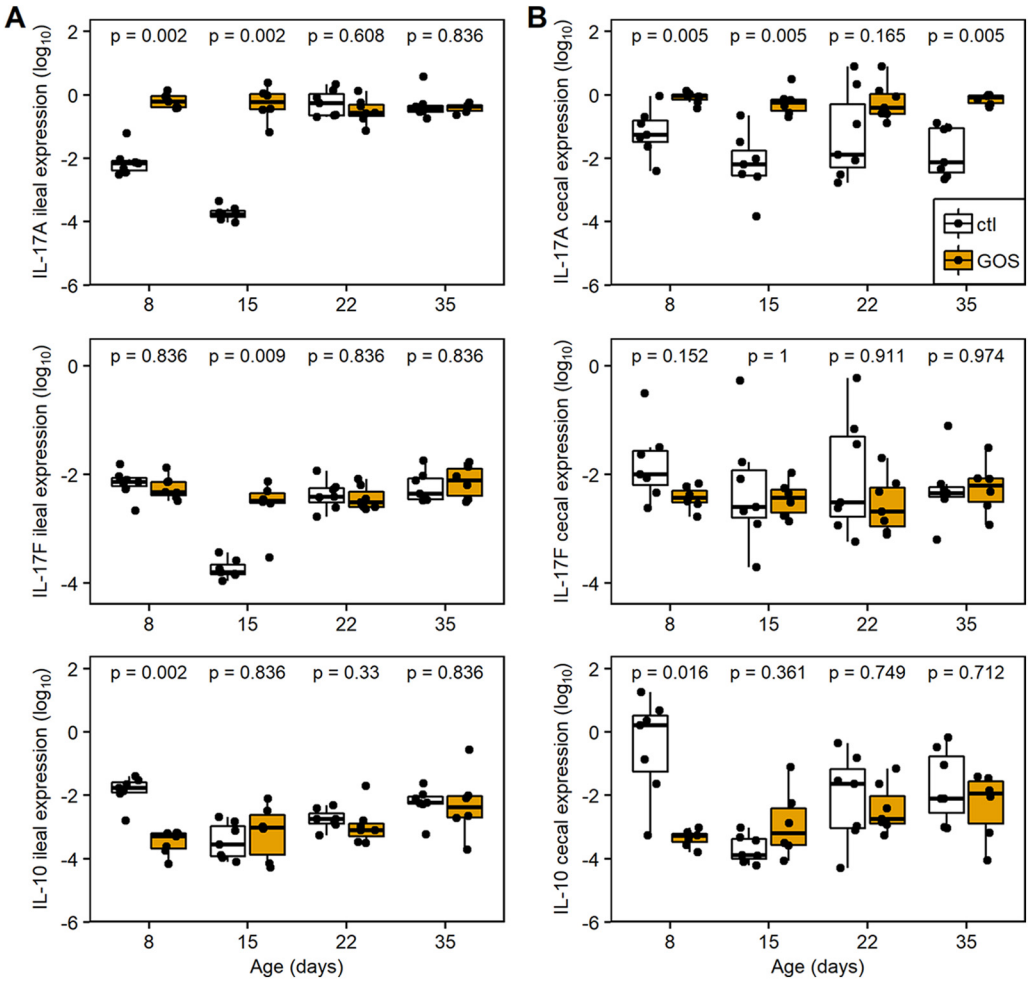

FIG 4 Changes in expression of IL-17A, IL-17F, and IL-10 from ileal (A) and cecal (B) tissues. Relative gene expression was determined by quantitative RT-PCR from total RNAs extracted from cecal tissues for GOS-fed birds (gold) and compared to the expression from birds on the control diet (black). Expression 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

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

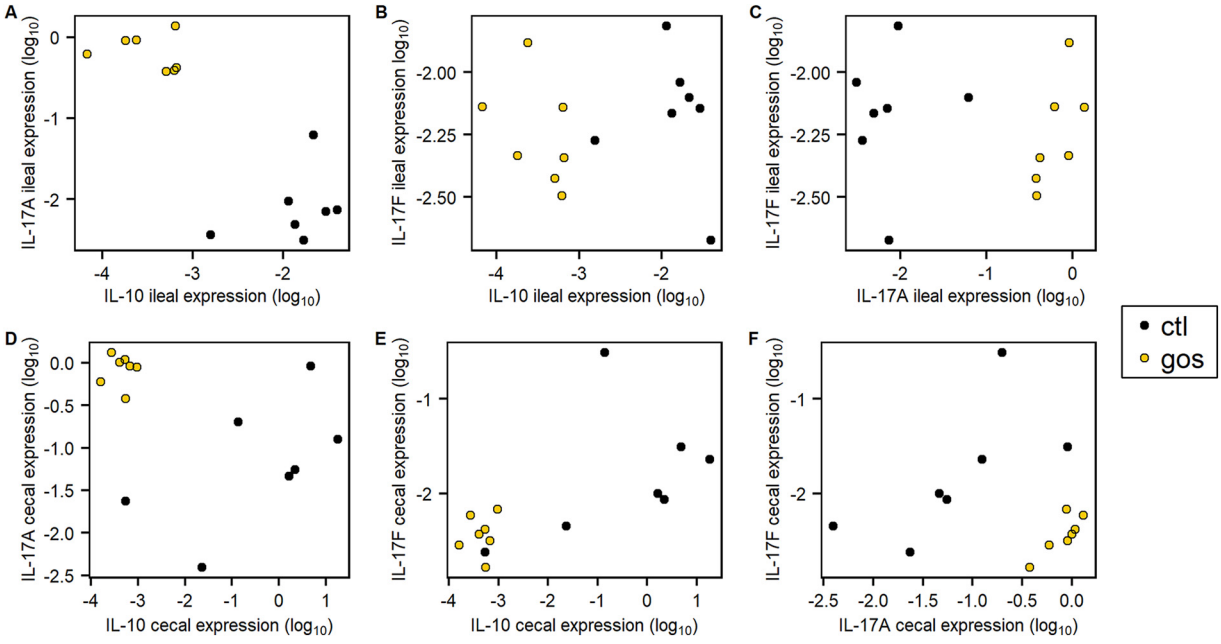

FIG 5 Differential cytokine gene expression in juvenile cecal and ileal tissues. Shown is juvenile IL-17A, IL-17F, and IL-10 differential gene expression relative to those of GAPDH and RPL4 at 8 da in ileal (A, B, and C) and cecal (D, E, and F) tissues of birds on the GOS diet compared to those on the 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 ($n = 7$).

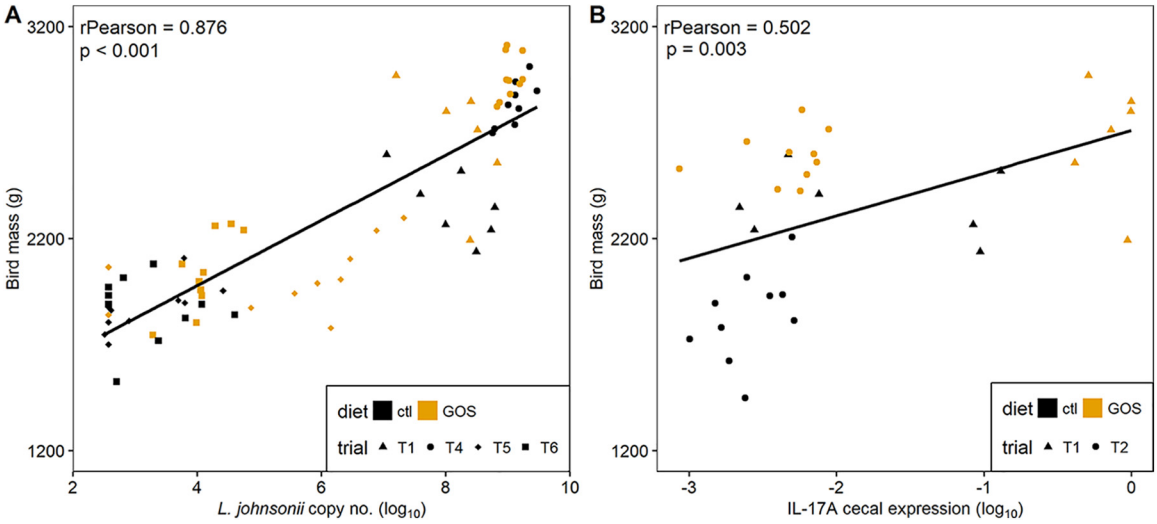

FIG 6 Correlation of growth performance to *L. johnsonii* abundance and IL-17A expression. Panel A shows the Pearson correlation of bird mass at 35 da against $\log_{10}$ *L. johnsonii* gene copy number per gram of cecal contents determined by quantitative PCR from DNA extracted from cecal contents. 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

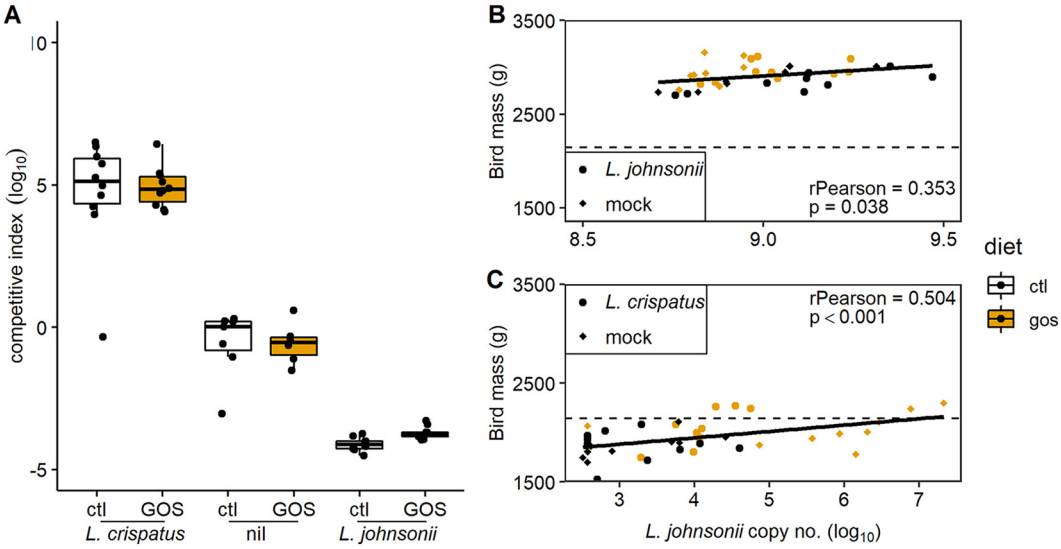

FIG 7 Modulation of the juvenile cecal microbiota. Panel A shows the competitive indices (*L. crispatus*/*L. johnsonii* ratios) at 35 da of broiler chickens either nontreated or administered 8 log_{10} CFU of either *L. crispatus* DC21.1 or *L. johnsonii* DC22.2 by cloacal gavage at 6 da. Competitive 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

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

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

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

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

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.

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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4 Chapter 4. Performance and gut health of broiler chicken challenged with *Campylobacter jejuni*

The first part of this chapter describes the effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens.

In the next section of the chapter, the effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens maintained on a GOS diet are presented.

4.1 The effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens

In this second paper, the importance of birds age at the time of *C. jejuni* challenge on the induction and persistence of the pro-inflammatory immune response in market-ready chickens was demonstrated. The study corroborated previous reports of time-dependent resistance to *Campylobacter* cecal colonisation that was transient for birds challenged at a young age (6 da). This resistance was concomitant with a temporary setback in juvenile weight gain, reduced villus height and crypt depth, increased ileal heterophils infiltration, microbiota shifts and induction of a transitory Th17 immune response, when compared to birds challenged older (20 da). Noticeably, the peak Th17 pro-inflammatory responses relating to the *C. jejuni* challenge coincided with reductions in the abundance of *Clostridium* XIVa for juvenile and older birds, whilst in

young birds the dampening of IL-6 expression paired with reduced relative abundance of *Lactobacillus* spp.

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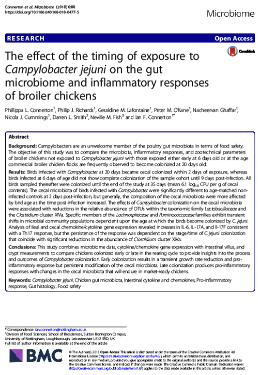
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The effect of the timing of exposure to *Campylobacter jejuni* on the gut microbiome and inflammatory responses of broiler chickens

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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

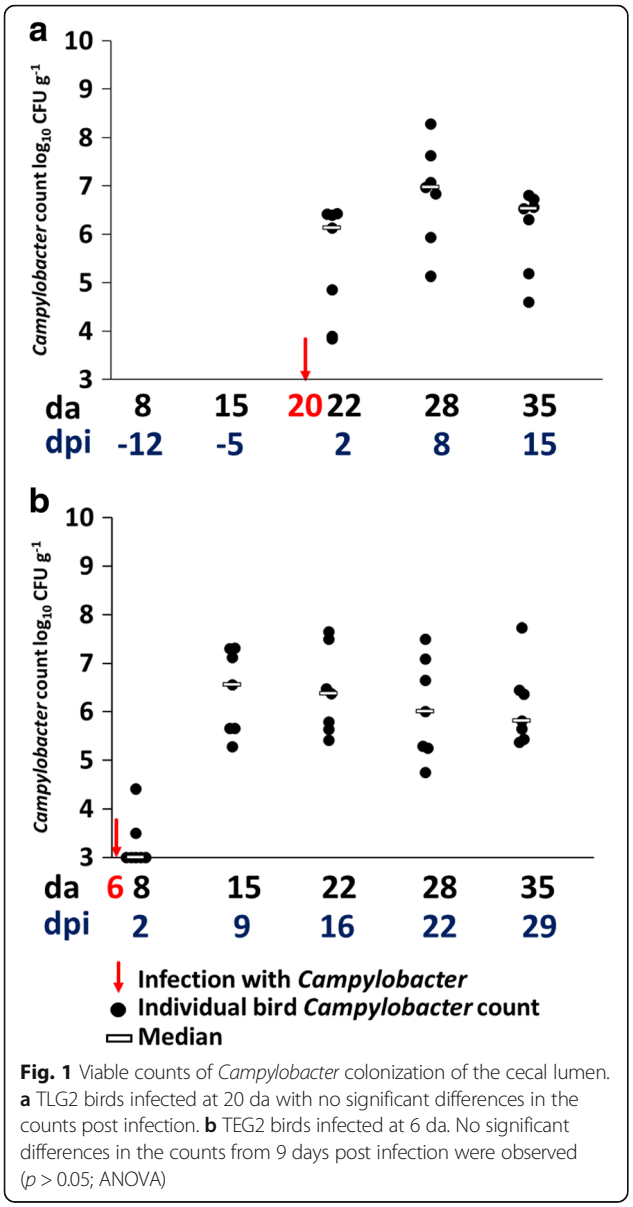


Fig. 1 Viable counts of *Campylobacter* colonization of the cecal lumen. **a** TLG2 birds infected at 20 da with no significant differences in the counts post infection. **b** TEG2 birds infected at 6 da. No significant differences in the counts from 9 days post infection were observed ($p > 0.05$; ANOVA)

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

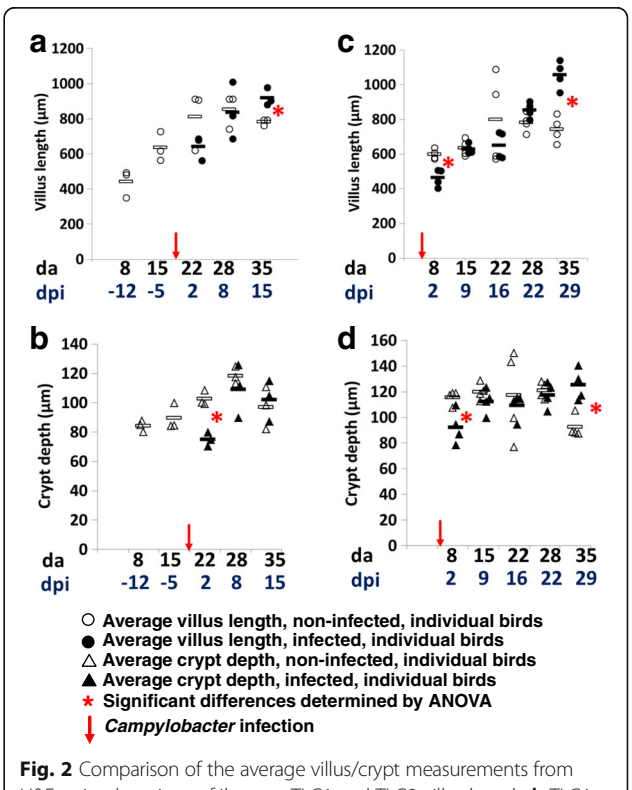


Fig. 2 Comparison of the average villus/crypt measurements from H&E-stained sections of ileum. **a** TLG1 and TLG2 villus length. **b** TLG1 and TLG2 crypt depth. **c** TEG1 and TEG2 villus length. **d** TEG1 and TEG2 crypt depth length. Measurements were taken from 10 villi or 10 crypts per bird. Three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

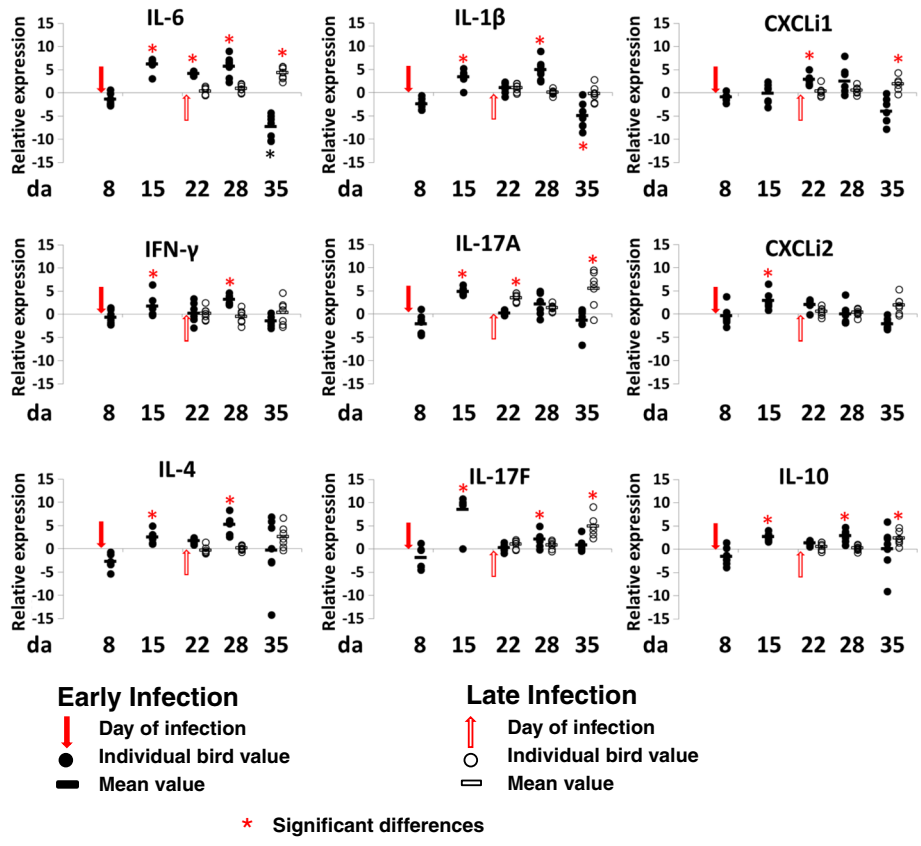


Fig. 3 Relative change in expression of cytokines and chemokines in ileum tissues. Relative gene expression represents $\log_2$ ratio infected/non-infected from qPCR of infected birds (TLG2 and TEG2) compared to expression in tissues from non-infected birds (TLG1 and TEG1). Significant differences between $2^{-\Delta Ct}$ 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

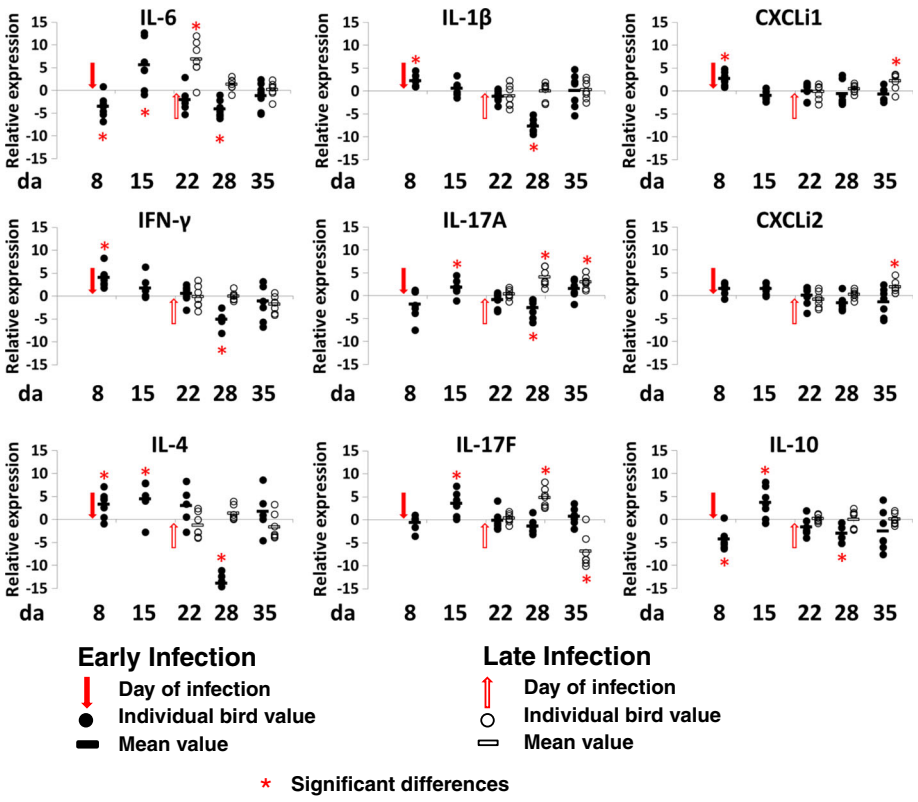


Fig. 4 Relative change in expression of cytokines and chemokines in cecal tissues. Relative gene expression represents $\log_2$ ratio infected/non-infected from qPCR of infected birds (TLG2 and TEG2) compared to expression in tissues from non-infected birds (TLG1 and TEG1). Significant differences between $2^{-\Delta Ct}$ 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

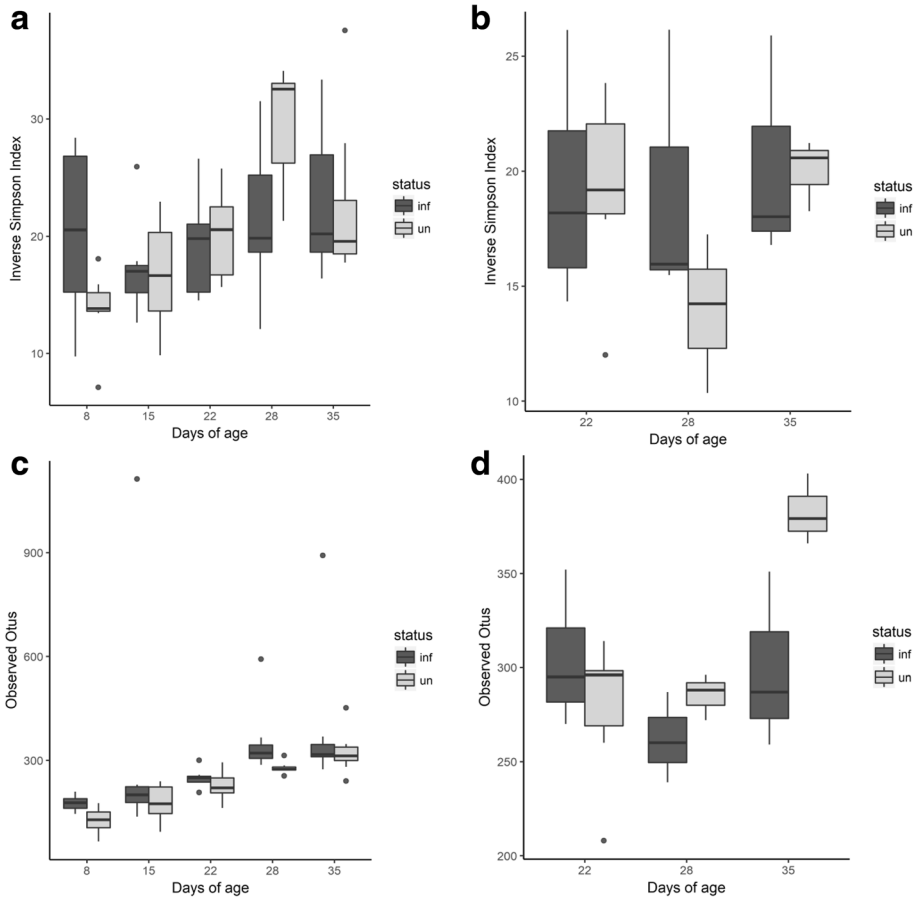


Fig. 5 Estimates of α-diversity and richness for TEG and TLG microbial communities from cecal contents. Inverse Simpsons indices (**a**, **b**) and species richness estimates (**c**, **d**) from 16,319 subsampled sequences for the non-colonized control (gray bars) and *C. jejuni*-colonized (dark gray 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

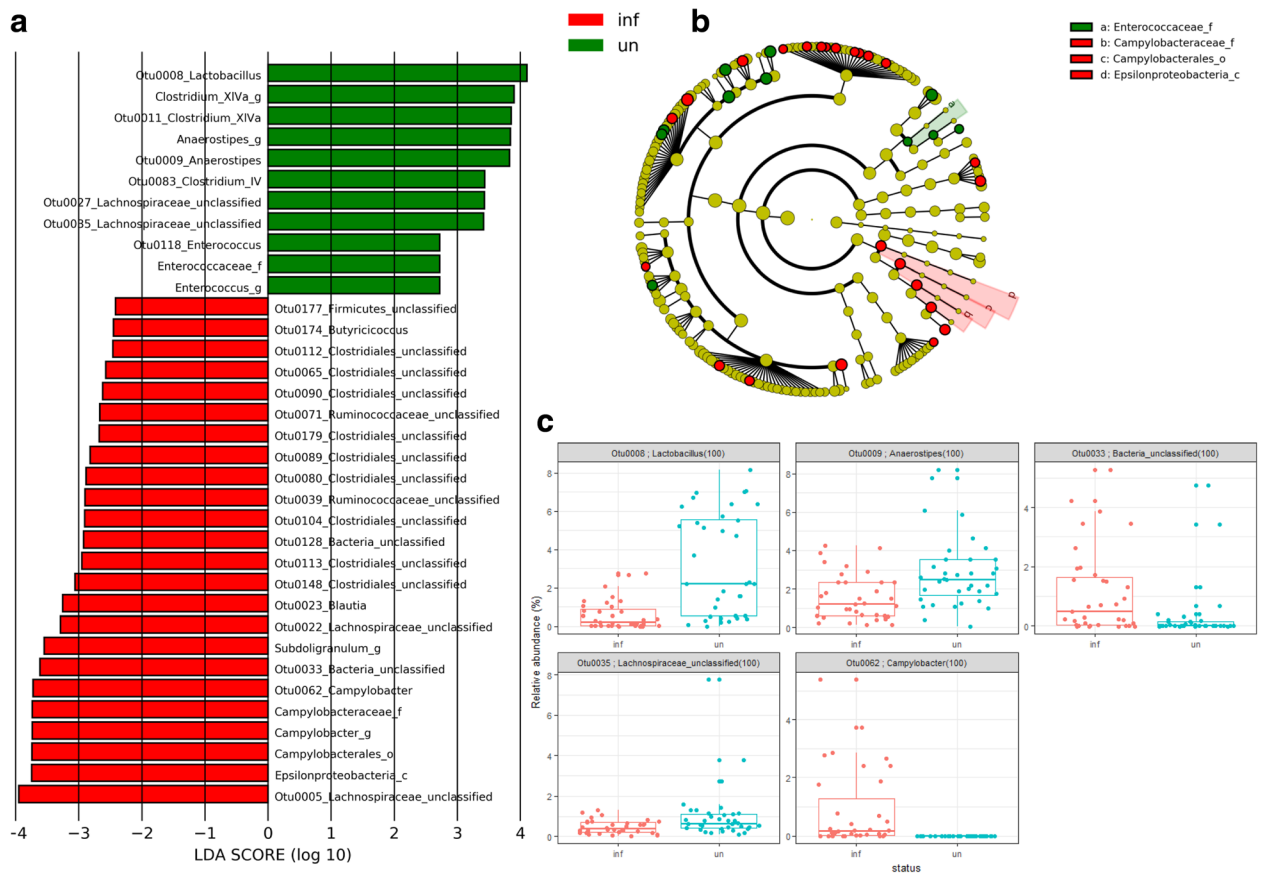


Fig. 6 Differential abundance of members of the cecal microbial communities of TEG *C. jejuni*-colonized and non-colonized broiler chickens. **a** Histogram of the LDA scores computed for features differentially abundant between *C. jejuni*-colonized broiler chickens from 6 da (denoted as "inf" by red 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

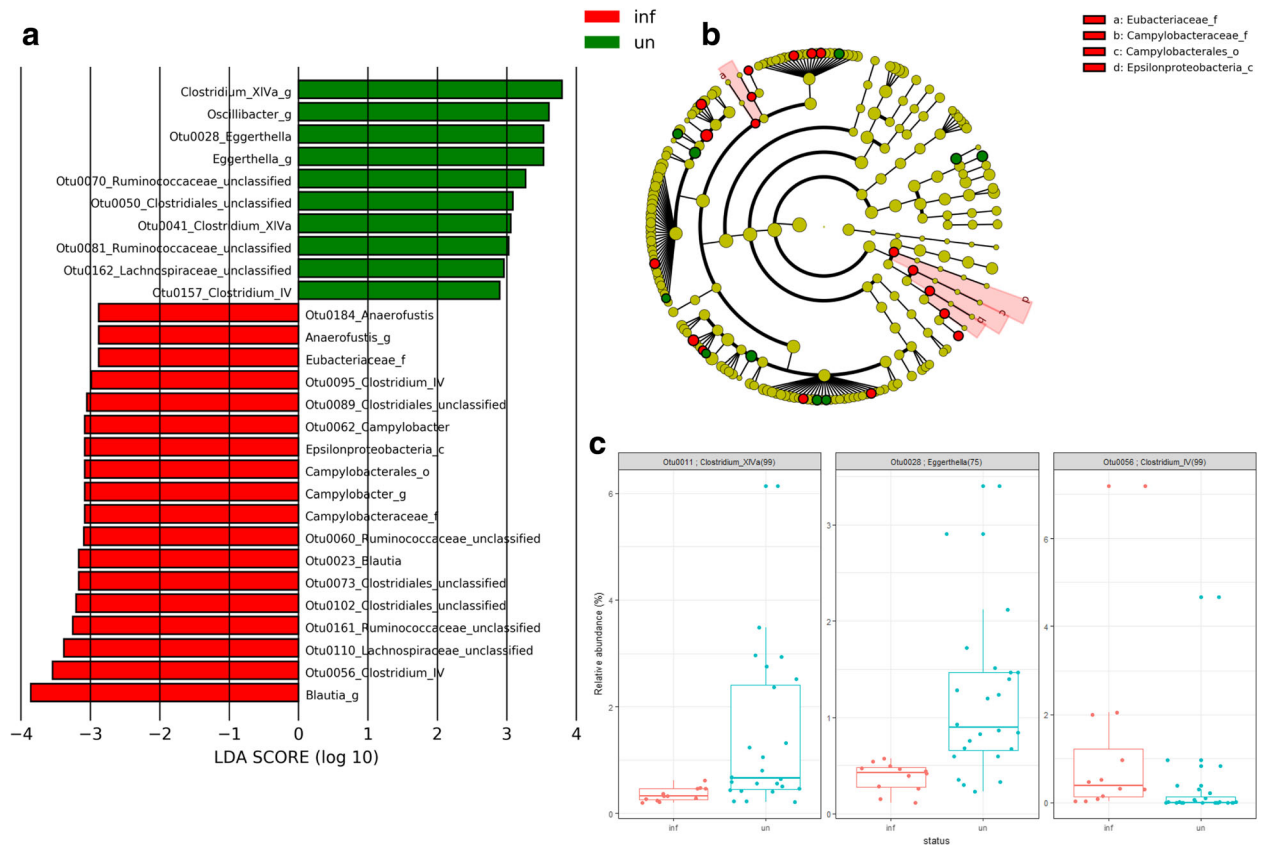


Fig. 7 Differential abundance of members of the cecal microbial communities of TLG *C. jejuni*-colonized and non-colonized broiler chickens. **a** Histogram of the LDA scores computed for features differentially abundant between *C. jejuni*-colonized broiler chickens from 20 da (denoted as "inf" by red 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).

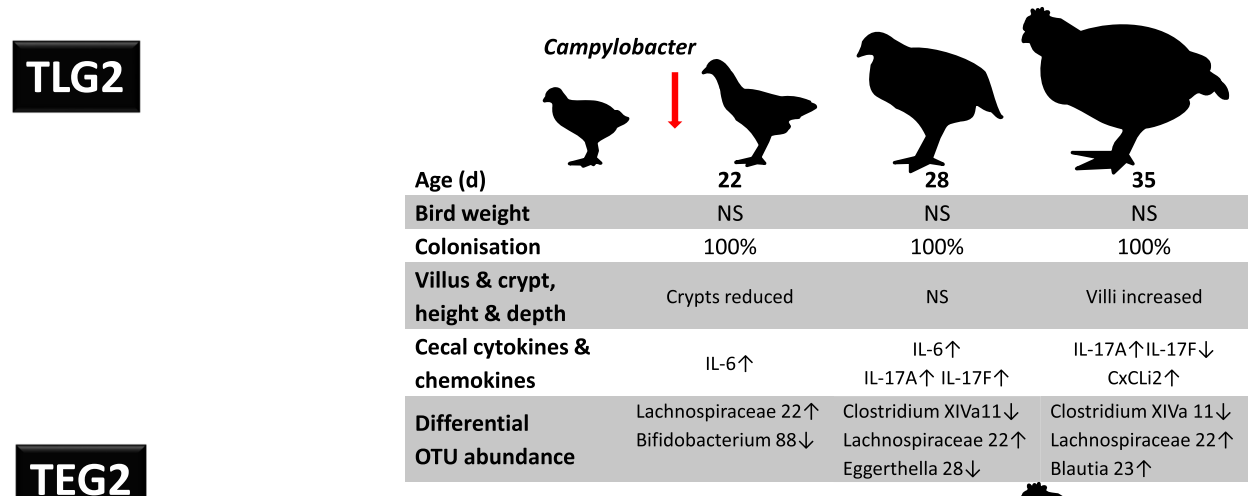
In response to *Campylobacter* colonization, we observed variable shifts in the abundance of members of the *Clostridiales*, which are largely unclassified but feature members of the *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococcaceae* families based on the consensus taxonomies. Increases in the 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



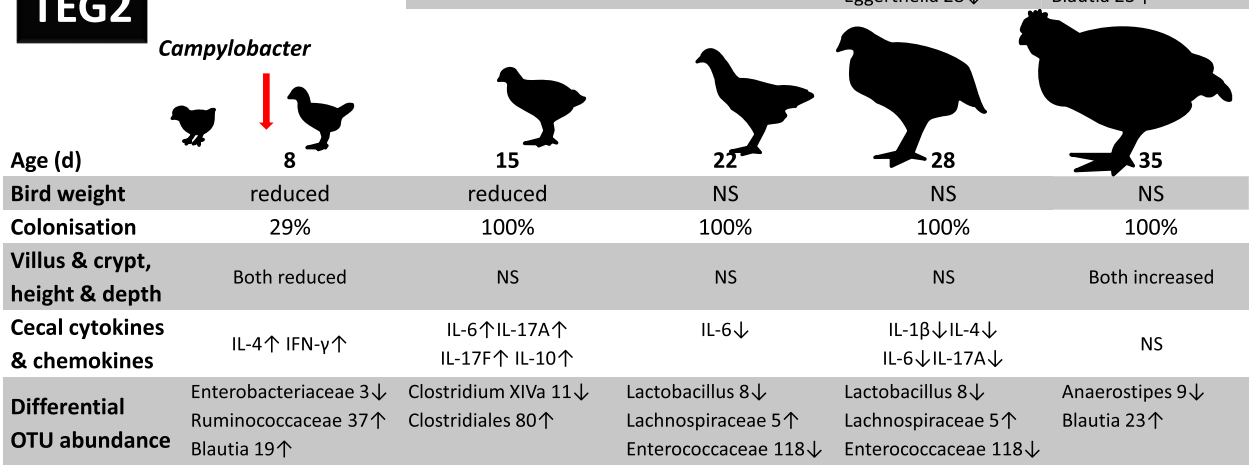


Fig. 8 Summary of age-dependent differences between *C. jejuni*-colonized broiler chickens and non-infected controls. Time dependent reductions in the mean weights of *Campylobacter*-infected chickens are indicated. Relative increases in the cecal cytokine/chemokine expression of *C. jejuni*-colonized 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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4.2 The effect of *C. jejuni* exposure on the gut microbiome and inflammatory responses of broiler chickens fed a GOS diet

The third paper described the effect of *C. jejuni* challenge of birds maintained on a GOS diet in terms of broilers' fitness, innate immune status and microbiota composition shifts. When comparing juvenile birds challenged at 6 da with older birds challenged at 20 da, this study demonstrated that broilers fed GOS exhibited improved growth performance with transient increased villus length and crypt depth in a challenge age-independent manner. Despite the prebiotic feed additive failing to reduce the levels of *C. jejuni* cecal colonisation, GOS-driven IL-17A (but not IL-17F) mucosal immune response priming and microbiota shifts towards increased Lactobacilli, with *L. johnsonii* outcompeting *L. crispatus*, were established for the first time to our knowledge.

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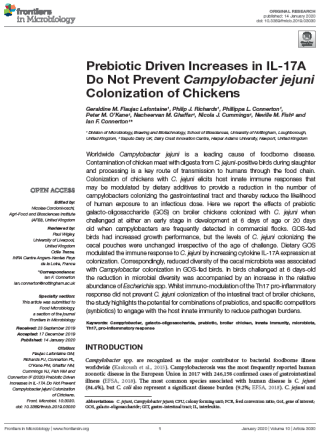

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ORIGINAL RESEARCH

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

Geraldine M. Flaujac Lefenstein[1], Philip J. Richards[1], Phillipca L. Connerton[1], Peter M. O'Kane[1], Nachervan M. Ghaffari[1], Nicola J. Cummings[1], Neville M. Fish[1] and Ian F. Connerton[1]*

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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 colonised 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* colonising 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 (symbiotics) to engage with the host innate immunity to reduce pathogen burdens.

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

INTRODUCTION

Campylobacter spp. are recognized as the major contributor to bacterial foodborne illness worldwide (Kaakoush et al., 2015). Campylobacteriosis 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 (16.3%, EFSA, 2018). *C. jejuni* and

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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

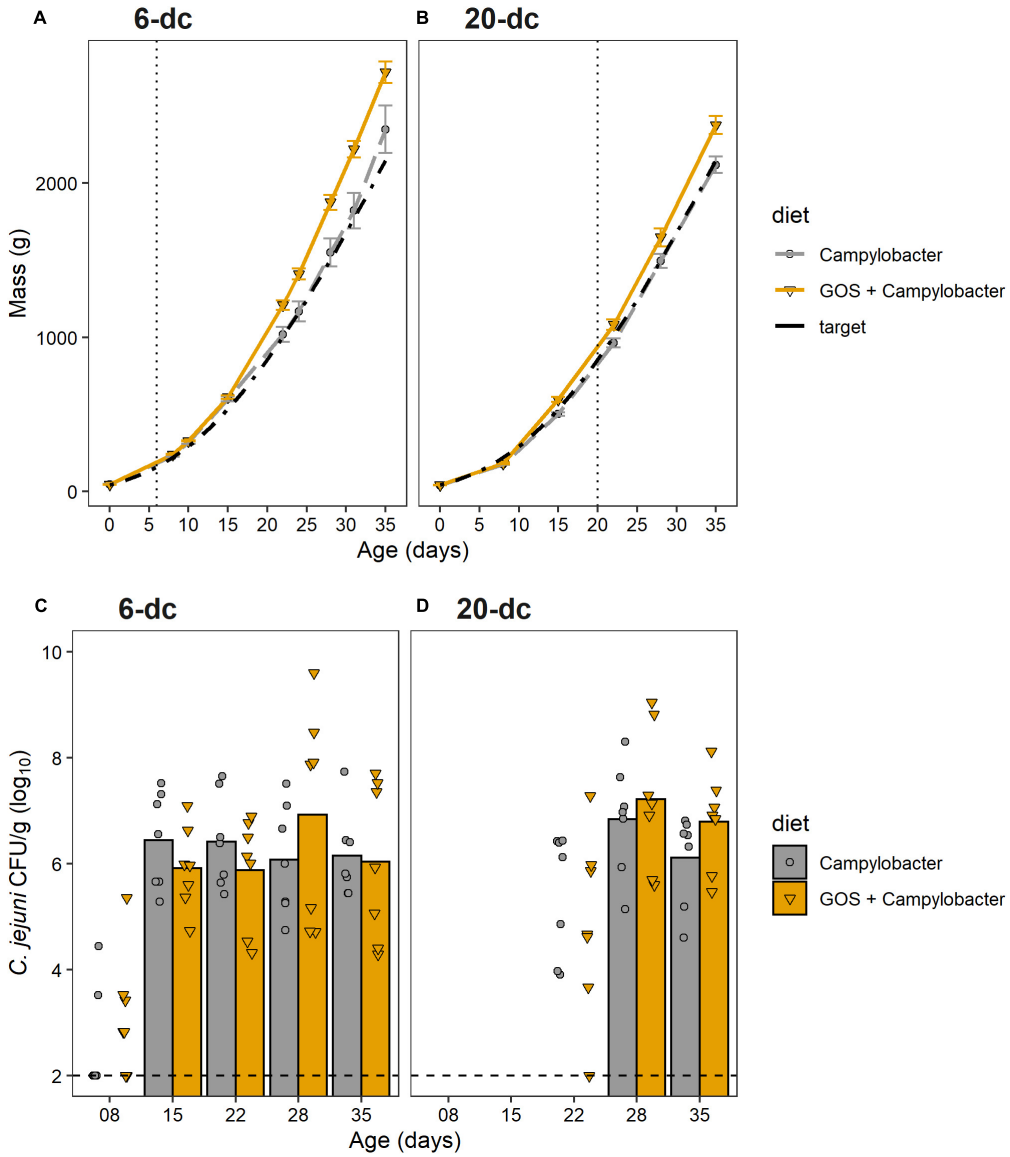

FIGURE 1 | GOS improves growth performance of broiler chickens, but does not affect *C. jejuni* colonization of the ceca. **(A,B)** Chicken live total mass from day of hatch to 35 days for 6-dc birds **(A)** and 20-dc birds **(B)**. The plots show the performances of the experimental treatments GOS + *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).

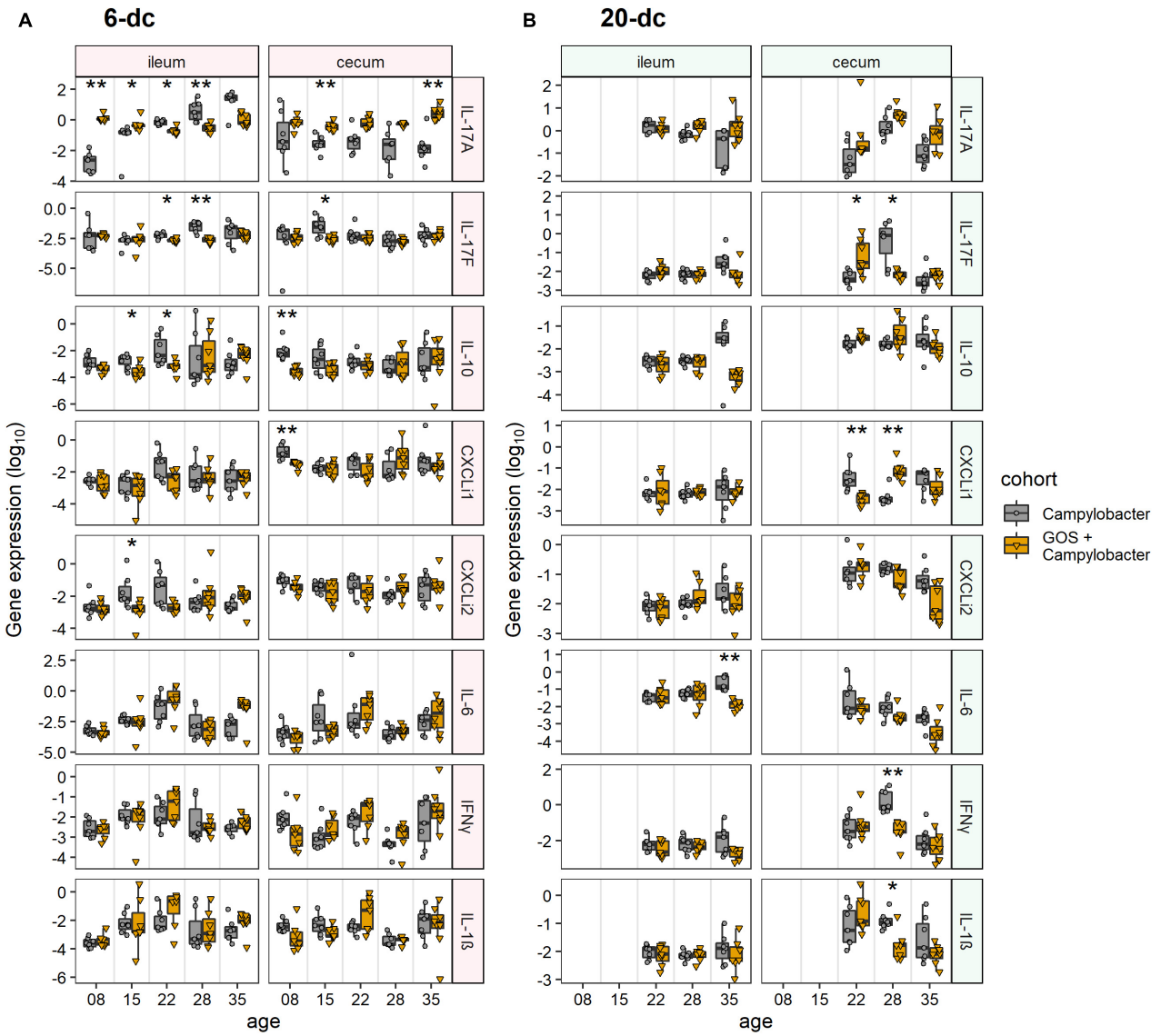

FIGURE 2 | Modulation of cecal and ileal innate immune responses to dietary GOS in *Campylobacter*-challenged broiler chickens. The figures report cytokine and chemokine normalized gene expression for 6-dc birds **(A)** and 20-dc birds **(B)** fed a control diet (*Campylobacter* treatment) or a GOS diet (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

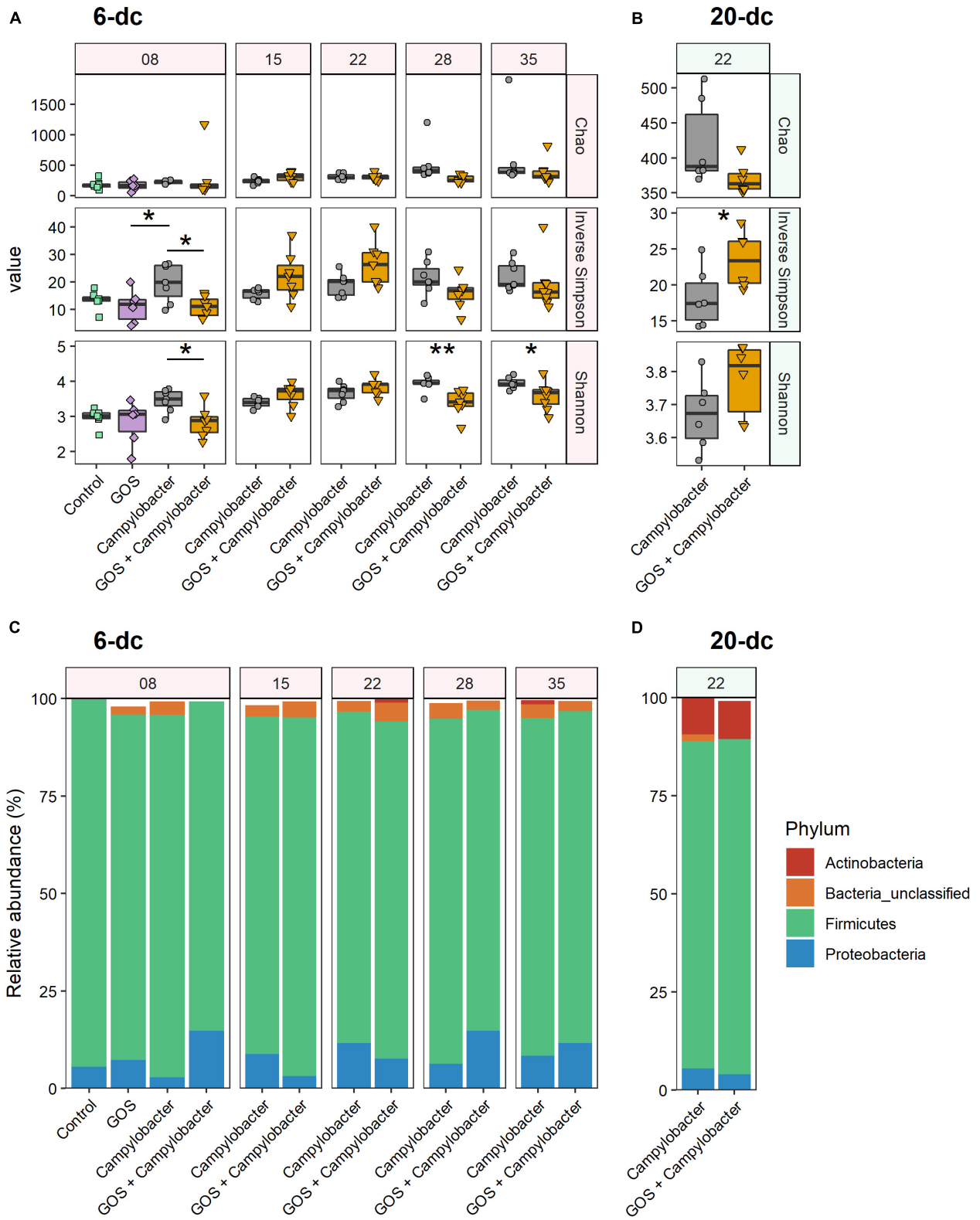

FIGURE 3 | Dietary GOS promotes differential shifts in the cecal microbiota post *Campylobacter* challenge. **(A,B)** Cecal α-diversity for 6-dc birds **(A)** and 20-dc birds **(B)** described as Chao richness, Inverse Simpson diversity and Shannon diversity (as indicated by panels on right hand side 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.

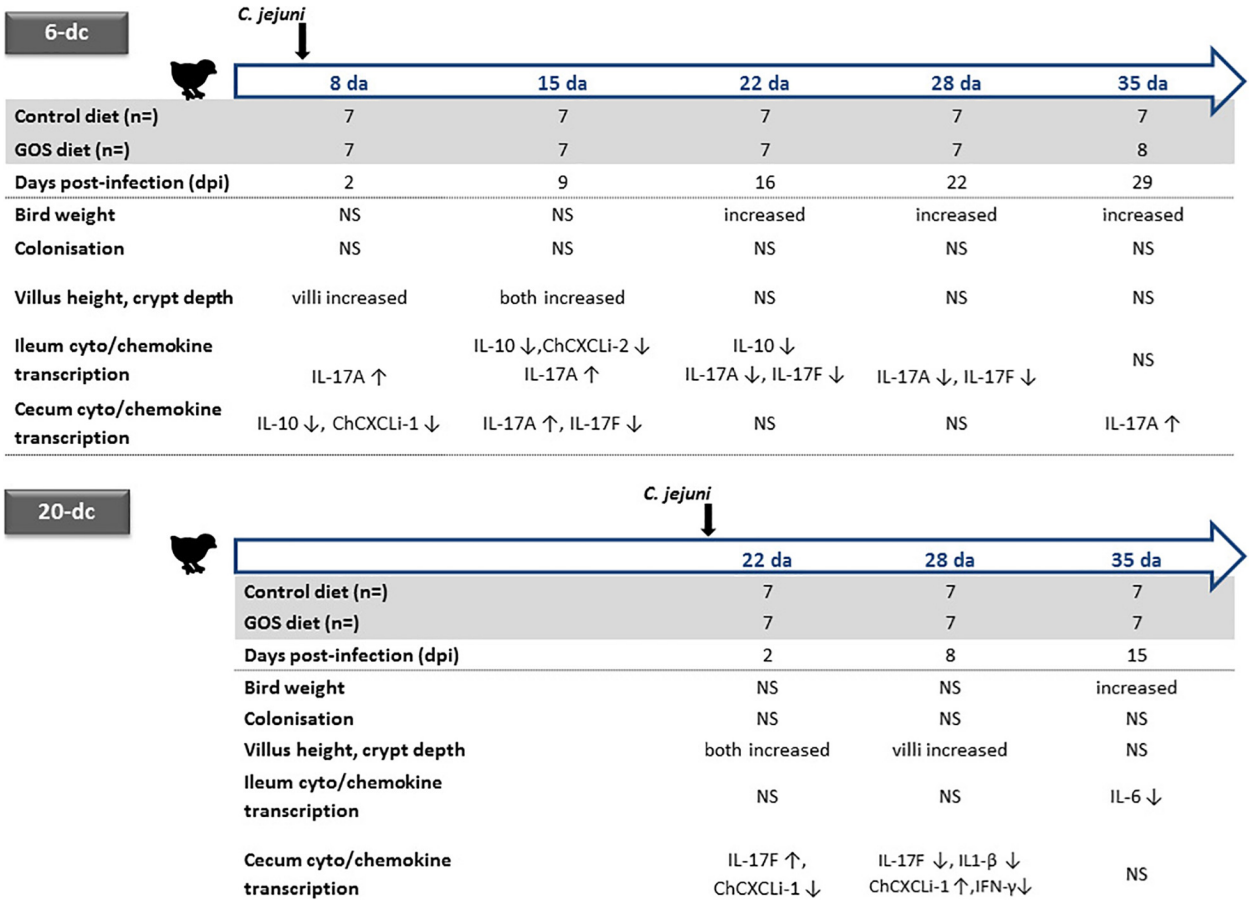

FIGURE 4 | Summary of age dependent differences for dietary GOS in *C. jejuni* colonized broiler chickens. Significant changes in the live weight, cecal *C. jejuni* colonization levels, ileal villus and crypt metrics are indicated for the GOS-fed birds compared to the birds on the control diet colonized 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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Conflict of Interest: NF is employed by Dairy Crest Ltd.

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

The authors declare that this study received funding from Saputo Dairy UK. The funder had the following involvement with the study: NF contributed to the study design.

5 Chapter 5. *In vitro* evaluation of prebiotics GOS and FOS on human colonic Caco-2 cells

Aforementioned studies demonstrated the beneficial effects of prebiotic GOS on the host through modulation of the gut microbiota during steady state and when the birds were challenged with *C. jejuni*, an effective coloniser of the chicken gut. Nevertheless, as non-digestible components of the intestinal lumen, little is known on the potential 'non-prebiotic' effects of well-studied prebiotics such as GOS and FOS on the colonic epithelial barrier. For this final piece we evaluated *in vitro* the potential direct effects of these non-digestible oligosaccharides on Caco-2 cell monolayers. In the next paper, increased trans-epithelial electrical resistance were revealed upon exposure to GOS, thus a similar trend was observed following FOS exposure. Analysis of the cells transcriptome by next-generation sequencing technology established GOS modulation involved a wider range of cell bioprocesses than FOS. Interestingly, some of GOS-induced gene transcripts were enriched in pathways related to steroids metabolism, digestion and absorption processes. By in large, our study generated a comprehensive valuable database that can be subsequently mined to further scientific knowledge and potentially facilitate product choice in human or animal intervention studies. The curated data are accessible through NCBI GEO Series accession number GSE145303 and NCBI Bioproject PRJNA606703, both accessible online via respective weblinks:

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145303, and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA606703.

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In Vitro Evaluation of the Effects of Commercial Prebiotic GOS and FOS Products on Human Colonic Caco-2 Cells

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Abstract: Prebiotic oligosaccharides are widely used as human and animal feed additives for their beneficial effects on the gut microbiota. However, there are limited data to assess the direct effect of such functional foods on the transcriptome of intestinal epithelial cells. The purpose of this study is to describe the differential transcriptomes and cellular pathways of colonic cells directly exposed to galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). We have examined the differential gene expression of polarized Caco-2 cells treated with GOS or FOS products and their respective mock-treated cells using mRNA sequencing (RNA-seq). A total of 59 significant differentially expressed genes were identified between GOS and mock-treated groups. For FOS treatment, a reduced number of 12 significant genes were observed to be differentially expressed relative to the control group. KEGG and gene ontology functional analysis revealed that genes up-regulated in the presence of GOS were involved in digestion and absorption processes, fatty acids and steroids metabolism, potential antimicrobial proteins, energy-dependent and -independent transmembrane trafficking of solutes and amino acids. Using our data, we have established complementary non-prebiotic modes of action for these frequently used dietary fibers.

Keywords: prebiotics; oligosaccharides; GOS; FOS; RNA-seq; transcriptome; functional pathway analysis; Caco-2; polarized monolayers

1. Introduction

Prebiotics are generally defined as substances that are selectively utilized by host microorganisms to produce a health benefit [1]. Prebiotic oligosaccharides conform to the definition as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon [2]. Such oligosaccharides are widely used as human and animal nutritional additives for their beneficial effects on the composition of the probiotic microbiota and gut health [3,4]. However, oligosaccharides that largely resist hydrolytic activities of salivary and intestinal digestive enzymes reach the colon virtually intact, thus prompting to question whether they could have a direct or "non-prebiotic" effect on the intestinal epithelium should they reach the mucosa.

Galacto-oligosaccharides (GOS or β-GOS) are produced through the β-galactosidase-catalyzed transgalactosylation of lactose creating oligosaccharides with degrees of polymerization (DP) ranging from 2 to 8 monomeric units with β-(1→3), β-(1→4) and β-(1→6) linkages between galactose units and are usually coupled to a terminal glucose [5]. Commercial GOS products typically also contain residual lactose, glucose and galactose moieties. Fructo-oligosaccharides (FOS) are commonly produced by acid- or enzyme-catalyzed hydrolysis of inulin [6], which results in fructan oligomers of 1 to 7 units

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1. Introduction

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(DP 1–7), sometimes with a terminal glucose [6,7]. Commercial FOS products typically also contains residual sucrose, fructose and glucose.

In vivo, GOS supplementation has been shown to increase iron absorption from micronutrient powder in Kenyan infants [8] or to reduce stress-induced gastrointestinal dysfunction and days of cold or flu symptoms in controlled trials of healthy university students [9]. In a healthy volunteer study, Schmidt et al. [10] described the reduction of the salivary cortisol awakening response associated with GOS administration, implying suppression of the neuroendocrine stress response. However, Liu et al. [11] suggested short-term intake of high-dose GOS and FOS prebiotics had an adverse effect on glucose metabolism despite increased *Bifidobacterium* in the fecal microbiota. In controlled animal studies, GOS has been associated with improved growth performance of broiler chickens [12] or during exposure to heat stress [13,14]. Dietary supplementation of GOS has also been linked with improvements in the transition to a mature intestinal microbiota in broiler chickens [12,15] and in suckling piglets [16,17]. In a study conducted with suckling male rats, in addition to changes in the intestinal microbiota, Le Dréan et al. [18] observed GOS and FOS supplementation impacted entero-endocrine cell maturation by bringing about transient increases in the density of GLP-1 cells and the production of the satiety-related peptides GLP-1 and PYY.

The direct cellular effects of prebiotics have been investigated using polarized intestinal epithelial cell models. For example, GOS exposure has been associated with improved tight junction formation and re-epithelialization post disruption, which coincided with increases in the expression of cell proliferation and cell differentiation pathways [19]. GOS was also reported to prevent deoxynivalenol-induced compromise of the integrity of Caco–2 cell monolayers and the corresponding decline in the expression of the tight junction encoding gene CLDN3 [20]. GOS is also reported to reduce the adherence of *Salmonella* Typhimurium to mucus- and to non-mucus-producing HT–29 cells [21].

Several studies have reported that GOS, FOS and inulin promote calcium absorption in the animal and human gut [22–24]. FOS has also been reported to have non-microbiome or non-prebiotic mediated effects on immune function. For example, Fransen et al. used germ-free mice to confirm microbiota-independent changes in immune function with short- or long-chain β2→1-fructans enhancing T helper 1 cells in Peyer's patches and short-chain β2→1-fructans, increasing regulatory T cells and CD11b−CD103− dendritic cells in the mesenteric lymph nodes [25]. FOS-inulin pre-incubation of a chicken macrophage cell line HD11 before challenge with *Salmonella* Enteriditis reduced cellular uptake of the pathogen and IL-1β gene expression, suggesting that inulin-enriched FOS had the ability to modulate the innate immune response [26].

Caco–2 cells are a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells that produce tight junctions, microvilli, enzymes and transporters characteristic of enterocytes. The Caco–2 monolayer is widely used in the pharmaceutical industry as an in vitro model of the human intestinal mucosa to predict the absorption of orally administered drugs. Caco–2 cells are commonly cultured as a polarized epithelial stratum by forming confluent monolayers on insert filters that provide a physical and biochemical barrier to the passage of ions and small molecules [27]. In this study, we have examined the effects of Nutrabiotic® GOS and Beneo® FOS (also referred to as "GOS" and "FOS" respectively) on the integrity of the monolayers by measuring trans-epithelial electrical resistance (TEER), and on differential gene expression relative to mock treated control using whole transcriptome sequencing technology. Our data establish complementary non-prebiotic modes of action for these frequently used dietary fibers.

2. Materials and Methods

2.1. Oligosaccharides

Galacto-oligosaccharides (Nutrabiotic® GOS, 66% w/w syrup) were provided by Dairy Crest Ltd (Esher, Surrey, UK) and Fructo-oligosaccharides (FOS Orafti® L95, 75% w/w syrup) were obtained from BENEO–Orafti (Oreye, Belgium). The detailed composition of the oligosaccharide treatments

applied are summarized in Table 1. The GOS treatment media contained 2% v/v of Nutrabiotic® GOS, equivalent to 1.4% w/v of DP 2–7+ galacto-oligosaccharides. The FOS treatment media contained 2% v/v of Orafti® L95 FOS, equivalent to 2% w/v of DP 2–8 fructo-oligosaccharides. To account for the presence of mono- and digestible di-saccharides contained in Nutrabiotic® GOS and Orafti® L95 FOS syrups used for treatment, we tailored the mock-treatments accordingly for GOS by incorporating galactose (0.03%), glucose (0.4%) and lactose (0.2%) in the GOS mock control, and by inclusion of fructose (0.06%), glucose (0.004%) and sucrose (0.04%) in the FOS mock control.

Table 1. Nutrabiotic® galacto-oligosaccharides (GOS) and Orafti® L95 fructo-oligosaccharides (FOS) syrup composition.

	Composition
Nutrabiotic® GOS	75% (w/w) dry solids (ds)
Galacto-oligosaccharides	66.5 (w/w ds)
Lactose	10.1 (w/w ds)
Glucose	21.8 (w/w ds)
Galactose	1.6 (w/w ds)
Orafti® L95 FOS	74.7% (w/w) dry solids
Fructo-oligosaccharides	94.8 (w/w ds)
Fructose	3 (w/w ds)
Sucrose	2 (w/w ds)
Glucose	0.2 (w/w ds)

2.2. Cells and Experimental Design

The microbiota-independent effects of GOS and FOS oligosaccharides were assessed in vitro using human Caco–2 cells cultured as a confluent monolayer and exposed to treatment for 24 h. Each treatment was undertaken as three technical replications of three independent biological replicates represented by different cell passages. The human colon adenocarcinoma Caco–2 cell line was obtained from the American Type Tissue Collection (ATCC® HTB–37™, passage 18; lot 62381028; LGC Standards, Teddington, UK,) and propagated according to established methods [28]. In brief, cells were cultured in Dulbecco's modified Eagle medium (DMEM) and seeded at a density of 10^5 cells onto 1.12 cm^2 Transwell® permeable support with a 0.4 μm tissue culture treated polyester membrane placed in a 12-well plate (3460; Costar, Kennebunk, ME, USA). Caco–2 cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Fresh culture medium containing DMEM (D6546, Sigma, Gillingham, UK), sterile Fetal Bovine Serum 10% v/v (F9665, Sigma), L-glutamine 2 mM (G7513, Sigma), MEM non-essential amino acid solution supplement 1x (M7145, Sigma), penicillin 100 U/mL and streptomycin 100 μg/mL (P0781, Sigma) was replenished every 2 to 3 days. Control mono- and di-saccharides-containing "GOS mock" and "FOS mock" treatment media were prepared as indicated in Table 2. GOS and FOS treatment media were prepared by dissolving 2% (v/v) of the oligo-saccharides syrups in FBS-free and antibiotic-free cell culture DMEM. All treatment culture media were free from serum and antibiotics to eliminate any interference from extraneous molecules, proteins or hormones.

After 18 days of culture, a confluent monolayer was obtained with a TEER exceeding 400 Ωcm^2 as measured by an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Independently, replicates of cells from passages ranging 22 to 26 were gently washed 3 times (at hourly intervals) with warm non-supplemented (serum-free, antibiotic-free) DMEM culture medium and finally replenished from apical and basolateral sides of the Transwell® inserts with oligosaccharides or mock treatment media for 24 h exposure before being harvested for total RNA extraction.

Table 2. Culture media composition.

Media	Basic media* supplementation
Cell growth & confluence	
	+ 10% (v/v) FBS + antibiotics 100 U/mL (penicillin, streptomycin)
Conditioning & treatment	
GOS 2%	+ 2% (v/v) Nutrabiotic® GOS
FOS 2%	+ 2% (v/v) Orafti® L95 FOS
GOS mock	+ 0.67 % (m/m) mono- and di-saccharides (glucose 4.796 g/L, galactose 0.352 g/L, lactose 2.126 g/L)
FOS mock	+ 0.104 % (m/m) mono- and di-saccharides (glucose 0.044 g/L, fructose 0.660 g/L, sucrose 0.421 g/L)

* basic media: Dulbecco's MEM medium (DMEM), L-glutamine 2mM, MEM non-essential amino acids supplement 1x, Fetal Bovine Serum (FBS).

2.3. Integrity of Tight Junctions

Development of tight junction formation was monitored at regular intervals during the monolayers expansion by measuring the TEER across each of the growth inserts with an EVOM voltohmmeter (World Precision Instruments) connected to a pair of chopstick electrodes. Ohmic resistance of a blank (culture insert with respective medium but without cells) was measured in parallel. To obtain the sample resistance, the blank value was subtracted from the total resistance of the sample-containing cells. Final unit area resistance (Ωcm^2) was calculated by multiplying the sample resistance by the effective area of the membrane (1.12 cm^2 for 12-wells Transwell® inserts). To assess the effect of oligosaccharides on the Caco–2 cell monolayers, the electrical resistance across each cell monolayer was measured prior to exposure (T_0) and after 24 h of exposure (T_{24h}) to the treatment media.

2.4. RNA Extraction and Qualification

Cells were gently washed in situ twice with ice cold sterile PBS followed by addition of 500 µl of TRIzol™ reagent (ThermoFisher Scientific, Paisley, UK) ready for harvesting by directly scraping the cells from the culture surface. Harvested cells were transferred to a 2 mL Eppendorf® safe-lock microcentrifuge tube for total RNA extraction according to TRIzol™ reagent manufacturer's protocol. Purified RNAs were quality-assessed using Agilent TapeStation 2200 system (G2964AA; Agilent, Stockport, UK) with RNA ScreenTape Assay reagents (5067–5576; 5067–5577; 5067–5578 Agilent). The RNA preparations had RIN values within the range 8.2 to 9.7 (mean = 9.24), which were subsequently aliquoted to limit freeze-thaw cycles and stored in −80 °C until cDNA library preparation.

2.5. Library Preparation for mRNA Sequencing

RNA concentrations were measured using Qubit Fluorometer and Qubit RNA BR Assay Kit (Q10211; ThermoFisher Scientific). A Biomek 4000 Automated Workstation (Beckman Coulter, High Wycombe, UK) was used to prepare sequencing libraries. mRNA was purified from 1 µg of total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490; New England Biolabs, Ipswich, UK). Indexed sequencing libraries were then prepared using the NEBNext Ultra directional RNA Library Preparation Kit for Illumina (E7760; New England) and NEBNext Multiplex Oligos for Illumina, Index Primers Sets 1 and 2 (E7335 and E7500; New England Biolabs). Libraries were quantified using Qubit Fluorometer and Qubit dsDNA HS Kit (Q32854; ThermoFisher Scientific). Library fragment-length distributions were analyzed using the Agilent TapeStation 4200 with the Agilent High Sensitivity D1000 ScreenTape Assay (5067–5584 and 5067–5585; Agilent). Libraries were pooled in equimolar amounts and final library quantification performed using KAPA Library Quantification Kit for Illumina (KK4824; Roche, Pleasanton, CA, USA). The library pool was sequenced on an Illlumina NextSeq500 over two High Output 150 cycle kits (FC-404-2002; Illumina, San Diego,

CA, USA), to generate over 40 million pairs of 75-bp paired-end reads per sample. The metadata and DNA sequences are available under the NCBI GEO series accession number GSE145303.

2.6. Quality Control and Mapping Analyses

High-throughput sequence reads were quality checked with FastQC (version 0.11.3). Raw reads in FASTQ format were subsequently processed using the Trim Sequences Module in CLC Genomics Workbench 12.03 (Qiagen, Aarhus, Denmark). In this step, clean data were obtained by removing reads from the raw data that contained adapter sequences and those of low-quality, all downstream analyses were performed with high-quality clean data. Using the RNA-Seq Analysis Module in CLC Genomics Workbench 12.03, clean trimmed reads were paired and mapped to *Homo sapiens* Hg38 (GRCh38 reference genome available in ENSEMBL) thus generating normalized counts of gene and transcript hits as RPKM (reads per kilobase of exon model per million mapped reads) and TPM (transcripts per million). Normalized counts were treated to compute P-values and fold changes (FC) for downstream differential expression analysis. Using the RNA-Seq Analysis Module from CLC Genomics Workbench 12.03, differential expression analysis between oligosaccharides-treated group and mock-treated group was determined using a negative binomial distribution model with the resulting P-values being adjusted to *p*-adj values using the approach of Benjamini and Hochberg [29] for controlling the false discovery rate (FDR). Genes with absolute FC $\geq$ 1.5 and *p*–adj value $<$ 0.05 were designated as significantly differentially expressed (DE). Volcano plots of genes that were differentially expressed were developed using CLC workbench 12.03. Principal Component Analysis (PCA) was carried out using the web-based tool ClustVis [30]. Heatmap diagrams were developed using the Morpheus web-based tool [31] where rows and columns were clustered using Pearson's correlation distance and average linkage. Venn diagram showing the overlap of differentially expressed genes between GOS and FOS treatments were calculated using the web-based tool InteractiVenn [32].

2.7. Functional Analysis of Differentially Expressed Genes

Functional analysis of significant differentially expressed genes was achieved by implementing bioinformatics tools on gene set enrichment and pathway databases analysis based on Gene Ontology (GO) [33,34] terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [35] collection of databases. We investigated statistically significant gene sets modulated by GOS and FOS with | FC value | $\geq$ 1.5 and FDR *p*–adj. $<$ 0.05 by implementing hypergeometric distribution within NIPA.Enrichment.R script (v0.6.7.R, https://github.com/ADAC-UoN/NIPA) and applying a minimum of 2 genes enriched, a cut-off *q*–value = 0.05 for KEGG and GO bases and an enrichment analysis established on "hsa" (*Homo sapiens*) Ensembl database. Briefly, NIPA.Enrichment.R script executed the bioconductor biomaRt [36,37] package to integrate Ensembl sequence data with data analysis using the GO tool, the Generally Applicable Gene set Enrichment for pathway (GAGE) package performed gene set analysis [38] and Pathview completed pathway-based data integration and visualization [39].

2.8. Quantitative Real-Time PCR Validation

Relative gene expression of DE genes computed by RNA-seq was established by RT-qPCR according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines [40]. Sequences of RT-qPCR primers (Eurofins Genomics, Ebersberg, Germany) were designed using National Center for Biotechnology Information (NCBI) primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [41] and are described in Table 3. cDNA was synthesized using Invitrogen™ SuperScript™ II (18064014, ThermoFisher Scientific) according to the random hexamers primer method previously described [42]. Quantitative RT-PCR was performed using the Applied Biosystems PowerUP™ SYBR™ Green PCR Master Mix (A25742, ThermoFisher Scientific) on a Real-Time PCR LightCycler® 480 System (Roche, Pleasanton, CA, USA). Each sample was processed in triplicate and the expression of genes of interest was normalized to endogenous housekeeping genes GAPDH, PUM1 and ACTB. Amplification protocol comprised one denaturation

cycle at 95 °C for 5 min (ramp rate 4.8 °C/s), forty amplification cycles at 95 °C for 15 s, 65 °C for 30 s and 72 °C for 30s (ramp rate 1.5 °C/s). Melting curves were assessed for each RT-qPCR reaction using LightCycler® 480 System software (v 1.5.1.62; Roche, Pleasanton). Resulting PCR product size was confirmed using Agilent TapeStation 2200 system (G2964AA; Agilent) with DNA D1000 ScreenTape Assay (5067-5582 and 5067-5583; Agilent). Primer sets were validated for specificity when melting curves analysis exhibited a single peak with Tm > 78 °C and amplified product size matched expected product size as established by the NCBI primer designing tool. Relative gene expression was calculated using the relative quantification method with amplification efficiency corrected calculation models [43,44]. Amplification rate and relative concentration was calculated using LightCycler® 480 System software (v 1.5.1.62; Roche, Pleasanton) based on a linear regression slope established by 2-fold dilution series of a pool of all RNA samples.

Table 3. Primer sequences for RNA-seq validation by qPCR.

Target Gene	Primer Sequence (5′–3′)	Product Size (bp)	NCBI Accession	RNA–Seq Identifier
CAPN8	F: GGTCTAGGTGACTGCTGGCT R: AGCAGCTGTCCATTCTTGGT	197	NM_001143962.2	ENSG00000203697
COL12A1	F: GGCAAGGCTATCCAGGTTCC R: TAAGCACGTGCGCAAACATC	106	NM_004370.6	ENSG00000111799
CYP1A1	F: CCCCCACAGCACAACAAGAG R: GGGTGAGAAACCGTTCAGGT	146	NM_000499.5	ENSG00000140465
F13B	F: GGACACTTCCTCCTGAGTGTGT R: CGTCTGCAACAGCCCCATTC	81	NM_001994.3	ENSG00000143278
GALNT16	F: CTGACCTTCGTGGAGGGTTC R: GGTCTGTCCGGGTCATCTTC	84	NM_020692.3	ENSG00000100626
GPX2	F: TTTCAATACGTTCCGGGGCA R: CTGACAGTTCTCCTGATGTCCA	169	NM_002083	ENSG00000176153
RGPD5	F: CAAGAAATTGCCTGTGCCCC R: TCCATCGAGGTGGTGTTTCG	215	NM_005054.3	ENSG00000015568
SLC5A3	F: ATGCAGCGGGGTTGGTACA R: AGCAACACAGCAGGGTCAAA	235	NM_006933.7	ENSG00000198743
SULT1A3	F: CGGTCTCCTACTACCATTTCC R: AGGACCCGTAGGACACTTC	108	NM_177552.3	ENSG00000261052
ACTB	F: CTGGAACGGTGAAGGTGACA R: AAGGGACTTCCTGTAACAATGCA	140	NM_001101.5	
GAPDH	F: GGAGTCCACTGGCGTCTTCAC R: GAGGCATTGCTGATGATCTTGAGG	165	NM_002046.7	
PUM1	F: TGAGGTGTGCACCATGAAC R: CAGAATGTGCTTGCCATAGG	187	NM_014676.2	

2.9. Statistical Analysis

TEER experimental results are expressed as mean ± standard deviation, the differences between groups were evaluated by one-way ANOVA using Genstat 19th edition (VSNI Ltd, Hemel Hempstead, UK), where p-values < 0.05 were considered statistically significant. Within CLC Genomics Workbench 12.03, differential gene expression statistical analysis was based on Baggerly's beta-binomial model [45] to account for between-library variability; two-sided p-values for the test are described as "p-value". p-values were subsequently corrected for false discovery rate FDR ("p-adj values") using the Benjamini and Hochberg's approach [29]; p-adj value < 0.05 was considered significant. For functional analysis, a hypergeometric distribution model generated q-values [46]; a minimum of 2 genes enriched with q-values < 0.05 for KEGG and GO enrichment were used as the criteria for reporting.

2.10. Data Availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO series accession number GSE145303 (https://www.ncbi.

nlm.nih.gov/geo/query/acc.cgi?acc=GSE145303). The project appears in the NCBI database within Bioproject PRJNA606703 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA606703).

3. Results

3.1. Influence of GOS and FOS on Epithelial Monolayer Tight Junction Integrity

To assess the direct effect of dietary GOS and FOS, cells were exposed to tailored media containing the oligosaccharides. The presence of mono- and di-saccharides contained in GOS and FOS syrups used for treatment was accounted for in the control mock media for Nutrabiotic® GOS 64% by incorporating galactose, glucose and lactose and by inclusion of fructose, glucose and sucrose for Orafti® L95 FOS. The treatment of Caco–2 cell monolayers with GOS generated a significant increase in the TEER values (Figure 1, TEER +33.62%, $p = 0.00037$) when compared to the mock-exposed cells. Similarly, FOS also induced greater TEER when compared to the mock-exposed cells (Figure 1, TEER +28.68%, $p = 0.054$). The rise in trans-epithelial electrical resistance of the monolayer suggest an acceleration of the tight junction dynamics that indicates an improvement of the monolayer integrity under the influence of the oligosaccharide treatments.

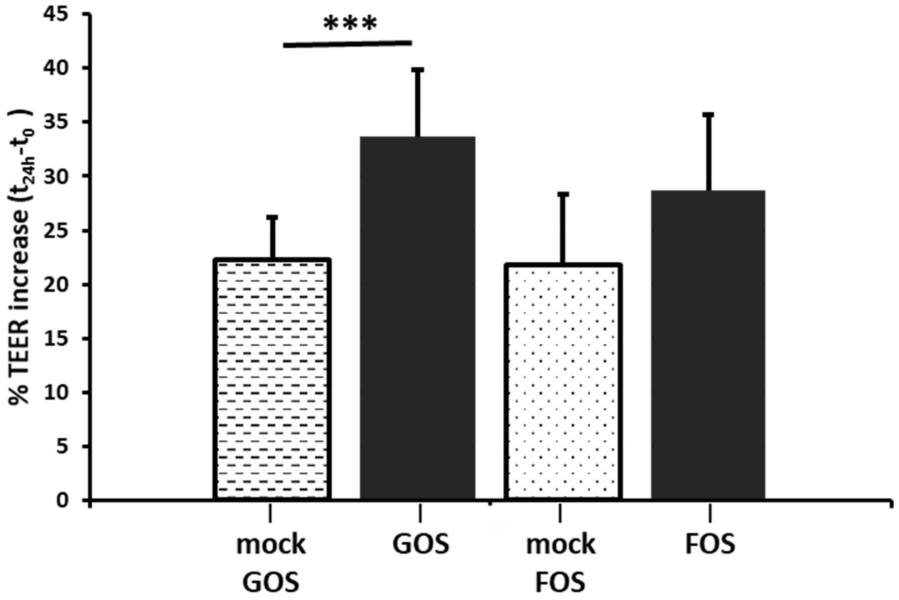

Figure 1. GOS and FOS effect on trans-epithelial resistance of Caco–2 monolayers. Polarized confluent Caco–2 monolayers were exposed for 24 h to GOS, FOS (2% v/v) or their respective mock media. GOS increased significantly the monolayer TEER (+33.62%, $p < 0.001$), while the TEER increase induced by FOS exposure was not statistically significant (+28.68%, $p = 0.054$). Data are expressed as means ± SD and were tested using ANOVA t-test, ***$p < 0.001$ ($n = 3$).

3.2. Transcriptome Sequencing and Functional Annotation

The effect of GOS and FOS preparations on the global transcriptome of Caco–2 cell monolayers were assessed by RNA-seq using the Illumina NextSeq sequencing platform. RNAs were harvested from three biological replicates for each treatment group and mRNA sequencing generated between 44 and 134 million paired-end reads of 75-bp per sample. An average of 88.67% good-quality paired-end reads per sample were mapped to Homo sapiens Hg38 reference genome. The summary of RNA-seq data and mapping for each sample are presented in Table 4. Gene and transcript normalized hit counts were used to generate FC and FDR adjusted *p*-values to implement differential gene expression analysis.

Table 4. Summary of sequencing reads mapped to the human reference genome. RNA sequencing was strand-specific and only reverse strand reads were mapped to the reference genome Homo sapiens Hg38. FOS (1–3) and GOS (1–3) are oligosaccharide-treated groups, the experiment specific control treatment groups are referred to as "mock" (1–3); each treatment consists of three independent biological replicates (labelled 1–3) that were created from a pool of three technical replicates (n = 3).

		Raw Read Count	Ignored Reads (Wrong Strand)	Reads Paired and Mapped	Fragments Mapped to Genes	Fragments Mapped as Intergenic	Protein Coding Genes
FOS experiment	**mock1**	47,141,326	615,415 (1.30%)	88.25%	95.87%	4.13%	96.47%
	mock2	49,710,422	771,068 (1.55%)	87.24%	95.81%	4.19%	96.54%
	mock3	44,983,458	717,544 (1.59%)	89.62%	95.86%	4.14%	96.64%
	FOS1	49,264,466	842,951 (1.71%)	85.56%	95.47%	4.53%	96.35%
	FOS2	51,905,580	749,101 (1.44%)	86.80%	95.64%	4.36%	96.46%
	FOS3	49,519,788	688,065 (1.39%)	89.41%	95.46%	4.54%	96.59%
GOS experiment	**mock1**	62,474,912	819,911 (1.31%)	89.96%	95.73%	4.27%	96.47%
	mock2	45,810,896	589,565 (1.29%)	91.01%	95.86%	4.14%	96.59%
	mock3	49,459,368	649,851 (1.31%)	88.43%	95.88%	4.12%	96.48%
	GOS1	134,054,024	2,460,936 (1.83%)	89.29%	95.78%	4.22%	96.54%
	GOS2	59,524,110	1,153,619 (1.94%)	87.68%	95.87%	4.13%	96.49%
	GOS3	56,118,898	809,112 (1.44%)	90.74%	95.53%	4.47%	96.61%

3.3. Analysis of Differential Gene Expression

Volcano plots were generated to visualize the distribution of differentially expressed genes following GOS and FOS exposure (Figures 2A and 3A respectively), which showed a limited number of genes modulated by FOS when compared to GOS. Principal component analysis of the normalized transcript counts (TPM) disclosed a tight relationship between biological replicates within each treatment group such that oligosaccharides-exposed samples clustered distinctly from their mock-treated counterparts. As a result, the first two principal components taken together explained 91.9% and 87.8% of the variability among the samples exposed to GOS and FOS when compared to mock treated cells (Figures 2B and 3B respectively). We further examined the distribution of differentially expressed genes exhibiting FDR p-adj < 0.05 and $|FC| \geq 1.5$ using heatmaps, which are presented in Figures 2C and 3C for the GOS and FOS treatments respectively. Following exposure to GOS, 89 genes were differentially expressed according to the criteria, of which 53 were up-regulated and 36 down-regulated when compared to control mock-treated cells (Figure 2C and Table S1). Whereas for FOS, the total number of genes fulfilling the criteria for differential expression was limited to 12, comprising of eight up-regulated and four down-regulated genes (Figure 3C and Table S1). GOS- and FOS-treated cells shared five differentially expressed genes as presented in the Venn diagram shown in Figure 3D: *TMEFF1*, *GPX2*, *SLC5A3*, *SULT1A3*, *AL139011.2*. Three of these genes were up-regulated upon exposure to GOS and FOS treatments (*GPX2*, *SLC5A3* and *AL139011.2*) and two genes showed the opposite modulation such that *TMEFF1* transcripts were reduced upon GOS exposure whilst increased with FOS treatment, and *SULT1A3* transcription was increased with GOS but reduced with FOS exposure.

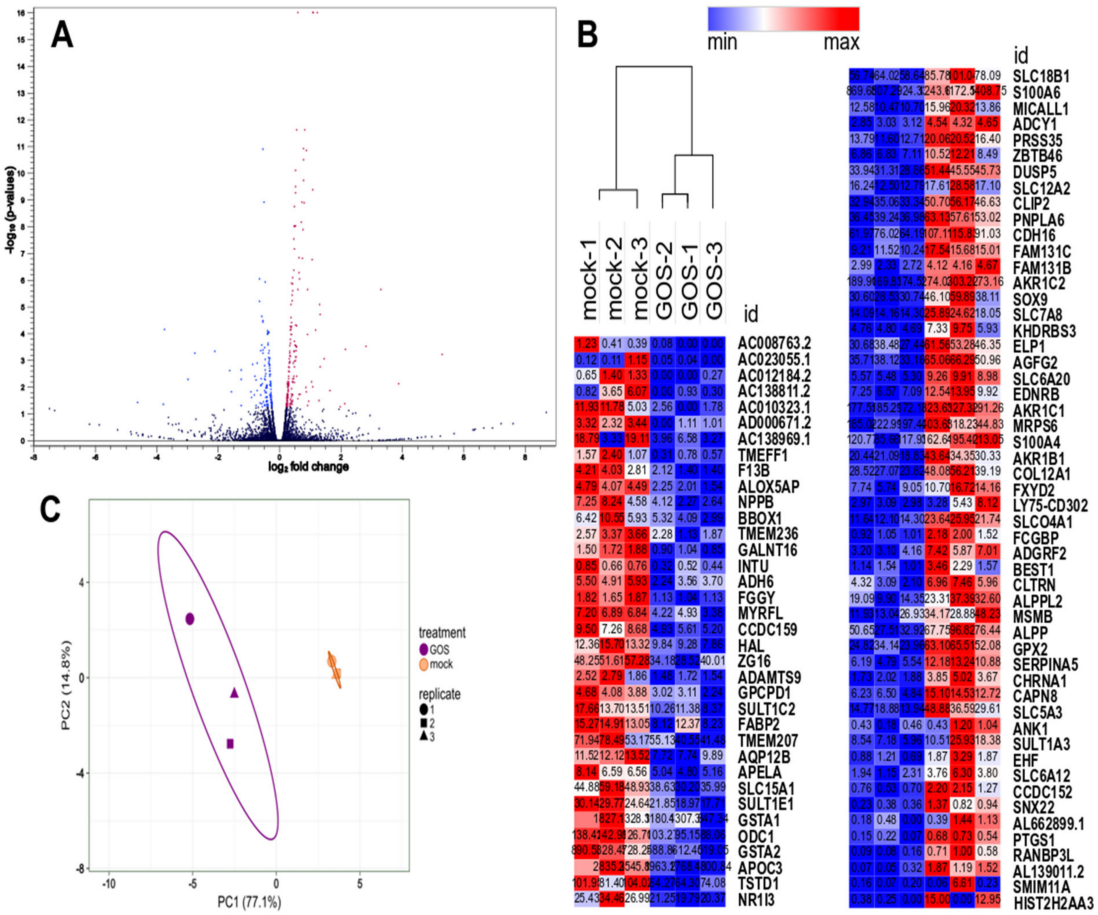

Figure 2. Differentially expressed genes associated with GOS treatment. (**A**) Volcano plot of genes differentially expressed between GOS and GOS mock treatment (n = 3). The abscissa represents $\log_2$ fold changes in gene expression, the ordinate represents statistical significance of the variations in gene expression, expressed as -$\log_{10}$(p-values). (**B**) Principal component scatter plot showing variance of the biological replicates for each treatment (n = 3). Axis x and y show principal components 1 (PC1) and 2 (PC2) that explain 77.1% and 14.8% of the total variance respectively. Prediction ellipses indicate probability 0.95 that a new observation from the same group will fall inside the ellipse; N = 6 data points. (**C**). Heatmap displaying differentially expressed genes for GOS and mock treatments. Rows and columns are clustered using Pearson's correlation distance and average linkage. Data are presented as normalized transcripts count (TPM) with |FC| ≥ 1.5 and q-value < 0.05.

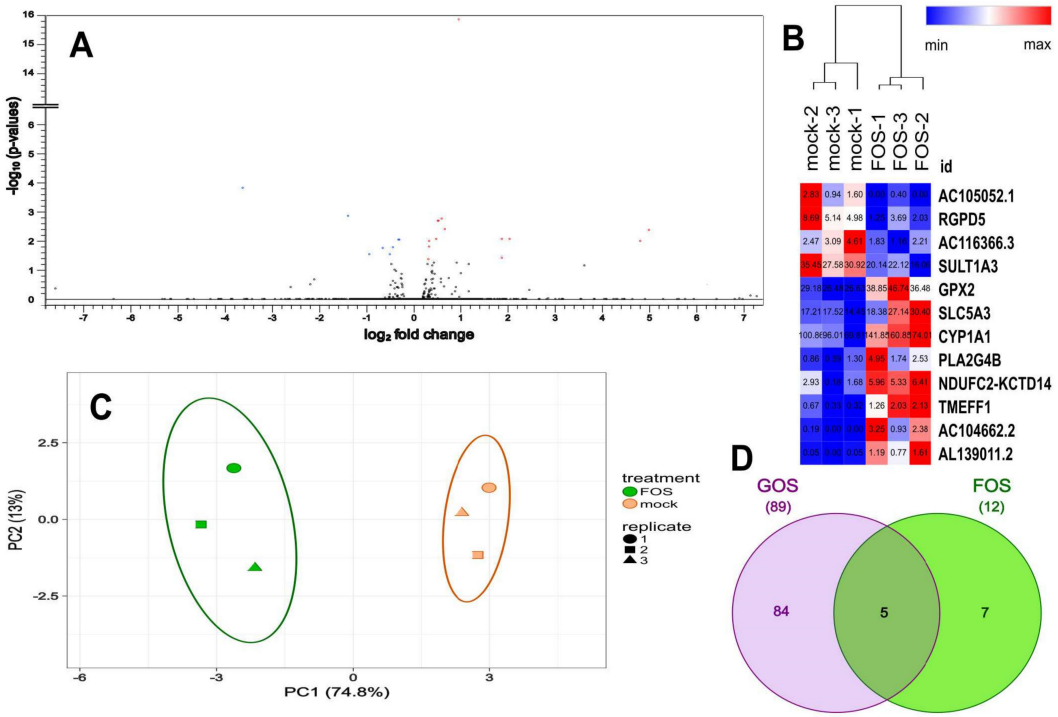

Figure 3. Differentially expressed genes associated with FOS treatment. (**A**) Volcano plot of genes differentially expressed between FOS and FOS mock treatment (n = 3). The abscissa represents $\log_2$ fold changes in gene expression, the ordinate represents statistical significance of the variations in gene expression, expressed as -$\log_{10}$ (p-values). (**B**) Principal component scatter plot showing variance of the biological replicates for each treatment (n = 3). Axis x and y show principal components 1 (PC1) and 2 (PC2) that explain 74.8% and 13% of the total variance, respectively. Prediction ellipses indicate probability 0.95 that a new observation from the same group will fall inside the ellipse; N = 6 data points. (**C**) Heatmap displaying differentially expressed genes for FOS and mock treatments. Rows and columns are clustered using Pearson's correlation distance and average linkage. Data are presented as the normalized transcripts count (TPM) with |FC| ≥ 1.5 and q-value < 0.05. (**D**) Venn diagram showing the overlap of differentially expressed genes between GOS and FOS treatments. Data are presented as the number of genes with |FC| ≥ 1.5 and q–value < 0.05.

3.4. Validation of Differentially Expressed Genes by RT-qPCR

RNA-seq data was independently validated by RT-qPCR measurement of the transcript levels of genes identified as differentially expressed (RT-qPCR raw data presented in Table S2). Results revealed strong correlation between the RNA-seq and RT-qPCR differential expression data for the GOS and FOS treatments (Figure 4; GOS R^2 = 0.9623 and FOS R^2 = 0.9173). Accordingly, genes *GPX2* (RT-qPCR GOS FC = +2.1; FOS FC = +1.4) and *SLC5A3* (RT-qPCR GOS FC = +2.7; FOS FC = +1.9) were found up-regulated simultaneously with GOS and FOS treatments. Consistent with the GOS RNA-seq data, genes *F13B* (RT-qPCR FC = −3.6) and *GALNT16* (RT-qPCR FC = −1.6) were found to be down-regulated whilst *COL12A1* (RT-qPCR FC = +1.9) and *CAPN8* (RT-qPCR FC = +2.3) were up-regulated. Similarly, congruency was observed for the FOS treatment-associated up-regulated gene *CYP1A1* (RT-qPCR FC = +1.7) and down-regulated genes *SULT1A3* (RT-qPCR FC = −1.3) and *RGPD5* (RT-qPCR FC = −1.4).

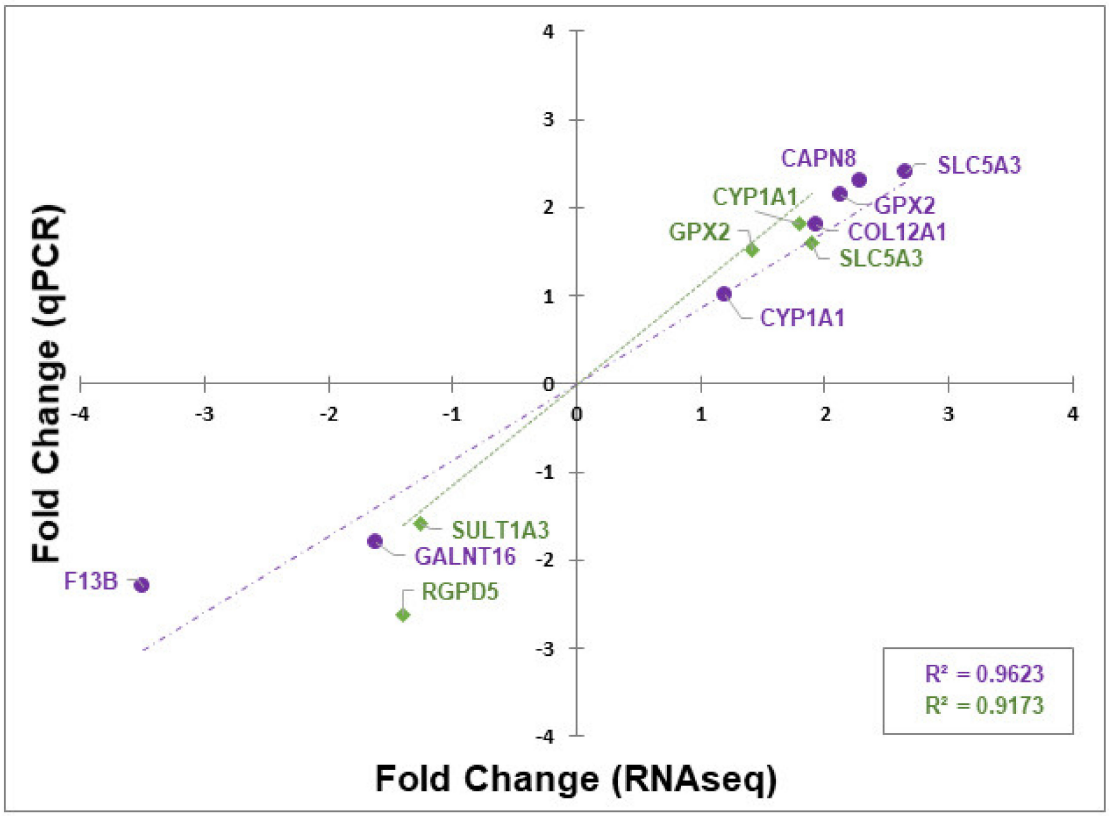

Figure 4. Validation of RNA-seq by RT-qPCR. The abscissa represents RT-qPCR Fold Change in gene expression, the ordinate represents RNA-seq Fold Change in gene expression. • GOS gene expression; –·–· GOS gene expression linear trend line; ♦ FOS gene expression; . . . . FOS gene expression linear trend line.

3.5. Functional Analysis of Differentially Expressed Genes

Biological attribute identification and functional interpretation of our data was achieved by implementing bioinformatics tools on gene set enrichment and pathway database analyses based on Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) collection of databases. The KEGG knowledge base links gene catalogues to functional information such as metabolism, membrane transport, signal transduction and cell cycle in pathway assemblies. GO resource provides computable knowledge for biological functions of genes and gene products structured within biological process, molecular function and cellular component classes.

GO classification of the 89 differentially expressed (DE) genes associated with GOS exposure placed the up-regulated genes within 10 GO terms (Figure 5) describing metabolic processes (daunorubicin, doxorubicin and progesterone metabolic processes, alkaline phosphatase activity and digestion), cell membrane transport (transepithelial chloride transport, amino acid transmembrane transport) and tissue homeostasis. KEGG analysis of the gene sets (Table 5) indicated the up-regulated gene-associated pathways were enriched for metabolic processes featuring folate biosynthesis (hsa00790), thiamine metabolism (hsa00730), thyroid hormone synthesis (hsa04918), protein digestion and absorption (hsa04974), membrane transport with salivary (hsa04970) and pancreatic (hsa04972) secretion. The GOS down-regulated genes were placed in five GO terms (Figure 5) that identified processes related to sulfation, 3′-phosphoadenosine-5′-phosphosulfate sulfotransferase, glutathione derivative biosynthesis, glutathione peroxidase activity and triglyceride catabolism. The down-regulated GO terms were further identified in KEGG (Table 5) as reduced glutathione metabolism (hsa00480), chemical carcinogenesis (hsa05204), drug and xenobiotics metabolism-cytochrome P450 (hsa00982 and hsa00980). A further seven DE genes were enriched in a unique GO cell compartment described as the collagen-containing

extracellular matrix (GO:0062023, *p*–adj.= 0.018) involving three down-regulated and four up-regulated genes (Table 6).

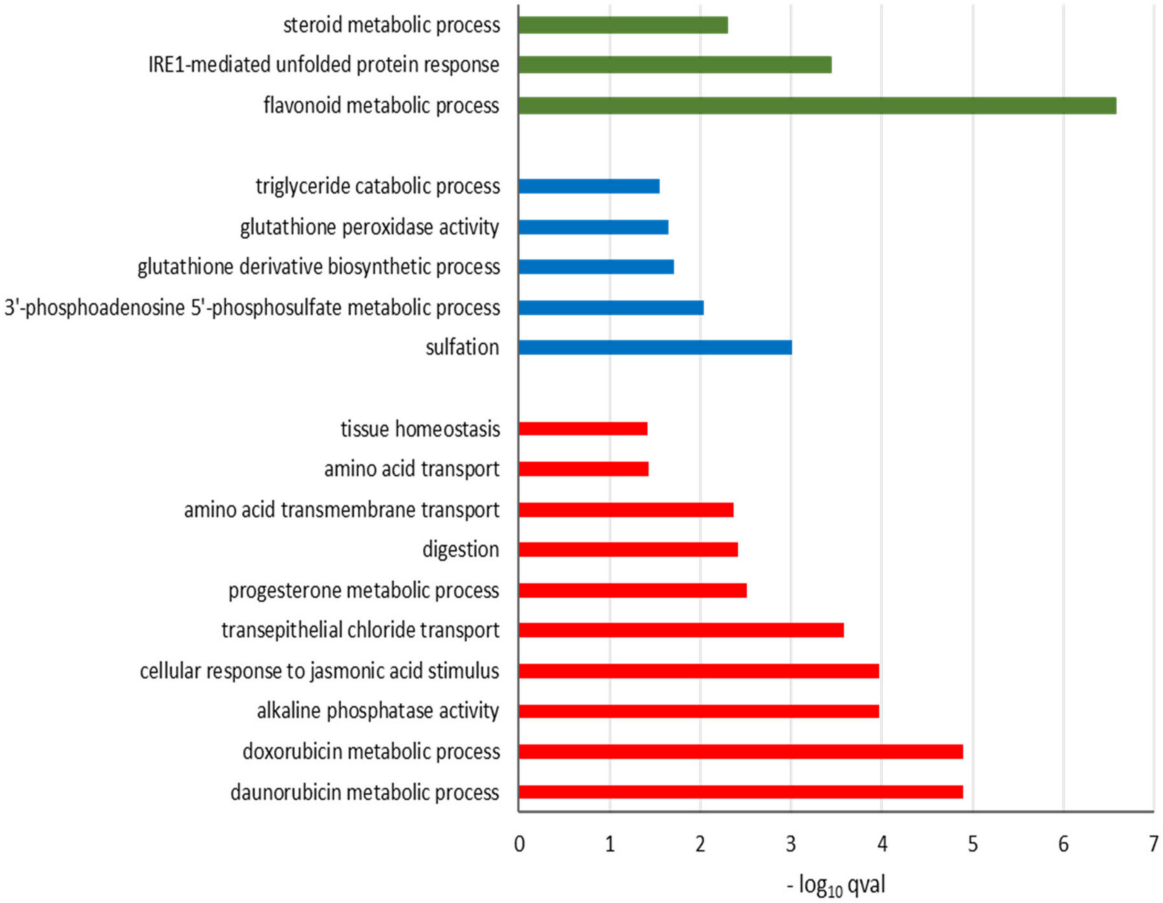

Figure 5. Gene Ontology (GO) enrichment analysis of differentially expressed genes of oligosaccharides treated compared to mock treated Caco–2 cells. Differentially expressed genes (FDR *p*–adj. < 0.05, $|FC| \geq 1.5$) were clustered according to GO bioprocesses classification. ■ GOS up-regulated; ■ GOS down-regulated; ■ FOS up-regulated.

Gene Ontology analysis of the 12 DE genes linked with FOS treatment revealed bioprocesses (Figure 5) involved in flavonoid metabolism, IRE1-mediated unfolded protein response and steroid metabolism represented by the genes *SULT1A3* (FC = –1.6, *p*–adj. = 0.0178), *CYP1A1* (FC = +1.8, *p*-adj. $< 1.10^{-12}$) and *PLA2G4B* (FC = +3.7, *p*–adj. = 0.008). Analysis of the KEGG pathways (Table 5) revealed increases in pathways linked with ovarian steroidogenesis (hsa04913), arachidonic acid metabolism (hsa00590) and general metabolic pathways (hsa01100). GO analysis also identified the mitochondrial inner membrane (GO:0005743) as a unique cell compartment, as represented by three up-regulated genes *CYP1A1*, *PLA2G4B* and *NDUFC2-KCTD14* (*p*–adj. = 0.002, Table 6).

Table 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Enriched pathways among the differentially expressed genes were identified by KEGG analysis. Differentially expressed gene false discovery rate (FDR) p-adj.< 0.05 and absolute value of fold change ≥1.5.

KEGG Pathway	FDR *q*.val	Enriched Gene	Protein Coding Alias	Fold Change (mRNAseq)	FDR *p*–Value (mRNAseq)
GOS (UP)					
hsa00790 Folate biosynthesis	0.0003	*AKR1B1*	Aldo-Keto Reductase	1.8	1×10^{-11}
		ALPP	Intestinal Alkaline Phosphatase	2.1	4×10^{-10}
		ALPG	Placental-Like Alkaline Phosphatase	2.1	2×10^{-7}
hsa00730 Thiamine metabolism	0.0025	*ALPP*			
		ALPG			
hsa04918 Thyroid hormone synthesis	0.0170	*GPX2*	Glutathione Peroxidase-Gastrointestinal	2.1	$<1 \times 10^{-12}$
		ADCY1	Ca^{2+}/Calmodulin-Activated Adenylyl Cyclase	1.5	5×10^{-4}
		FXYD2	Sodium/Potassium-Transporting ATPase Subunit Gamma	1.8	0.010
hsa04970 Salivary secretion	0.0359	*SLC12A2*	Basolateral Na-K-Cl Symporter	1.5	0.004
		ADCY1			
		FXYD2			
hsa04974 Protein digestion and absorption	0.0359	*SLC7A8*	L-Type Amino Acid Transporter 2	1.6	9×10^{-7}
		COL12A1	Collagen Type XII Alpha 1 Chain	1.8	2×10^{-12}
		FXYD2			
hsa04972 Pancreatic secretion	0.0495	*SLC12A2*			
		ADCY1			
		FXYD2			
GOS (DOWN)					
hsa00480 Glutathione metabolism	0.002	*ODC1*	Ornithine Decarboxylase 1	−1.5	1×10^{-11}
		GSTA2	Glutathione S-Transferase Alpha 2	−1.5	4×10^{-5}
		GSTA1	Glutathione S-Transferase Alpha 1	−1.5	0.008
hsa00982 Drug metabolism - cytochrome P	0.005	*GSTA2*			
hsa00980 Metabolism of xenobiotics by cytochrome P	0.007	*GSTA1*			
hsa05204 Chemical carcinogenesis	0.009	*ADH6*	Alcohol Dehydrogenase 6 (Class V)	−1.7	0.004
FOS (UP)					
hsa04913 Ovarian steroidogenesis	0.0001	*CYP1A1*	Cytochrome P450 Family 1 Subfamily A Member 1 (Aryl Hydrocarbon Hydroxylase)	1.8	$<1 \times 10^{-12}$
		PLA2G4B	Phospholipase A2 Group IVB	3.7	0.008

Table 5. *Cont.*

KEGG Pathway	FDR *q*.val	Enriched Gene	Protein Coding Alias	Fold Change (mRNAseq)	FDR *p*–Value (mRNAseq)
hsa00590 Arachidonic acid metabolism	0.0002	*PLA2G4B*			
		GPX2	Gastrointestinal Glutathione Peroxidase	1.5	0.002
hsa01100 Metabolic pathways	0.0130	*NDUFC2-KCTD14*	NDUFC2-KCTD14 Readthrough Transcript Protein (NADH Dehydrogenase (Ubiquinone) 1 Subunit C2, Isoform 2)	3.7	0.039
		AC104662.2	predicted type II PI4 kinase protein family (PI4K2B)	28.2	0.009
		PLA2G4B			
		CYP1A1			

Table 6. Cell compartment GO enrichment analysis of differentially expressed genes.

GO Cell Compartment Enrichment	FDR q.val	Enriched Gene	Protein Coding Alias	Fold Change (mRNAseq)	FDR *p*–Value (mRNAseq)
GOS					
GO:0062023 Collagen-containing extracellular matrix	0.018	*ZG16*	Zymogen Granule Protein 16 (Jacalin-Like Lectin Domain Containing)	−1.6	9×10^{-7}
		APOC3	Apolipoprotein C3	−1.5	3×10^{-5}
		ADAMTS9	A Disintegrin And Metalloproteinase with ThromboSpondin Motifs 9	−1.5	4×10^{-2}
		SERPINA5	Serine (Or Cysteine) Proteinase Inhibitor, clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 5	2.1	$<1 \times 10^{-12}$
		COL12A1	Collagen type XII Proteoglycan	1.8	2×10^{-12}
		S100A4	S100 Calcium Binding Protein A4	1.7	2×10^{-10}
		S100A6	S100 Calcium-Binding Protein A6 (Calcyclin)	1.5	5×10^{-5}
FOS					
GO:0005743 Mitochondrial inner membrane	0.002	*CYP1A1*	Cytochrome P450 Family 1 Subfamily A Member 1 (Aryl Hydrocarbon Hydroxylase)	1.8	$<1 \times 10^{-12}$
		PLA2G4B	Phospholipase A2 Group IVB	3.7	0.008
		NDUFC2-KCTD14	NDUFC2-KCTD14 Readthrough Transcript Protein (NADH Dehydrogenase (Ubiquinone) 1 Subunit C2, Isoform 2)	3.7	0.039

4. Discussion

Non-digestible oligosaccharides are prebiotics able to selectively stimulate the components of gut microbiota beneficial to host health [47]. A consequence of non-digestion is that they will reach the colon intact to exert any non-prebiotic effects on the intestinal epithelium. In this study, we addressed the hypothesis that oligosaccharides could directly affect the transcriptome of epithelial cells representative of the intestinal mucosa and have exploited RNA-seq methodology to describe the transcriptome of confluent Caco–2 cell monolayers exposed to the GOS and FOS.

The Caco–2 cell line model was chosen for its ability to form tight junctions and microvilli, thus expressing enzymes and transporters characteristic of enterocytes [48,49]. A number of in vitro studies have evaluated the impact of various prebiotics on the cell barrier integrity when subject to pathogen-induced barrier disruptions [50,51], inflammation-inducing cytokines and lipopolysaccharides [52] or toxin-induced challenge with the mycotoxin deoxynivalenol [53]. Here, exposure to GOS (1.4% w/v) increased trans-epithelial electrical resistance of the monolayer suggesting GOS can improve the integrity of the tights junctions. FOS (2% w/v) exposed cells similarly increased trans-epithelial electrical resistance despite marginally failing to meet significance. We sought to understand these effects and any wider transcriptional responses to GOS and FOS by establishing differential gene expression using RNA-seq. Differential expression was determined in prebiotic-treated Caco–2 cell monolayers with respect to tailored mock exposure to the saccharides present in the GOS and FOS preparations.

Differentially expressed genes were used as the basis for in silico approaches to assess the functional consequences. The biological significance of a given fold-change is likely to depend on the gene and on the experimental context, and for this reason, universal thresholds are not applied for the bioinformatic interrogation of transcriptomic data [54–56]. We have adopted a stringent statistical approach through the use of Benjamini and Hochberg's false discovery rate correction for multiple testing to establish the criteria of FDR adjusted probability p-adj < 0.05 and an inclusive absolute fold change ≥ 1.5 to identify differential gene expression. We opted for the inclusive value of ≥ 1.5-fold change since it has been demonstrated that the enrichment of biologically relevant functions can occur at low fold changes in RNA levels when appropriate statistical methods are employed [57]. However, despite steady-state observations that protein levels are generally determined by transcript concentrations, there are frequent differences observed between transcriptomic and proteomic assessments of expression during cellular transitions [58]. A total of 89 DE genes were identified between GOS and mock treated cells, whereas for FOS exposure the number was reduced to 12 DE genes. The RNA-seq data from both experiments were validated by RT-qPCR using specific primer sets. Strong correlations were observed between the fold-change determined for the two methods with $R^2 = 0.96$ for GOS and $R^2 = 0.92$ for FOS treatments. The DE data were used for GO term enrichment and KEGG analyses to identify transcriptional pathways responding to prebiotic oligosaccharide exposure. Modulation of the pathways identified are discussed below and are summarized in Figure 6 for reference.

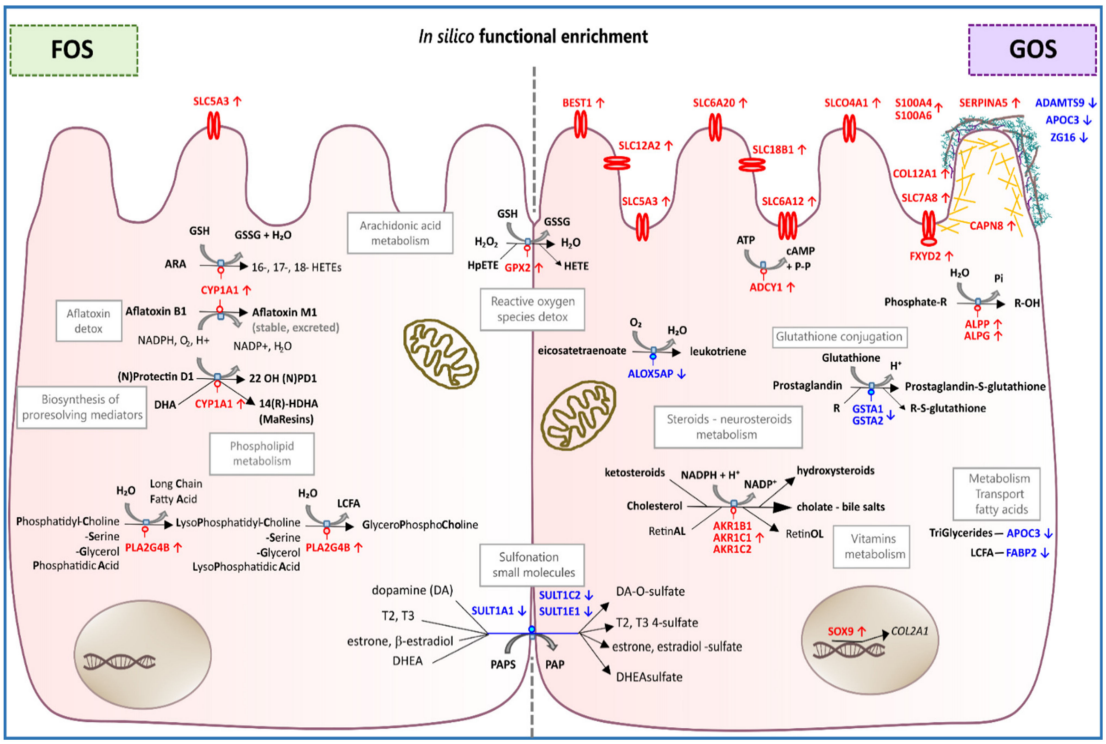

Figure 6. Summary of the pathway associations of the differentially expressed genes (FDR p-adj. < 0.05, |FC| ≥ 1.5) identified as responding to the prebiotic oligosaccharides FOS or GOS in confluent colonic Caco–2 cells. Pathways were deduced from GO enrichment and KEGG analyses. Up-regulated functions are marked in red text and the down-regulated, in blue text.

4.1. FOS Treatment Associated with 12 Differentially Expressed Genes

FOS treatment resulted in 12 DE genes comprising of four down-regulated and eight up-regulated genes. GO terms identified the up-regulated genes *SULT1A3*, *CYP1A1* and *PLA2G4B* as related to flavonoid metabolism, IRE1-mediated unfolded protein response and steroid metabolism. Interrogation of the KEGG database confirmed these three genes are functional in ovarian steroidogenesis and arachidonic acid metabolism (FDR *q*–val < 0.001) and general metabolic pathways (FDR q–val = 0.013). *CYP1A1* encodes a Cytochrome P450 monooxygenase exhibiting high catalytic activity for steroid hormones [59] and hydroxylation of certain polyunsaturated fatty acids (PUFA) [60], whereas *PLA2G4B* encodes phospholipase A2 that preferentially catalyzes hydrolysis of arachidonoyl phospholipids to release lysophospholipids and fatty acids, with a preference for arachidonoyl phospholipids. The products of these genes also form a nexus with the phosphatidylinositol 4-kinases products of *AC104662.2* and *PI4K2B* around cell signaling, lipid and membrane trafficking pathways [61]. In the absence of direct evidence for any transcriptional changes of the genes encoding tight junction proteins to affect the increases in TEER observed, it is conceivable that FOS exposure results in compositional changes in membrane sterols and phospholipids that affect the physical properties of the cell membrane, altering rigidity/fluidity leading to stabilization or disruption [62]. This contrasts with the direct effect, although not attributed to oligosaccharides, reported on the expression of *CLDN4*, encoding the epithelial cell tight junction protein Claudin 4, in Caco–2 cells exposed to complex food homogenates of apple, broccoli and button mushroom [63].

4.2. GOS Treatment Associated with 53 up-Regulated Genes

GOS treatment led to 53 up-regulated genes that associated with 10 GO terms. Genes *AKR1C1*, *AKR1C2* and *AKR1B1* were related to the terms daunorubicin and doxorubicin metabolic process, cellular response to jasmonic acid stimulus, progesterone metabolic process and digestion (FDR q-val < 0.005).

These genes encode enzymes harboring oxidoreductase and aldo-keto reductase activities that target steroids and prostaglandins with varying levels of bile acid-binding affinity. Progesterone metabolic process and cellular response to jasmonic acid stimulus relate also to up-regulation of *AKR1C1* and *AKR1C2* genes due to their catalytic activity related to androgens inactivation (progesterone and testosterone respectively). Despite its plant origin, jasmonic acid is an oxylipin-signaling molecule often considered a structural and functional analogue to prostaglandins in animals. Mammalian eicosanoids and oxylipins such as prostaglandins and leukotrienes are active signaling molecules derived from oxygenated PUFAs and are potent regulators of host immune responses. Notably, mammalian prostaglandins and leukotrienes (products of arachidonic acid metabolism) can polarize macrophages, modulate T helper cell immune responses, stimulate chemokine production, phagocytosis, lymphocyte proliferation and leukocyte chemotaxis [64].

GO also associated *AKR1C1* and *AKR1C2* with *CAPN8* within digestion processes. *CAPN8* encodes tissue-specific calpain 8a (cytosolic calcium-dependent cysteine protease), which in mice is reported to be expressed specifically in the mucus-secreting pit cells of the gastric mucosa as well as in a subset of goblet cells in the small intestine [65]. Calpains are also implicated in membrane trafficking processes due to their localization in endoplasmic reticulum, Golgi and lipid rafts [66,67]. The KEGG protein digestion and absorption pathway (hsa04974) identified the genes *SLC7A8*, *COL12A1* and *FXYD2*, which respectively encode L-type amino acid transporter 2, collagen type XII alpha 1 chain and Na/K-transporting ATPase subunit gamma suggesting specific amino acid transport and focused structural protein remodeling. The KEGG pancreatic secretion (hsa04972) and salivary secretion (hsa04970) pathways featured the up-regulated genes *SLC12A2* encoding a basolateral Na-K-Cl symporter and *ADCY1* encoding Ca^{2+}/calmodulin-activated adenylyl cyclase. These activities are linked as Ca^{2+} and cAMP can regulate intestinal Na-K-Cl symporter activity [68]. Related to this, the GO terms transepithelial chloride transport (FDR q-val < 0.001) and amino acid transport (FDR q-val < 0.05) identified genes for ion channel and solute carrier proteins (*BEST1*, *SLC12A2*, *SLC6A20*, *SLC7A8* and *SLC6A12*). Several solute carrier encoding genes were up-regulated (*SLC7A8*, *SLC6A20*, *SLC6A12*, *SLCO4A1*, *SLC12A2*, *SLC5A3* and *BEST1*) that feature in Reactome pathway analyses related to amino acid transport across the plasma membrane and the transport of small molecules. Two bioprocesses relevant to the digestion pathway were enriched in KEGG folate biosynthesis (hsa00790) and thiamine metabolism (hsa00730) pathways. Genes *ALPP* and *ALPG* encoding intestinal and placental-like alkaline phosphatase respectively were identified with *AKR1B1* encoding aldo-keto reductase. *AKR1B1* in association with *SOX9* was identified as enriched constituents of the GO tissue homeostasis pathway. *SOX9* encodes SRY-Box Transcription Factor 9 that binds to the *COL2A1* promoter to activate cartilaginous tissue-specific Collagen Type II Alpha 1 Chain protein expression, which has an essential role in skeletal development and contributes to the ability of cartilage to resist compressive forces. Collectively, these data are consistent with the hypothesis that GOS elicits energy-dependent transmembrane trafficking of solutes in Caco–2 cell monolayers accompanied by collagen and cytoplasmic membrane remodeling that may be linked to the observed increases in TEER.

4.3. GOS Treatment Associated with 36 Down-Regulated Genes

Of 36 DE genes found down-regulated following GOS exposure, seven DE genes were annotated in enriched GO bioprocess terms associated with sulfation and 3′-phosphoadenosine 5′-phosphosulfate metabolic process (genes *SULT1E1* and *SULT1C2*), glutathione peroxidase activity and derivative biosynthetic process (genes *GSTA2*, *GSTA1* and *ALOX5AP*), and triglyceride catabolic process (genes *APOC3* and *FABP2*). All these genes encode proteins related to fatty acid and arachidonic acid metabolism. Genes *SULT1E1* and *SULT1C2*, encode sulfotransferases responsible for sulphate conjugation of many hormones (such as estrogens), neurotransmitters and xenobiotic compounds. *GSTA2* and *GSTA1* encode alpha-glutathione S-transferases (GSTs) involved in the synthesis of prostaglandins (PGs) and leukotrienes and are thus responsible for the metabolization of electrophilic compounds including carcinogens, therapeutic drugs and the by-products of oxidative stress. Notably,

through eicosanoid metabolites GSTs can play a central role as regulators of transcription factor peroxisome proliferator-activated receptor γ (PPARγ) [69,70]. In mammals, overexpression of GSTs in tumor cells has been implicated with resistance to various anti-cancer agents and chemical carcinogens and in microbes, plants and mammals, expression of GSTs are up-regulated by exposure to pro-oxidants, thus suggesting that induction of GST is an evolutionary conserved response of cells to oxidative stress [71]. *ALOX5AP* encodes arachidonate 5-lipoxygenase activating protein required for leukotriene biosynthesis by 5-lipoxygenase (ALOX5). The products of LOX metabolism either represent biologically active eicosanoid metabolites such as hydroperoxy–eicosatetraenoic acids (HPETEs) or give rise to the production of reactive oxygen species (ROS). A functional role has also been attributed to lipoxygenase-catalyzed arachidonic and linoleic acid metabolism in cancer development [72,73]. *APOC3* encodes apolipoprotein C3, a protein component of the triglyceride (TG)-rich lipoproteins (TRLs) and FABP2 encodes intestinal-type Fatty Acid-Binding Protein—an intracellular fatty acid-binding protein that participates in the uptake, metabolism and transport of long-chain fatty acids. This lends further support to the reconfiguration of fatty acid metabolism in response to GOS treatment whilst maintaining homeostasis.

GO cell compartment analysis identified the collagen-containing extracellular matrix based on the down-regulation of *APOC3*, *ZG16* and *ADAMTS9* and up-regulation of *COL12A1*, *S100A4*, *S100A6* and *SERPINA5*. Gene *APOC3* encodes a liver and small intestine-secreted apolipoprotein C-III (apoC-III) found with triglyceride-rich lipoproteins, such that increases in apoC-III levels induces the development of hypertriglyceridemia and overexpression can contribute to coronary heart disease in humans [74,75]. Following exposure to GOS, *APOC3* was down-regulated thus suggesting that a reduction in apoC-III levels could be beneficial for triglyceridemia. However, *ZG16* and *ADAMTS9*, which have been ascribed roles in the control of cancer development, were also down-regulated. Zymogen granule protein 16 (ZG16) is one of the most significantly down-regulated genes in colorectal cancer (CRC) and harbors a jacalin-like lectin domain. Lectins are carbohydrate-binding proteins able to detect subtle differences between complex carbohydrate structures, thus recognizing specific sugars to carry out various functions including cell attachment, migration and invasion. Amongst these, some galectins share an affinity for simple β-galactoside moieties [76]. ZG16 may be an important component of the protective mucus layer, which helps separate host epithelium from commensal bacteria in the colon [77]. The high similarity of ZG16 to jacalin suggests that the human homologue may play an important role in colon cancer immunity. The loss of ZG16 associated with CRC development has led to the hypothesis that ZG16 reduction may disrupt well-organized bacterial surveillance systems to facilitate bacterial invasion of host tissues and cause local inflammatory changes, which may constitute an increased risk for the development of cancer [78]. *ADAMTS9* encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family, a secreted mammalian metalloprotease that localizes to the cell-surface and/or extracellular matrix. The extracellular matrix is continuously remodeled by coordinated biosynthesis and proteolysis of its components. Active proteolytic ADAMTS9 has been shown to interact with fibronectin and disrupt fibril networks [79]. Furthermore, it has been suggested ADAMTS9 contributes to the suppression of tumorigenesis by decreasing cell proliferation, inducing cell apoptosis and inhibiting angiogenesis through regulating the AKT/mTOR signaling pathway, whilst methylation of *ADAMTS9* genes is associated with poor survival of gastric cancer patients [80].

Collagen-containing extracellular matrix enriched genes *S100A4* and *S100A6* were found to be up-regulated following treatment with GOS. Proteins S100A4 and S100A6 are members of the S100 calcium binding protein family that exert biological functions via interactions with target proteins. Many kinds of cells including fibroblasts, immune cells, and cancer cells can produce S100A4, which are released into the extracellular space in response to various stimuli such as activated normal T cell expression and secreted factors (RANTES) produced by the tumor cells. S100A4 could be involved in the regulation of cell proliferation and differentiation, apoptosis, Ca^{2+} homeostasis, and energy metabolism [81]. Sun et al. demonstrated that mice deficient in S100A4 exhibited impaired humoral and

cellular immunity after mucosal immunization using Cholera toxin as adjuvant, revealing a reduced production of Th1, Th2 and Th17 cytokines [82]. Protein S100A6 has been described in a limited number of cell types in adult normal tissues and in several tumor cell types. As an intracellular protein, S100A6 has been implicated in the regulation of several cellular functions, such as proliferation, apoptosis, cytoskeleton dynamics and cellular response to different stress factors. S100A6 can be secreted/released by certain cell types which points to extracellular effects of the protein such as antimicrobial activity [83]. Similar up-regulation was observed for gene *SERPINA5* encoding Protein C inhibitor (PCI), a serine protease inhibitor of serpin type that is found in most tissues and fluids, including blood plasma, seminal plasma and the urine of humans [84]. As an antimicrobial agent, PCI has the ability to disrupt the bacterial cell wall to cause death by interacting with lipid membranes leading to permeabilization of bacterial pathogens [85]. PCI also inhibits proteases of the blood coagulation and fibrinolysis system, whilst in cancer cells it suppresses tumor invasion by inhibiting urokinase-type plasminogen activators and inhibits tumor growth and metastasis, which are independent of its protease-inhibitory activity [86]. Lastly, *COL12A1* encoded collagen XII is a member of the family of fibril-associated collagens with an interrupted triple helix (FACIT) structure. Mutations in the collagen XII gene associated with extracellular matrix-related myopathy, as the protein functions to preserve muscle and bone architecture through collagen fibril organization [87,88].

5. Conclusions

In this study, the direct effects of GOS and FOS on colonic epithelial cells were assessed through changes in monolayer transepithelial resistance and transcriptome analysis. Results suggest GOS have the potential to directly increase integrity of the epithelial barrier. Transcriptome data suggest TEER increased independent of specific tight junction protein expression but could be due to sterol/fatty acid compositional changes associated with transporter-dependent remodeling of the cell membrane. Specific candidate pathways group the genes involved in digestion and transepithelial transport, which contribute to intestinal cell integrity and function.

Carbohydrates have enormous potential to encode biological information. It is conceivable that GOS and FOS can crosstalk with cells using lectin molecules as the interface. Typically, lectins and their complimentary carbohydrate are located on the surfaces of opposing cells, which can be of the same type or different types. Such interactions are required for cell differentiation, development and pathological states. These results highlight concerted effects on transmembrane trafficking, differences in xenobiotic biotransformation, and the production of antimicrobial agents.

GOS and FOS treatments shared relatively few differentially expressed genes, suggesting they have different modes of action. Our strategy has produced a comprehensive database of gene expression profiles of Caco–2 cell monolayers exposed to oligosaccharide food ingredients, allowing further work to link gene expression signatures of cultured cells to their mode of action, and thus potentially facilitating product choice in human or animal intervention studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/5/1281/s1, Supplementary Table S1: GOS and FOS gene expression browser, Table S2: RT-qPCR raw data.

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6 Chapter 6. Concluding remarks

6.1 Summary

To infer valuable knowledge on the impact of selected prebiotic oligosaccharides as natural functional food ingredients that are aimed at animal and human applications, this study addressed two functional actions. The first was to the evaluate the impact of the prebiotic GOS on the performance and gut health of broiler chickens, an important monogastric farm animal. The second objective was to characterise the direct impact of the prebiotic oligosaccharides GOS and FOS on polarised human colonic epithelial cells.

Under controlled biosecure conditions, we demonstrated that the inclusion of GOS in the diet of broiler chickens significantly enhanced their growth rate and body weight at a market age of 35 days (Richards et al. 2020). This observation was corroborated in challenge experiments with *C. jejuni*, an effective and frequent coloniser of the gastrointestinal tract of chicken and the causative agent responsible for human campylobacteriosis (Flaujac Lafontaine et al. 2020). Notably, *C. jejuni* challenge did not impede the growth performance of broilers maintained on a control-calorie matched diet (Connerton et al. 2018). The growth performance enhancement of GOS-fed chickens correlated with the augmented abundance of *L. johnsonii* in the cecal microbiota. In turn, *L. johnsonii* abundance was found to be positively correlated with increased intestinal expression of IL-17A, a Th17 cytokine. In contrast *L. crispatus* was found to be most prevalent in control-fed birds. We demonstrated that a *L. johnsonii* isolate (DC22.2) matching the OTU identified from 16S rRNA microbiome could be selected from the cecal contents of high performing GOS-fed birds, and was able to out-compete *L. crispatus* when administrated to GOS-fed chickens via cloacal gavage. Notably, *L.*

johnsonii DC22.2 did not efficiently utilize GOS *in vitro*, probably due to a deficient lactose permease system, as revealed by genome sequence analysis, hence implying the bacterium relies on the metabolic capacity of other members of the chicken gut microbiome to thrive. These findings were remarkable in light of a study by Torok *et al*. establishing that the absence of significant body weight gain of broilers fed antimicrobial additives was associated with the reduced abundance of *L. johnsonii* concomitant with increases in *L. crispatus* and *L. reuteri* abundance in the ileal microbiota (Torok, Allison, et al. 2011). Thus, endorsing the premise that prebiotic selection of an autochthonous symbiotic species can improve animal growth performance. Interestingly, *L. johnsonii*-correlated enhanced IL-17A expression in unchallenged chickens suggests similarities with mice symbiont segmented filamentous bacteria (SFB) due to their immunomodulatory capabilities, their tight adherence to the intestinal mucosa and their reliance on imported nutrients generated by the intestinal milieu (Mollet et al. 2004; Bergonzelli et al. 2006; Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). Likewise, it is also conceivable that *Lactobacillus*, an abundant member of the gastro-intestinal tract in chicken, encompass genus members exhibiting diverse immunomodulatory potencies.

C. jejuni challenge of birds maintained on a normal diet, unveiled a transient resistance to the cecal colonisation of juvenile birds, possibly due to protection conferred by the residual presence of maternal antibodies. The effect of such antibodies may not only be limited to the challenge organism, but other components of the microbiota may be hindered in their ability to compete and colonize the intestine. As the titres of the maternal antibodies diminish then any growth check will be released, which would be consistent with the transitions in the intestinal microbiota composition observed at 15 days of age. The transition was associated with a transient set-back in weight gain and short-lived changes in villus-crypt measurements (Connerton et al. 2018; Han, Pielsticker, et al. 2016).

Thereafter from two weeks old, birds exhibited high levels of *Campylobacter* cecal load, this was despite a transient polarized Th17 response associated with IL-6, IL-17A, and IL-17F increased expression. The kinetics of the innate immune response for older birds challenged at 20 da with *C. jejuni* was different but similarly led to increases in IL-17A, and IL-17F. The ability of *Campylobacter* to colonise the intestinal tracts of birds was not impeded by an increase in the expression of IL-17A and IL-17F cytokines. When *C. jejuni* challenged birds were maintained on a GOS diet, *Campylobacter* remained a successful cecal coloniser. However, this did not prevent a sustained Th17 immune response or the differential increase in abundance of *L. johnsonii* associated previously with dietary GOS. Our findings were consistent with the hypothesis that *L. johnsonii* outcompetes *L. crispatus* in GOS-fed birds, and consistent with the premise that *L. johnsonii* contributes to the stability of the innate immune response observed in GOS-fed birds. Dietary GOS inclusion maintained the transcript levels of IL-17A and suppressed *C. jejuni*-driven IL-17F expression whist alleviating intestinal impediment and promoting the expansion of goblet cells populating the intestine. These changes were accompanied by improvements in the growth rate of broiler chickens independent of *C. jejuni* colonization.

Investigation of the direct effect of GOS and FOS on confluent human colonic epithelial cells revealed different transcriptional responses. Whilst both oligosaccharides improved epithelial tight junctions as indicated by trans-epithelial resistance, GOS and FOS shared relatively few differentially expressed genes suggesting they have different modes of action. Transcriptional response to FOS was reduced compared to GOS, however functional analysis of the differentially expressed genes revealed that both oligosaccharides increased expression of genes associated with hormone functions through fatty acids and steroid metabolism. Notably, transcriptome examination revealed GOS directly up-regulated genes involved in prostaglandin-associated eicosanoids and oxylipin signalling

molecules, digestion and transmembrane trafficking processes, which together contribute to intestinal cell integrity and function. Our study established the first comprehensive database on complementary non-prebiotic modes of action for these frequently used dietary fibres.

6.2 Impact of the Research

Undoubtedly the outcomes of our research have increased academic knowledge and understanding of the interplay between prebiotic oligosaccharides, the intestinal microbiome and the status of host intestinal immune function. The novelty and importance of these findings have been validated by peer reviews and published in scientific journals, hence enabling dissemination to the wider community through open access articles. Our conclusions have progressed our mission in finding alternatives to AGPs for the poultry industry, and resulted in patent application GB2558021A for a "Composition for treatment and/or nutrition of poultry" (Connerton I., Connerton P., Fish N.M., Lafontaine G. and Richards P.; 2016).

The outcome of our new knowledge benefits feed suppliers through valorisation of by-products into commercial patented beneficial feed additives directly impacting farmers and customers. The wider impact on the farming community will bring about improved animal performance whilst reducing the impact of antimicrobial growth promoters in animal feed. This may require adjustments in farm practice much of which can be achieved through feed supplier's information, veterinarian reports and information delivered at farmers discussion groups. In the long term, we can foresee improved satisfaction of consumers drawn to high quality meat produced in respect of improved animal welfare, and a positive long-term environmental impact through by-product valorisation, reduction of

effluent antimicrobials and reduced greenhouse gas owing to enhanced animal health and feed conversion indices.

6.3 Suggestions for Future Research

In the future, work should be developed to further our knowledge in terms of animal welfare and performance. Provision of dietary GOS increased chicken cumulative FCR and growth rate. Our studies were conducted in clean controlled biosecure facilities exempt from the farm endemic pathogens frequently endured by commercial broilers and associated with degraded flock health and growth performance. Under these circumstances, the impact of prebiotic priming of intestinal immunity may be more significant in field applications. To further evaluate the impact of commercial GOS on economic performance, additional trials should be performed on larger scale, ideally at farm level.

Despite promising intestinal immune function stimulation, cecal microbiota changes and improved growth rate performance of *Campylobacter*-colonized broiler chickens, our findings show dietary GOS inclusion did not prevent nor reduce *C. jejuni* gut colonization within the 35 days life span of the birds. Further trials should assess the encouraging potential of GOS feedstuff to reduce gut colonisation of other poultry farm endemic bacterial pathogens such as *Salmonella* Enteritidis.

Regarding growth performance, essential amino acids are required for the biosynthesis of indispensable proteins, and increased protein synthesis can enhance the rate of poultry growth. To understand the mechanism by which the GOS-infeed additive led to increased growth rate and performance, it would be pertinent to evaluate *in vivo* whether chicken intestinal proteases such as aminopeptidase activity were intensified by the GOS diet, thus leading to increased amino acid availability for transmembrane trafficking. Enzyme activity could be further assessed as

previously described by Maroux et al. (Maroux, Louvard, and Barath 1973). Similarly, intestinal assimilation of carbohydrate is essential for energy supply in animals. Activities of carbohydrates hydrolases, such as host sucrase and maltase located in the brush border of intestinal cells and the functional activities of the intestinal microbiota, are modulated by the availability of their substrates (Dahlqvist 1984). It could be hypothesised the level of carbohydrates available in the luminal content either through diet supply or through microbiota degradation of complex carbohydrates into mono- and di-saccharides would modulate intestinal carbohydrase activity. To this aim, carbohydrase gene expression could be evaluated by qPCR targeting mRNA from ileal and cecal biopsies for sucrase–isomaltase (SI), which is critical in disaccharide digestion, together with that of Na–glucose transporter (SGLT)-1 (Sklan et al. 2003; Uni, Ganot, and Sklan 1998). Furthermore, enzyme activity could be assayed as previously described by Dahlqvist et al. (Dahlqvist 1984).

Our *in vitro* model has demonstrated that GOS can directly modulate intestinal cell gene expression with specific increases in the transcriptional expression of amino acid transporters, hormone functions, digestion and transmembrane trafficking processes. Exploiting the database generated, it would be pertinent to evaluate *in vivo* whether similar genes or pathways were modulated for chickens fed GOS feedstuff. To do so, qPCR primers designed for orthologous genes of interest of *Gallus gallus* species could be considered to detect and quantify levels of transcripts in chicken intestinal biopsies collected during feed trials.

Animal growth rate is also linked to the absorption capacity of the intestinal mucosa. Enterocyte proliferation and maturation are influenced by the hormone glucagonlike-peptide 2 (GLP-2) produced by enteroendocrine L-cells of the intestinal epithelium. Thus, under various diets regimens and/or pathogen challenge, the density of enteroendocrine L-cell could be investigated in the ileal mucosa via immunohistochemical staining.

As part of the effect of dietary oligosaccharides on chicken intestinal health we have shown that improvement in intestinal barrier architecture and the modulation of a mucosal immune function were associated with compositional shifts in the microbiota. To understand how these associations arise, the next step would be to evaluate the links between microbiota fermentation of oligosaccharides and the production of SCFAs and their relationship with the intestinal function. SCFAs are important modulators of immune function and intestinal cell metabolism. Indeed, butyrate and propionate are known to be taken up by the intestinal epithelium through receptor mediated processes. Fatty acids are volatile molecules routinely characterised and quantified by gas chromatography to high levels of precision when coupled with mass spectrometry. In addition, high performance liquid chromatography analysis of the luminal content could further extend investigation of potential non-volatile polar metabolites involved in cellular modulation. More recently, the development of machine learning tools to predict retention time of small molecules has increased their identification rate thus leading to improved biological interpretation of metabolomics data. By in large metabolic profiling could be developed in our animal model to elucidate patterns and connections between diet-related changes in microbiota composition and intestinal function. It is conceivable that the molecular signatures from collective biomarkers could be associated with specific microbial communities to deduce the roles adopted to modify intestinal functions.

The gut microflora is considered one of the key factors influencing the host response and pathogen antagonization. More work should be carried out to understand which components of gut microbiota may influence local immune development, and the redundancies involved in these processes. Here, we have shown high numbers of goblet cells were detected in GOS-fed birds. Mucus and mucins of goblet cells are known to provide one of the first lines of defence for the intestinal mucosa. Since gut microflora changes can affect mucin production, further animal studies should

investigate potential GOS-led modulation in mucin composition in the presence or absence of pathogen colonisation such as *Campylobacter* and *Salmonella*.

To advance our knowledge of the chicken mucosal innate immune response, it would be relevant to assess whether the Th17 responses driven by GOS feed additive and *Campylobacter* challenge were associated with modulation patterns of T- and/or B- intraepithelial lymphocyte populations. The density of ileal and cecal CD4+, CD8+, CD3+ CD8+ intestinal NK cells, and B cells could be assessed from tissue immunohistochemical staining using anti-chicken monoclonal antibodies anti-CD4, anti-CD8, mAB 28-4, and anti-Bu1 respectively (Han et al. 2017; Walliser and Göbel 2018). Further flow-cytometry cell sorting would enable establishment of an *in vitro* model to characterise the immuno-modulatory potency of specific members of the microbiota such as *Lactobacilli* DC21.1 and DC22.2 in presence or absence of selected oligosaccharides fractions.

Moreover, additional studies are required to elucidate the dichotomy in IL-17A and IL-17F expression as revealed by the provision of dietary GOS or the challenge with *Campylobacter,* and whether IL-17A and IL-17F were produced by similar or distinct intestinal cell subsets. Better understanding of the IL-17 family of pro-inflammatory cytokines may lead to new approaches in dealing with protection against bacterial pathogen colonisation, and more widely leaky gut and inflammation disorders. We have proposed that probiotic strains of *L. johnsonii* have the ability to adhere to gut epithelial cells and shape the innate immune responses to induce the expression of IL-17A. *Ex vivo* exposure of chicken ileal and cecal biopsies to *L. johnsonii* with or without GOS could address the role of tight adherence of the bacterium in the modulation of gene expression and the influence of prebiotic oligosaccharides on these processes revealed by RNA sequencing. This strategy would open prospects for identifying key genes involved in the control of digestive and immune functions in chickens.

Data generated by new spatial transcriptomics technology would allow cell mapping and constitute a comprehensive spatial functional genomic resource from tissue sections that would be accessible for future studies of biological functions of the chicken gut.

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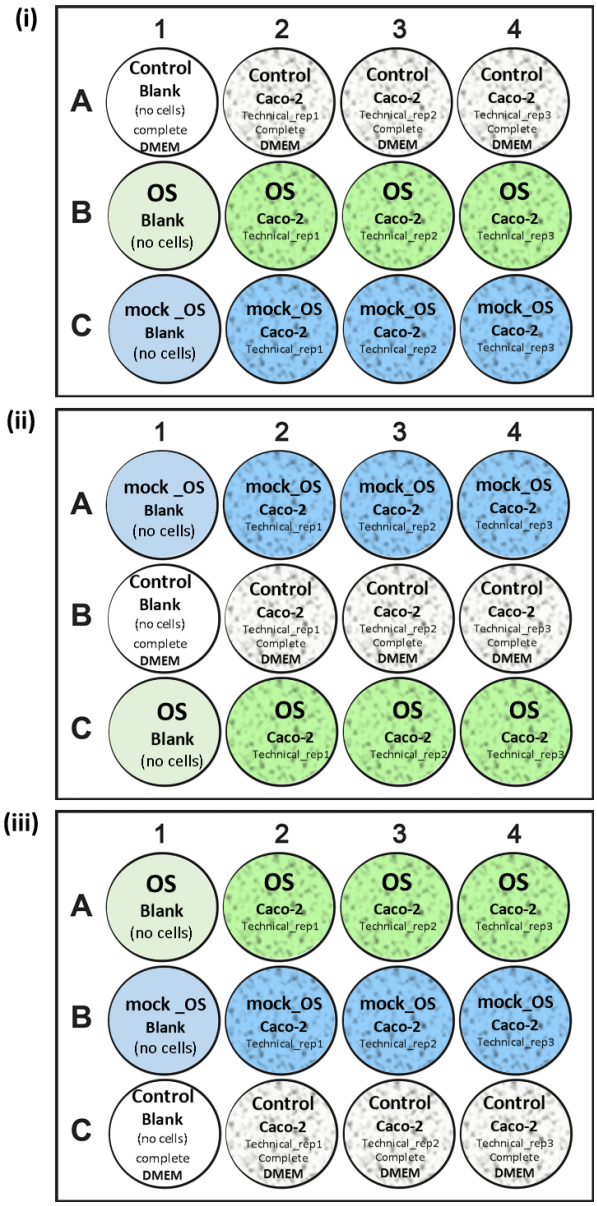
APPENDICES

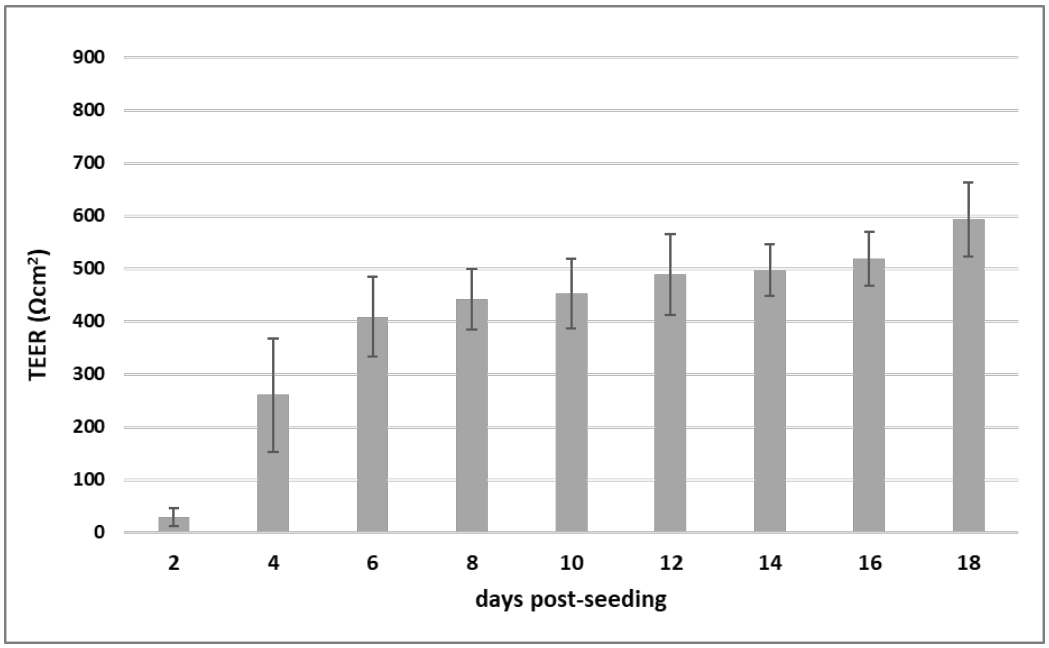
Appendix 1. Caco-2 cells experimental plate layout.

A total of three plate layouts were designed, (i), (ii) and (iii). Column 1 was maintained without cells (blank) for TEER measurements; columns 2, 3 and 4 were seeded with cells at 10^5 cells.cm^{-2} for technical replicates. Lanes A, B and C were dispensed with the following treatments: control, complete DMEM (white); mock media (blue); oligosaccharides GOS or FOS media, OS (green).



Appendix 2. TEER monitoring of Caco-2 cell monolayers.

Cells were seeded at 10^5 cells.cm^{-2} in 12 wells Transwell® plates and maintained in complete DMEM until treatment at day 18.



Appendix 3. Exemplary primers set validation.

RT-qPCR of GOS, FOS and respective mocks samples. Top panel, reference gene PUM1; bottom panel, gene of interest GALNT16; (A): electrophoresis gel snapshot; (B): amplicon size measured; (C): RT-qPCR melting curves. Electrophoresis showing samples pool, melting curve viewing individual samples.