GUT HEALTH: THE NEW PARADIGM IN FOOD ANIMAL PRODUCTION

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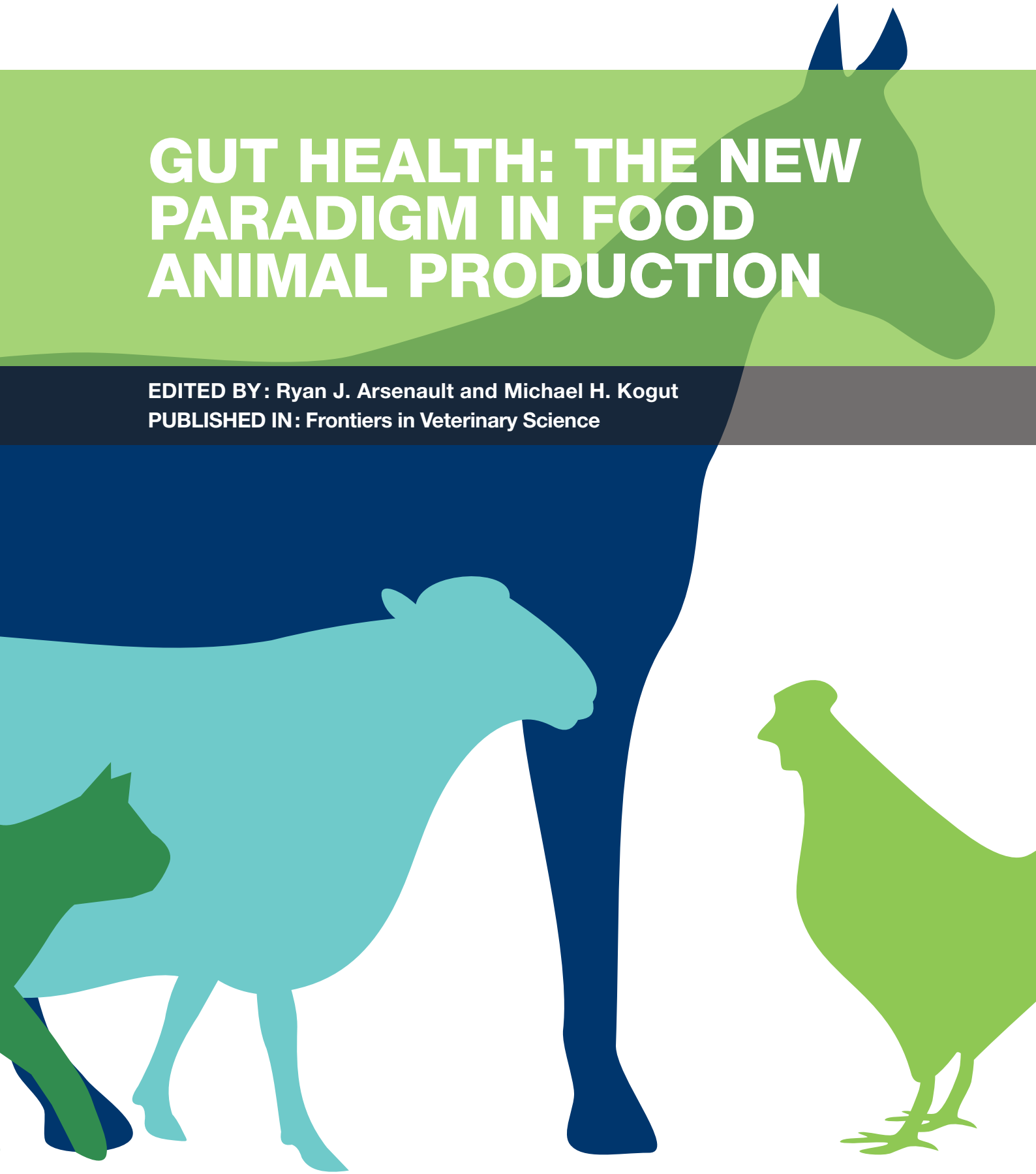

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GUT HEALTH: THE NEW PARADIGM IN FOOD ANIMAL PRODUCTION

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Gut health and specifically the gut microbiome-host interaction is currently a major research topic across the life sciences. In the case of animal sciences research into animal production and health, the gut has been a continuous area of interest. Production parameters such as growth and feed efficiency are entirely dependent on optimum gut health. In addition, the gut is a major immune organ and one of the first lines of defense in animal disease. Recent changes in animal production management and feed regulations, both regulatory and consumer driven, have placed added emphasis on finding ways to optimize gut health in novel and effective ways.

In this volume we bring together original research and review articles covering three major categories of gut health and animal production: the gut microbiome, mucosal immunology, and feed-based interventions. Included within these categories is a broad range of scientific expertise and experimental approaches that span food animal production. Our goal in bringing together the articles on this research topic is to survey the current knowledge on gut health in animal production. The following 15 articles include knowledge and perspectives from researchers from multiple countries and research perspectives, all with the central goal of improving animal health and production.

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EDITORIAL
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Editorial: Gut Health: The New Paradigm in Food Animal Production

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The Editorial on the Research Topic

Gut Health: The New Paradigm in Animal Production

Optimal gut health is of vital importance to the performance of production animals. Gut health is synonymous in animal production industries with animal health. Although there does appear to be a direct relationship between animal performance and a "healthy" gastrointestinal tract (GIT), there is no clear definition for "gut health" that encompasses a number of physiological and functional features, including nutrient digestion and absorption, host metabolism and energy generation, a stable microbiome, mucus layer development, barrier function, and mucosal immune responses (1–8). The GIT is responsible for regulating physiological homeostasis that provides the host the ability to withstand infectious and non-infectious stressors (9–19). Understanding the interactions between these diverse physiological features emphasizes the extent of areas encompassed by gut health and the ability to regulate animal production. For our part, we will define gut health as the absence/prevention/avoidance of disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressors. Furthermore, worldwide public concerns about the production animal industries' dependency on the use of growth-promoting antibiotics (AGPs) have resulted in the ban of AGPs by the European Union and a reassessment of their use in the United States. Thus, current research is focused on alternatives to antibiotics for sustainable food animal production (20).

A recent Research Topic in Frontiers in Veterinary Infectious Diseases was on gut health and wondering whether we should consider gut health as the new standard when considering animal production. The objective of this Editorial is not to review the literature on gut health in production animals, but, rather, it is our attempt to summarize findings of the 15 papers that were published within this Research Topic. Obviously, the Topic was not comprehensive in the production animal commodity reported, but it was a very good overview of the current status of the ongoing work in gut health and physiology within the veterinary community.

GUT MICROBIOME

The complex gut microbiome is not a silent organ or a collection of passenger microorganisms; but rather, the intestinal microbial community represents active participants in vertebrate immunity and physiology. The gut microbiota confers health benefits to the host, including aiding in the digestion and absorption of nutrients, contributing to the construction of the intestinal epithelial barrier, the development and function of the host immune system, and competing with pathogenic microbes to prevent their harmful propagation (18, 21). Unlike the host genome, which is rarely manipulated by xenobiotic intervention, the microbiome is readily changeable by diet, ingestion of antibiotics, infection by pathogens, and other life events [Danzeisen et al.; Ballou et al.; Mon et al.; Malmuthauge et al.; (8)].

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Antibiotics have a great effect on the host normal microbiota upsetting the balance and inducing a dysbiotic state (8). The use of sub-therapeutic doses of antibiotics in animal diets have been a common practice for promoting growth due to their ability to increase feed efficiency or preventing diseases. Danzeisen et al. used a sub-therapeutic concentration of penicillin to define beneficial members of the microbiome in turkeys that resulted in increased feed efficiency and enhanced growth. By identifying the specific bacterial populations responsible for improved performance, the authors hypothesize that these bacteria can then be used as probiotics.

The microbiome has a direct effect on the development and function of the mucosal immune system. Malmuthauge et al. found significant associations between the microbiome and the expression of genes regulating the mucosal barrier and innate immunity in neonatal cattle. Regional differences in the microbiome were associated with regional differences in innate immune gene transcription. Similar findings were described between the microbiome of broiler chickens and the expression of avian cytokine RNA transcripts (Oakley and Kogut). A negative correlation between pro-inflammatory cytokine genes and the phylum Firmicutes was found; whereas a positive correlation was identified with the pro-inflammatory cytokines and the phylum Proteobacteria.

Wigley and Ballou et al. asked the questions: what constitutes a normal or healthy microbiome and what effects do treatments that are being used to improve gut health (vaccines and probiotics) have on the development of the gut microbiome? Wigley pointed out that certain bacteria, such as *Escherichia coli, Clostridium perfringens*, and *Campylobacter*, are often considered commensals and part of the cecal microbiome. The removal of AGPs, manipulation of the cecal microbiome, changing husbandry practices, and other internal and external factors lead to changes in the host responses that result in "new" infections (22–25). Using a live attenuated *Salmonella* vaccine or a lactic acid bacteria probiotic, Ballou et al. characterized the effects of gut health treatments have on the microbiome. Alterations in microbial diversity in the microbiome of young chicks given the vaccine and, to a less extent with the probiotic, were found, which were independent of bacterial colonization by the treatments. The microbiome alterations were maintained through 28 days of age, suggesting that early exposure to certain bacteria may permanently influence the microbial diversity in the microbiomes. Similar results were described by Mon et al. where a *Salmonella* infection in day-old chicks induced a profound decrease in microbial diversity in the cecum. Specifically, there was an increase in *Enterobacteriaceae* and a decrease in butyrate-producing bacteria in the *Lachnospiraceae* family implying that exposure to a *Salmonella* infection early after hatch can impact the composition of the developing microbiome that affects colonization resistance to microbial pathogens.

Yeast-derived dietary supplements are increasingly being used as pre- and probiotics to improve gut health (26). Roto et al. detailed the effects of yeast-derived compounds in livestock diets and their effect of the microbiome. The use of yeast-derived compounds as supplements in livestock diets improved performance, increased beneficial bacteria in the microbiome, and increased immune responsiveness. Additionally, the yeast-derived products are cost-effective, do not induce antimicrobial resistance in pathogens, and, because of their multiple mechanisms of action, can be used in the variety of environments found in livestock industries.

MUCOSAL IMMUNE RESPONSE

The intestinal tract is an active immunological organ with more resident immune cells than anywhere else in the body. They are organized in lymphoid structures called Peyer's patches and isolated lymphoid follicles, such as the cecal tonsils. Macrophages, dendritic cells, various subsets of T cells, B cells, and secretory IgA all contribute to the generation of a proper immune response to invading pathogens, while keeping the resident microbial community in check without generating an overt inflammatory response.

In addition to the immune cells, the intestinal epithelial cells contribute to mucosal immunity (21). A single layer of epithelial cells separates the densely colonized and environmentally exposed intestinal lumen from the sterile subepithelial tissue, maintains homeostasis in the presence of the enteric microbiota, and contributes to rapid and efficient antimicrobial host defense in the event of infection with pathogens. Both epithelial antimicrobial host defense and homeostasis rely on signaling pathways induced by innate immune receptors demonstrating the active role of epithelial cells in the host–microbial interplay. Lastly, a layer of mucus overlying the intestinal epithelium forms a physical barrier between the mucosa and the resident microbiota, minimizing both microbial translocation and excessive immune activation by the resident microbes.

Intestinal integrity is fundamentally important for the growth and performance of food animals. One of the main advantages of AGPs in animal feed was the reduction in the low-grade, food-induced chronic inflammation that would otherwise be detrimental to animal growth (27). Removal of AGPs from animal feeds results in an increase in enteric disorders, infections, and diseases (24, 25, 28, 29). One of the issues with determining dysfunction of the gut barrier is the lack of specific biomarkers. Two papers in the Research Topic described new methods that: (a) identify serum and tissue biomarkers of gut barrier function (Chen et al.) and (b) identify a non-invasive means to measure gut inflammation as a marker of gut leakage (Kuttappan et al.). Additionally, Ayoola et al. found that the addition of supplemental enzymes (β-mannanase, a blend of xylanase, amylase, protease) to the diet of turkeys reduced food-induced inflammation.

One of the main immune functions of the epithelial cell surface is the production of antimicrobial peptides or host defense peptides [HDPs; Ref. (30)]. HDPs are a diverse group of small molecules that possess antimicrobial, immunomodulatory, and barrier function enhancing activities. Robinson et al. described several classes of small-molecule compounds that induce specific induction of endogenous HDP. Furthermore, supplementation of these HDP-inducing compounds enhanced bacterial clearance, improved enteric barrier integrity, and improved animal production efficiency with minimal intestinal inflammation.

The host/pathogen interactome leads to a number of immune and biochemical changes at the infection site as the

pathogen tries to derive nutrients from the host, while the host uses immunometabolic countermeasures against the pathogen. Arsenault and Kogut developed a novel tool that characterizes the immunometabolic phenotype of infected cells/tissues. The kinome peptide array identifies alterations in phosphorylation events in both immunity and metabolism simultaneously. The kinome array was used to identify the immune changes occurring in the cecum of chickens during the establishment of a persistent, asymptomatic *Salmonella* infection (Kogut and Arsenault). A number of immune signaling pathways were activated at the site of infection that indicates the development of a tolerogenic response allowing the bacteria to establish a persistent infection.

DIRECT FED MICROBIALS

The increased use of grains as alternative energy sources in poultry diets has led to an issue with higher levels of less digestible carbohydrates that result in an increase in digesta viscosity and food-induced inflammation. One alternative to optimize digestibility of these complex carbohydrates is the inclusion of dietary enzyme supplements. Latorre et al. took this concept a step further and described the selection of a *Bacillus* spp. direct fed microbial (DFM) candidate based on their capacity to produce enzymes that breakdown these complex carbohydrates. *Bacillus* spp. that produced cellulose and xylanase were used as DFM and were found to reduce digesta viscosity and reduce *C. perfringens* growth in a number of different diets containing different complex carbohydrates.

A group of natural products known as phytobiotics have been the focus of several studies in recent years as antibiotic alternatives (31). Phytobiotics are plant-derived products used in feed that possess antimicrobial activity, provide antioxidative effects, enhance palatability, improve gut functions, and promote growth. Murugesan et al. compared the effects of a commercial phytogenic feed additive on growth, intestinal morphology, and microbial composition in chickens to the effects of an AGP. Improved growth, increased intestinal villus height, and decreased total cecal numbers of *Clostridium* and anaerobic bacteria were comparable between the two treatments. However, birds fed the phytobiotic additives had a significant reduction in coliforms and an increase in *Lactobacillus* spp. implying an environment that was more suitable for the establishment of growth-promoting bacteria in the microbiome.

Although the GIT is frequently described simply as "the gut," it is actually made up of (1) an epithelium; (2) a diverse and robust immune arm, which contains most of the immune cells in the body; and (3) the commensal bacteria, which contain more cells than are present in the entire host organism. Understanding of the crosstalk between ALL of these interrelated components of the gut is what cumulatively makes the gut the basis for the health of animals and the motor that drives their performance.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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OPINION
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Blurred lines: pathogens, commensals, and the healthy gut

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The Chicken Microbiome and Health

Detailed studies of the chicken microbiome have emerged in recent years, largely due to the impact of next-generation sequencing (NGS). We increasingly understand how the microbiome is important in health, in development of the gut and the immune system, and in maintenance of homeostasis. Manipulation of the microbiota directly through probiotics or antimicrobials or indirectly through feed and feed additives has long been used by the poultry industry to increase growth rates and feed conversion, to improve gut health, and to reduce the burden of pathogens and, in particular, to reduce the load of foodborne zoonotic pathogens such as *Salmonella* and *Campylobacter*. We can now begin to mechanistically determine how these treatments affect the microbiota and the wider host, and this understanding will allow us to use more targeted approaches in the future. In terms of food security, increasing yield is clearly a good thing. However, it is far from clear what represents a "healthy" microbiome, and the lines between what is a "harmless" commensal and what is a pathogen are often blurred. As such an understanding of the microbial ecology of the gut and how this is affected by manipulation of the microbiome or indeed treatment of "pathogens" is essential in ensuring that treatments intended to improve health and productivity do not in fact cause more problems.

Is it a Pathogen or a Commensal?

The chicken microbiome consists of around 1,000 bacterial species, though the composition varies over time, between breeds and lines of birds, between flocks, individuals, and at different sites within the gut (1–5). Proteobacteria make up a relatively small amount of species in the microbiome, but among these species are a number that may cause disease in the chicken, notably *Escherichia coli* and *Clostridium perfringens*, and as such are often considered pathogens (4–6). In contrast, the foodborne zoonotic pathogen *Campylobacter jejuni* is also found frequently as a component of the cecal microbiome but is often considered to be a "harmless commensal." However, in reality, these species can have the properties of either pathogen or commensal depending on the bacterial pathotype, host immune status, diet, and coinfection.

Of these three exemplars, *E. coli* has perhaps the least direct impact on gut health. However, extraintestinal infections are a considerable health problem in both broiler and layer chicken production. Isolates associated with disease are termed avian pathogenic *E. coli* or APEC. Much effort has been directed at understanding the virulence factors and pathogenesis of APEC, and there are clearly a number of pathotypes that can cause disease (7, 8). However, wider analysis of isolates associated with systemic infection or colibacillosis of broiler chickens and those associated with a healthy gut show that disease may be caused by isolates that harbor few, if any, APEC-associated virulence factors while apparently "commensal" isolates carry numerous virulence factors (9). The implication is that in many clinical cases of colibacillosis, commensal bacteria act as an opportunistic pathogen due to host factors, environmental stress, poor management, or as a secondary infection.

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As such infections are rarely investigated in detail such as genotyping of isolates; the more generic term of APEC has become associated with all *E. coli* isolated from diseased chickens rather than those *E. coli* isolates that are primary pathogens *per se*.

Campylobacter jejuni is the most common cause of foodborne human gastrointestinal infection worldwide. Chicken is the main reservoir of infection with around 70% of UK retail chicken contaminated in recent surveys (10, 11). *C. jejuni* colonizes the lower gastrointestinal tract of the chicken to a high level and has been considered to be a commensal due to the absence of clinical disease in experimental infection studies (12). However, in recent years, we have begun to reassess this paradigm. Experimental infection of broiler breeds with *C. jejuni* leads to an inflammatory response and changes to gut structure (13–16). Generally, it would appear that this inflammation is regulated by IL-10-producing cells, but in some broiler breeds, regulation appears to be dysfunctional and infection may lead to prolonged inflammation, damage, and diarrhea. Does this mean that *C. jejuni* is truly a pathogen of the chicken or more a reflection of dysregulation of mucosal immune regulation? Indeed, poor gut health is often considered as a problem for broiler chickens. Wet litter, due to loose feces mixed with the bedding substrate, and dysbacteriosis are frequent problems in broiler production that affect productivity and animal welfare both directly and through resulting problems such as pododermatitis and hock burn (17–19). Modern broiler chickens have been successfully bred to efficiently convert grain into protein and grow rapidly, reaching slaughter weight at 6–7 weeks of age and we increasingly understand the genetic basis for this (20, 21). This, however, may have consequences; well-documented musculoskeletal problems are being addressed, but problems with gut health may be less obvious and harder to deal with. One may pose the question to what extent are these problems related to the composition of the microbiota and development of a healthy gut or more a consequence of a defect in their gut physiology or immune function? Additionally, to what extent could inappropriate or poorly regulated responses to the "normal" microbiota be contributing to poor gut health? The example of *C. jejuni* illustrates how the balance in maintaining a healthy gut is likely to be influenced by a large number of both host and microbial factors.

Clostridia are a major component of the proteobacteria in the chicken microbiome (5). Of these species, *C. perfringens* is the most important in poultry health. Variants of *C. perfringens* are associated with the gut of many species, and it can be generally considered as a commensal. Yet, it may produce toxins associated with disease including human food poisoning or in necrotic infections of the gut or deep tissue. In the chicken, the *C. perfringens* toxin group A has become most associated with necrotic enteritis (NE), these isolates producing alpha and particularly netB toxins (22). Despite *C. perfringens* producing these toxins being closely associated with NE, it had proved very difficult to fulfill Koch's postulates as such isolates are frequently found in healthy birds and reliable experimental infection models for NE based on *C. perfringens* infection alone have proved hard to develop. This is largely due to most clinical disease being multifactorial involving predisposing factors such as coinfection particularly with species of the apicomplexan protozoan parasite *Eimeria* or due to dietary factors such as diets high in non-starch polysaccharides (NSPs; wheat, rye, and barley) or animal proteins that provide favorable conditions for the growth of *C. perfringens* A and stress on birds in production (23). Again it is difficult to define *C. perfringens* as a true gut pathogen, but more of an opportunist that frequently makes up part of the microbiome.

Manipulation of the Microbiome: Past and Future Implications for Gut Health

Historically, currently and likely into the future, the chicken microbiome has been manipulated perhaps more than any other vertebrate species through the use of growth-promoting antimicrobials, prebiotic and probiotic treatments, and dietary additives (24–27). Feed additives such as enzymes have been used to increase productivity. For example, the use of phytases to allow the breakdown of plant phytates to be utilized in diet (28). Other additives such as plantain NSPs have been proposed to reduce the burden of infections such as *Salmonella* (29). The use of growth-promoting antimicrobials has been banned in the European Union since 2006 and their use in the USA is under increasing pressure due to their role in development of antimicrobial resistance. Not unexpectedly, the use of antimicrobial growth promoters affects the composition of the microbiota (30, 31), and equally the withdrawal of both growth-promoting and anticoccidial drugs will lead to change in the microbiota composition in commercial flocks. Interestingly, a recent study on drug-free broiler production systems in Canada showed an increase in *C. perfringens* (32). Anecdotally, the increased prevalence of both NE and colibacillosis in Europe has been blamed, at least in part, on the withdrawal of growth promoters. While the overriding problems associated with the emergence of antimicrobial resistance rightly mean that growth-promoting antibiotics have been or are being withdrawn, it clearly illustrates how the manipulation of microbiota can have positive effects on health of the chicken. Equally, we need to be aware that changing the microbiota or modulating host responses that are affected by or effect changes upon the microbiome may have undesirable effects. In the case of growth promoters, this was their role in the development of resistant bacteria and potential drug residues in the food chain. As such a better understanding of microbial ecology and how interventions impact on the microbiota and the host is needed before we adopt such changes wholesale. Manipulation of the microbiome may be used to improve productivity, although the consequences of removing "detrimental" or enriching "beneficial" taxa are likely to go beyond improving feed conversion. A perturbed microbiome may reveal commensals as having pathogenic potential and lead to problems in development of the gut and immune system and poor gut homeostasis. Manipulation of the gut, the microbiome, and the immune response has all been proposed in reducing the burden of carriage of foodborne bacterial pathogens. Our work on feed supplementation with plantain NSP showed successful inhibition of *Salmonella* invasion (29), but rather unexpectedly lead to increased colonization of the intestinal tract with *Salmonella gallinarum*. *S. gallinarum* has evolved with several defective metabolic pathways that make it a poor colonizer of the chicken gut, but supplementation with plantain NSP

increased colonization either through a direct nutritional source or more likely utilization of breakdown products of microbiota components (33). Equally immunological manipulation may have unexpected consequences. Both colonization of *Salmonella* and *Campylobacter* are accompanied by regulation of innate responses to these bacteria in the gut (13, 34, 35). It has been proposed that depletion of the regulatory $CD4^+$ $CD25^+$ T-cell population will enhance clearance and thereby reduce the public health risk due to these pathogens (36, 37). A downside of this may be increased inflammation and more significantly loss of regulation to components of the microbiome, again blurring lines between pathogen and commensal and leading to poor gut and poor health.

Conclusion

Ultimately, the "take-home" message in this article is that the power of NGS and metagenomic approaches allow us to understand the composition of microbiomes in multiple individuals of a livestock species quickly and relatively easily. We can associate individual taxa and species with good or poor outcomes in productivity or health. Yet, this power needs to be tempered with (often substantial) gaps in our understanding of microbial ecology within the gut. Can changing the microbiota lead to perturbation of gut regulation? As we have seen, there are blurred lines between pathogens and commensals, and so can changes to remove apparent pathogens have negative consequences on other aspects of gut health or could the promotion of "good bacteria" lead to emergence of "new pathogens." The historical example of growth-promoting antimicrobials illustrates the point. Their use was successful in increasing productivity yet almost certainly has contributed to antimicrobial resistance (38). Their subsequent withdrawal is now resulting in problems in our faster growing modern broiler chickens. Our understanding of the microbiome and its manipulation offers a wealth of opportunities, though may not be without risk.

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REVIEW
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The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract

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A diverse microbial population colonizes the sterile mammalian gastrointestinal tract during and after the birth. There is increasing evidence that this complex microbiome plays a crucial role in the development of the mucosal immune system and influences newborn health. Microbial colonization is a complex process influenced by a two-way interaction between host and microbes and a variety of external factors, including maternal microbiota, birth process, diet, and antibiotics. Following this initial colonization, continuous exposure to host-specific microbes is not only essential for development and maturation of the mucosal immune system but also the nutrition and health of the animal. Thus, it is important to understand host–microbiome interactions within the context of individual animal species and specific management practices. Data is now being generated revealing significant associations between the early microbiome, development of the mucosal immune system, and the growth and health of newborn calves. The current review focuses on recent information and discusses the limitation of current data and the potential challenges to better characterizing key host-specific microbial interactions. We also discuss potential strategies that may be used to manipulate the early microbiome to improve production and health during the time when newborn calves are most susceptible to enteric disease.

Keywords: gut microbiota, neonatal ruminants, gut development, mucosal immune system, enteric infections

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Introduction

The *in utero* sterile mammalian gastrointestinal tract (GIT) is rapidly colonized by an array of microbiota during and after birth. This process of colonization has been described as a co-evolution due to the two-way interaction between host and microbes (1). Host (luminal pH, food retention time in the gut, and immune defense mechanisms), microbial factors (adhesion, survival mechanisms under oxygen gradient, and mechanisms to obtain nutrients from the host), and external factors, such as maternal microbiota, delivery mode, diet, and antibiotic treatment during early life, all combine to influence gut colonization (2–4). The initial colonizers (*Streptococcus* and *Enterococcus*) utilize available oxygen in the gut and create the anaerobic environment required for strict anaerobic gut residents, such as *Bifidobacterium* and *Bacteroides* (2, 5, 6). *Bifidobacterium* and *Bacteroides* are two of the main gut bacteria present in the majority of human infants (3) that have a beneficial impact on mucosal immune system. The presence of *Bacteroides* in the gut plays a vital role in the development of immunological tolerance to commensal microbiota (7), while the composition of

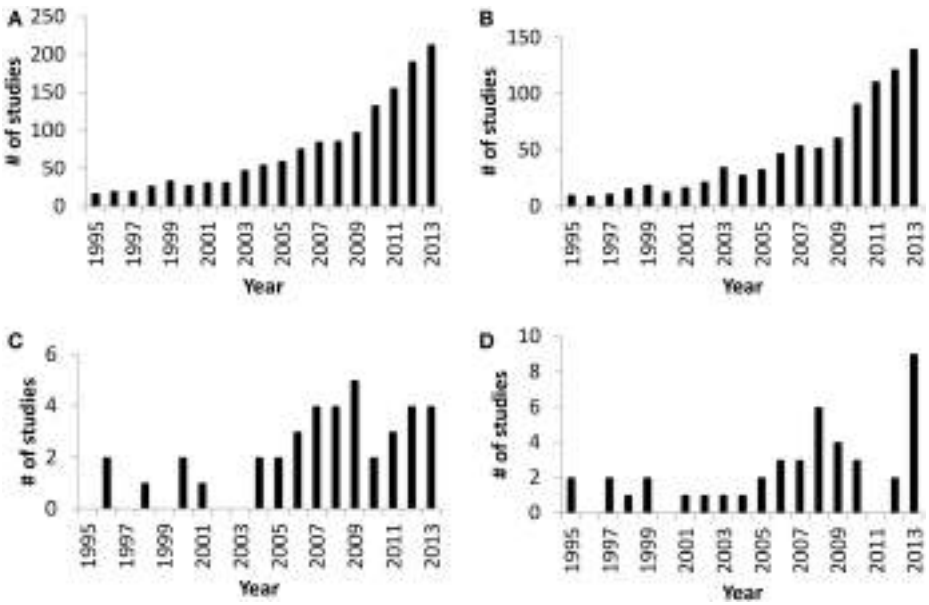

FIGURE 1 | Number of publication entries in Medline (PubMed) trend* from 1995 to 2013. **(A)** Publication entries searched with query "gut colonization." **(B)** Publication entries searched with query "gut colonization and human." **(C)** Publication entries searched with query "gut colonization and ruminant." **(D)** Publication entries searched with query "rumen colonization." *Medline Trend, URL: http://dan.corlan.net/medline-trend.html.

Bifidobacterium in the infant gut is linked to a reduced incidence of allergy (8). Therefore, neonatal gut colonization is a crucial period for the developing gut and naïve immune system (9, 10) and may have long-term health effects (5). Although research focused on understanding gut colonization of mammals has increased dramatically over the last decade (**Figures 1A,B**), there are still very few studies focused on domestic livestock species, especially ruminants (**Figures 1C,D**). Information is extremely limited on ruminant gut colonization, especially when focusing on the role of the microbiota in the early development of the GIT during the pre-ruminant period. Therefore, the present review builds on the information available for early colonization of the ruminant GIT to identify challenges in understanding the complex interaction between host and microbiome. We also use this information to speculate on possible strategies to engineer the microbiome and improve ruminant health and production.

Gut Microbiota in Ruminants

Gut microbes of ruminants, mainly the rumen microbiota, provide 70% of their daily energy requirement via the fermentation of undigestible dietary substrates (11). Therefore, studies in ruminant gut microbiota have focused mainly on the rumen to understand how this microbiome impacts meat and milk production. Rumen microbiota consists of bacteria, archaea, protozoa, and fungi involved in the fermentation of complex carbohydrates, and their composition is influenced by a number of factors. For example, distinct microbial populations have been identified for the particle-attached, fluid-associated, and tissue-attached fractions of the rumen (12). Rumen microbial composition can also vary significantly depending on the ruminant species, diet, host age, season, and geographic region (13). Bacteria dominate the rumen microbiome and contribute mainly to the production of volatile fatty acids (VFAs) and microbial protein (14). Despite numerous human and mouse studies reporting the importance of early gut microbiota on host health, there are few attempts to understand the role of early gut/rumen colonization on GIT development or host health in neonatal ruminants. Furthermore, rumen/gut development and establishment of the microbiota have always been studied as separate aspects of ruminant biology and there have been few attempts to understand possible interactions between these two events.

Rumen Colonization in Pre-Ruminants

Colonization of pre-ruminant rumen was first studied using light microscopy and Gram-staining to visualize bacteria in the late 1940s (15, 16). In the 1980s, Gerard Fonty started to investigate the establishment of the rumen microbial community in lambs by using culture-dependent approaches and was the first to report age-dependent changes in the appearance of different microbial populations (17). Anaerobic bacteria dominate in the rumen of neonatal ruminants by the second day of life (10^9 CFU/ml of rumen fluid) and the density of cellulolytic bacteria stabilized (10^7 CFU/ml of rumen fluid) within the first week of life (17). This study revealed that the dominant bacterial species in the neonatal lamb rumen was different from those species colonizing the adult rumen. When the establishment of other microbial groups was investigated, their appearance was delayed until after bacteria were established (17). Anaerobic fungi and methanogens appear in the neonatal rumen between 8 and 10 days postpartum (17), while protozoa appear only after 15 days postpartum (18). Furthermore, comparison of conventionalized lambs with conventionally reared lambs suggested that the establishment of protozoa required a well-established bacterial population (18).

The early rumen microbiota consist of bacterial species from *Propionibacterium*, *Clostridium*, *Peptostreptococcus* and *Bifidobacterium* genera, while *Ruminococcus* species dominated the cellulolytic bacterial population (17). Restricted exposure of lambs to their dams or other animals also delayed the establishment of cellulolytic bacteria, when compared to lambs reared in close contact with their dams during the first few weeks of life (19). This observation revealed the important role of early environmental exposure for the establishment of a host-specific microbiota. Fonty and colleagues have also extended their studies to explore the establishment of tissue-attached (epimural) bacteria in the ovine rumen (20). Similar to the fluid-associated community, the complexity of the epimural community and homogeneity among individuals increased with increasing age (20). A recent study revealed, however, that the rumen epimural bacterial community in pre-weaned calves differs significantly from the content-associated community (21). This observation suggests that host–microbial interactions might play an important role in defining these two distinct microbial communities.

Rumen microbiota has a significant impact on pre-ruminant management, especially the weaning process, which depends on rumen development and the ability of the microbiome to ferment complex carbohydrates (22). The presence of VFAs (acetate, propionate, and butyrate) in the rumen plays an important role in rumen development, especially the development of rumen papillae (23). The fermentation of undigestible dietary substrates by rumen microbiota is the major source of VFAs in ruminants (11, 14), and it is generally believed that feeding a solid diet accelerates this process in pre-ruminants (22). Although the establishment of rumen microbiota has long been studied and their importance in the rumen development has been suggested, the mechanisms by which bacteria influence rumen development remain poorly defined. Moreover, culture-based studies can only identify around 10% of the total rumen microbiota, leaving the majority of the microbiome undefined (24).

Recently, enhanced molecular-based technologies, such as next generation sequencing (NGS), provide an excellent platform to identify both culturable and non-culturable microbes as well as characterizing their potential functions (25). It is now possible to generate a comprehensive profile of both microbial diversity and functions and explore potential associations between the microbiome and early rumen development. Using NGS, a comparison of the rumen bacteriome and metagenome in 2-week-old and 6-week-old calves, fed a milk replacer diet, revealed a taxonomically and functionally diverse rumen microbiome in pre-ruminant calves with significant age-dependent changes (26). This study revealed that *Bacteroidetes*, followed by *Firmicutes* and *Proteobacteria*, colonized in the rumen content of pre-weaned calves, which displayed age-dependent variations in their relative abundance. For example, the abundance of *Bacteroidetes* increased from 45.7% at 2 weeks to 74.8% at 6 weeks of age, despite calves receiving the same diet over time. Such age-related differences were more prominent at the bacterial genera level, where the predominant *Prevotella* (33.1%) at 2 weeks was replaced by *Bacteroides* (71.4%) at 6 weeks.

Since the study by Li and colleagues (26), there have been further studies analyzing changes in the composition of the rumen microbial community from birth to weaning. Rumen fluid or content was used as a proxy for the rumen microbiome and 16S rRNA amplicon-based sequencing approaches were used to identify and quantify bacteria (21, 27–29). These studies revealed marked heterogeneity in the rumen bacterial composition of individual animals immediately postpartum, but greater similarity in bacterial composition was observed with increasing age (26–29). There were, however, a number of discrepancies in terms of rumen bacterial composition when comparing among studies. For example, Jami and colleagues (27) reported a higher abundance of *Streptococcus* belonging to the phylum *Firmicutes* in 1–3-day-old calves. In contrast, Rey and colleagues (28) reported a higher abundance of *Proteobacteria* in 2-day-old calves. Furthermore, both Jami et al. (27) and Rey et al. (28) reported a higher abundance of *Bacteroides* in rumen fluid at 2 weeks of life, while Li and colleagues (26) observed a greater abundance of *Prevotella* in rumen content. Targeting different variable regions of 16S rRNA gene (V1/V2 versus V3/V4) for amplicon-based sequencing and differences in the environment, in which these calves were raised, may have influenced the apparent bacterial composition of rumen fluid.

A study comparing content-associated versus epimural bacterial populations in 3-week-old calves revealed that bacterial phylotypes belonging to *Bacteroidetes* (43.8%) and β-*Proteobacteria* (25.1%) dominated the epimural community. In contrast, phylotypes from *Bacteroidetes* (54.8%) and *Firmicutes* (29.6%) dominated the rumen content-associated community (21). Using 16S rRNA amplicon-based sequencing, temporal changes in the epimural bacterial community have also been reported in goat kids during the first 10 weeks of life (30). The predominant *Proteobacteria* (>85%) during the first week of life were gradually replaced by an increasing abundance of *Bacteroidetes* (~10%) and *Firmicutes* (>15%) (30). Similar to previous culture-based approaches, these recent studies have confirmed that dynamic changes occur in the rumen bacterial community during early life, with significant differences between the epimural and fluid-associated communities in the pre-weaned rumen.

Associated with the age-dependent changes in rumen microbial composition (**Figure 2**), there are also changes in the activity of the rumen microbiota. These functional changes occur in the absence of dietary changes during the first 6 weeks of life (26). Currently, this is the only study using a metagenomic approach to assess the metabolic potential of pre-ruminant rumen microbiome. Li and colleagues (26) revealed that ATP-binding cassette family transporters are more abundant at 2 weeks than 6 weeks of age but TonB-dependent receptors are more abundant at 6 weeks. Glycoside hydrolases (GH2, GH3, GH42, and GH92), which breakdown complex carbohydrates, were also detected in the pre-ruminant rumen, even when the diet did not contain complex carbohydrates. These observations suggest that early rumen microbiota has the capacity to ferment dietary fiber prior to being exposed to this material. Moreover, a recent study investigating the activity of the early rumen microbiome revealed that VFA production and xylanase and amylase, enzymes that breakdown complex carbohydrates, were active within 2 days postpartum (31). The observed glycoside hydrolase activity, in conjunction with VFA production, reveals establishment of a metabolically

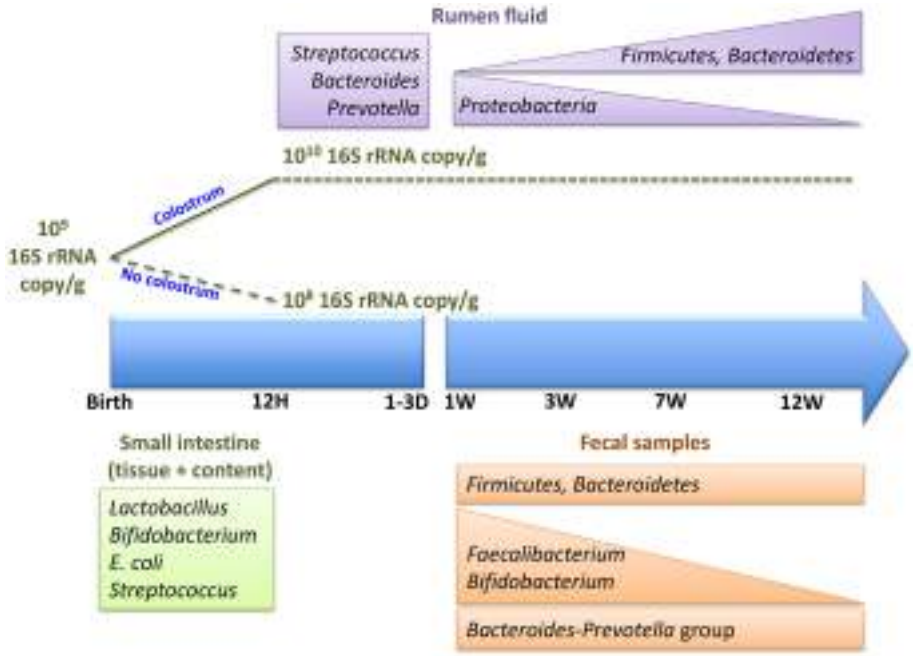

FIGURE 2 | Colonization of neonatal calf rumen/gut, immediately postpartum and within the first 12 weeks of life.

active adult-like microbiome in the neonatal rumen prior to exposure to appropriate dietary substrates. Thus, the establishment of metabolically active microbiome may occur along with the transfer of microbiome from the dam to newborn calf and the colonization of a species-specific microbiome.

Diet is one of the main factors that influences the composition of gut microbiota and may also play an important role in the observed temporal changes of the rumen microbiome in neonatal calves (27, 28). The rumen content of 3-week-old calves fed milk replacer, supplemented with a calf starter ration (20% crude protein, 3% crude fat, and 5.7% crude fiber), contained a similar abundance of *Prevotella* (15.1%) and *Bacteroides* (15.8%) (21). Calves that received milk replacer only, however, displayed a shift in the predominant rumen content-associated bacteria from *Prevotella* to *Bacteroides* (26) within the first 6 weeks of life. Thus, the observed similar abundance of these two bacterial genera in 3-week-old calves fed milk supplemented with calf starter suggests that the age-dependent shift in the dominant bacteria may have been triggered by the dietary supplement that contained fiber. In general, it is believed that the introduction of solid diet plays a key role in promoting the establishment of rumen microbiota as milk bypasses the rumen to enter the abomasum (22). Moreover, pre-weaning diet and feeding methods have been reported to have more pronounced and long-lasting impacts on rumen microbial composition (29, 32, 33). Altering feeding practices during the pre-weaning period were reported to significantly alter methanogen composition after weaning (32) as well as the density of bacteria and protozoa in pre-weaned lambs (33). Therefore, managing pre-weaning feeding may be as important as managing feeding during the weaning period in terms of microbiota establishment as well as development of the microbial fermentation capacity of the rumen.

Currently, characterization of the rumen microbiota is based primarily on the sequencing of DNA, which represents both active and dead microbiota. Therefore, the use of RNA-based metatranscriptome approaches may provide a better understanding of the biological activity of the early rumen microbiome. Understanding the activity of the rumen microbiota may help designing multidisciplinary approaches to engineer the early rumen microbiome with the objective of promoting both rumen development and function that better supports the critical transition that occurs when ruminants are weaned.

Intestinal Tract Colonization in Pre-Ruminants

Early studies on bacterial colonization of the pre-ruminant intestine focused primarily on pathogenic *Escherichia coli* in calves and described the pathogenesis of neonatal diarrhea (34–37). Microscopic imaging revealed that pathogenic *E. coli* preferably attached to and effaced the mucosal epithelium in the ileum and large intestine, but not the duodenum and jejunum of neonatal calves (36). Feeding of probiotic strains isolated from the intestine of calves reduced enteric colonization of pathogenic *E. coli* O157:H7 in pre-weaned calves (38). Furthermore, the administration of *Bifidobacterium* and *Lactobacillus* to newborn calves during the first week of life increased weight gain and the feed conversion ratio, while decreasing diarrhea incidences (39). These effects were most pronounced in pre-weaned calves than weaned calves (39), suggesting the probiotic supplements are more effective when the gut microbiota is being established and less effective when the microbiome has stabilized.

Supplementation of *Lactobacillus* in young calves was also reported to increase the total serum immunoglobulin G concentration (40), providing evidence of a host–microbiome

interaction that may influence calf health. More recently, supplementation of newborn calves with prebiotics (galactooligo saccharides) was associated with an increased abundance of *Lactobacillus* and *Bifidobacterium* in the colon of 2-week-old calves (41). However, this effect was less pronounced in 4-week-old calves (41), suggesting that as with probiotics, it may be easier to manipulate the microbiome during the early colonization period (39). In an attempt to reduce antibiotic usage during the pre-weaning period, studies continue to investigate the impact of both probiotics and prebiotics on calf growth and health (42). The full impact of these approaches on gut microbial colonization and composition throughout the pre-ruminant period has yet to be understood and studies are lacking on how altering the gut microbiome may impact mucosal immune defenses in the GIT.

In 1965, Williams Smith used culture-dependent approaches for the first time to study bacterial colonization in the pre-ruminant GIT, beginning immediately postpartum. He reported colonization by *E. coli* and *Streptococcus* in all gut regions (stomach, small intestine, and cecum) of calves within 8 h after birth, while *Lactobacillus* colonization was only observed 1 day after birth. *Lactobacillus* then predominated throughout all regions of the GIT tested within the first week (43). *Bacteroides* were observed only in the cecum and feces after the second day of life (43). The colonization of *Clostridium perfringens*, previously known as *Clostridium welchii*, was also observed in the cecum within 8 h after birth; however, it was not detected in other gut regions until 18 h after birth (43). This study suggested that the newborn GIT was first colonized by facultative anaerobes, which then created the anaerobic conditions required for colonization by obligate anaerobic gut microbiota, such as *Lactobacillus* and *Bacteroides*. A similar evolution of bacterial colonization of the GIT has been reported for other newborn mammals (6).

Subsequent studies have revealed a higher abundance of *Bifidobacterium* and *Lactobacillus* in fecal samples and throughout the GIT of newborn calves (44, 45). A higher abundance of *Bifidobacterium* in 3–7 days old calves was also associated with a lower abundance of *E. coli* (44). More recently, culture-independent approaches have been employed to better understand the diversity and abundance of bacteria throughout the neonatal ruminant GIT (46, 47). RNA-based, sequence-specific rRNA cleavage analysis of bacteria throughout the first 12 weeks postpartum revealed a higher abundance of the *Bacteroides–Prevotella* and *Clostridium coccoides–Eubacterium rectale* groups in the feces of dairy calves (46). *Faecalibacterium* was one of the most abundant bacteria in 1-week-old calves (21.7%), but then declined with increasing calf age (46). *Ruminococcus flavefaciens* and *Fibrobacter*, fibrolytic bacteria, were only observed after 5 weeks postpartum, while *Streptococcus* and *Lactococcus* could not be detected after the fifth week (46). These studies confirmed that there were significant age-dependent changes in the composition of the GIT microbiome and revealed substantial differences between the rumen and lower GIT microbiome.

Regional variations in bacterial phylotypes richness, diversity, density, and composition throughout the GIT of newborn calves have been described, using both culture-dependent and independent approaches (21, 45, 48, 49). When bacterial populations throughout the GIT of 20-week-old calves were analyzed, *Bifidobacterium* and *Lactobacillus* displayed greater survival of stomach passage than coliforms and *E. coli* (45). The density of these beneficial bacteria was high throughout all GIT regions (rumen, abomasum, duodenum, jejunum, cecum, and colon) of the 20-week-old calves (45). Using culture-independent approaches, higher bacterial phylotype richness was observed in the rumen and large intestinal regions than the small intestinal regions of lambs and calves (21, 48, 49). Collado and Sanz (48) reported, however, a similar bacterial richness throughout the GIT, when using a culture-dependent approach. This observation is consistent with there being many more unculturable bacterial species in the rumen and large intestine than the small intestine. A longer retention time, higher availability of nutrients, and reduced scrutiny by the host mucosal immune system have all been suggested to contribute to the increase in bacterial diversity and density in the rumen and large intestine of mammals (1).

When bacterial composition throughout the GIT is explored, the rumen and large intestinal regions consist primarily of *Bacteroidetes* and *Firmicutes*, while >95% of the bacteria in the small intestine contents are composed of *Firmicutes* (21). In contrast, the mucosa-associated bacterial community in the small intestine is composed of primarily *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, including 17 genera that are unique to this region of the GIT (21). The presence of bacteria unique to the small intestine (21) suggests that fecal sample-based studies do not reveal the true GIT microbiome and may not reveal important regional host–microbial interactions. A recent study in human infants reported similar observations and it was also concluded that feces was not representative of host–microbiota interactions throughout the gut (50).

There is increasing evidence that mucosa-attached microbiota are significantly different from those associated with ingesta and present in the intestinal lumen. Collado and Sanz (48) first studied mucosa-attached bacteria and reported that *Bifidobacterium* and *Lactobacillus* were predominant throughout the GIT (rumen, duodenum, and colon) of calves (9–11 months) and lambs (6–9 months). They did not, however, compare mucosa-associated versus intestinal content communities. Studies by Malmuthuge and colleagues (21, 49) compared mucosa-attached and content-associated bacterial communities throughout the GIT of calves and reported that at 3 weeks of life, distinct mucosa-attached bacterial phylotypes had been established. Furthermore, bacterial richness in mucosa-attached communities, especially in the ileum, was higher than the content-associated community (49). These distinct and richer mucosa-attached bacterial communities were subsequently confirmed by using pyrosequencing of 16S rRNA gene amplicons (21). Although the majority of mucosa-attached bacteria could not be assigned at a genus level, the use of a NGS approach provided a greater understanding of region- (rumen, small intestine, and large intestine) and sample type- (content and mucosa) specific bacteria throughout the GIT of pre-weaned calves (21).

Based on the previously cited studies, it is clear that the composition, diversity, and richness of rumen and intestinal microbiota in pre-weaned ruminants can vary depending on various factors,

TABLE 1 | Factors influencing pre-weaned calf rumen/gut microbiota.

Factor	Study
Age	(17, 26–28, 30, 46, 47, 49)
Diet (colostrum, calf starter)	(28, 29, 32, 33, 46, 51, 52)
Feeding method (suckling, bottle feeding)	(53)
Probiotic, prebiotics	(39, 41)
Exposure to dam	(19, 53)
Sample site	(21, 43, 48, 49)
Sample type (fluid, content, mucosa)	(20, 21, 49)
Host (individuality)	(27)
Infections	(47)

such as age, diet, feeding method, feed additives, sampling location (content, mucosa, and feces), and gut region (rumen, large intestine, and small intestine) (**Table 1**; **Figure 2**). Furthermore, variation in microbial composition among individual animals is higher in young than adult ruminants (27). The high variation in bacterial diversity and density (27, 49) among individual ruminants during early life also suggests that the gut microbiome may be more easily changed at this time of life than in adults. This may explain why probiotics and prebiotics have been reported to have a much greater effect in young animals than older calves (39, 41). Of particular interest are the recent studies conducted by Abecia and colleagues, which revealed long-lasting consequences when dietary interventions were used to manipulate the rumen microbiota in young calves. Thus, a much greater understanding of early gut microbial colonization and the factors influencing establishment of microbiota may provide the basis for rational strategies to manipulate the gut microbiome and improve the growth and health of ruminants throughout the entire production cycle.

Influence of Microbiome on Gut Development and Mucosal Immune Functions

Gut microbiota are essential for the development and differentiation of the intestinal mucosal epithelium as well as the mucosal immune system (54). Most of our knowledge regarding host–microbiome interactions in the GIT has been obtained from a variety of mouse models. Comparisons between gnotobiotic and conventionally reared mice revealed decreased development of the intestinal epithelium and the mucosal immune system in the absence of gut microbiota. Thickness of the mucus barrier is reduced in germfree mice, but administration of microbe-derived lipopolysaccharides and peptidoglycans to the colonic mucosal surface stimulated mucus production and within 40 min restored the thickness of the mucus layer to that of conventional mice (55). This observation supports the conclusion that the gut microbiota is essential for the secretion of intestinal mucus, an important physical barrier throughout the GIT. In addition, the generation rate of epithelial cells in germfree mice is lower than that of the conventionally raised mice (56), revealing the importance of gut microbiota for maintaining intestinal epithelial cells proliferation and ensuring recovery of the mucosal barrier following injuries.

The presence of gut microbiota in mice is also necessary for the development of secondary lymphoid structure, such as Peyer's patches (PPs), mesenteric lymph nodes, and isolated lymphoid follicles (54). The establishment of host-specific microbiota, especially bacterial species belong to phylum *Firmicutes*, is essential for the development of a variety of intestinal immune cells (57). For example, when human microbiota colonized the mouse intestine there were low numbers of $CD4^+$ and $CD8^+$ T cells, and fewer proliferating T cells and dendritic cells when compared to mice colonized with mouse microbiota (57). Interestingly, the immune cell profile of human microbiota colonized mice was similar to that of germfree mice (57), suggesting the presence of a host-specific microbiota is fundamental for mucosal immune system development. Thus, host–microbial interactions in the developing gut of newborn animals must be studied within relevant host species to accurately understand the role of early microbiota on gut development.

In ruminants, development of mucosa-associated lymphoid tissues (MALTs) in the GIT begins *in utero* and there is active proliferation of B cells in lymphoid follicles of the PP in the complete absence of the gut microbiome (58, 59). Furthermore, oral delivery of antigens *in utero* has confirmed that these MALTs are fully functional and can generate specific immune responses with the production of secretory IgA (60). In the absence of an *in utero* infection, however, the appearance of IgG^+ and IgA^+ cells in PPs is delayed until after birth (59). Since immunoglobulin class switching occurs in the germinal centers of PPs (54), this suggests that the full development of germinal centers requires exposure to the gut microbiota. However, information regarding the role of the gut microbiota in the early postnatal development of MALT in ruminants is scarce. There is a single report that preventing exposure of the ileal PPs to gut microbiome results in premature involution of lymphoid follicles in the PPs of newborn lambs (61). However, restoration of the gut microbiome at 4 weeks after birth reversed lymphoid follicle involution in the ileal PPs (61). Thus, the gut microbiome appears to provide critical signals that maintain the production of the pre-immune B cell repertoire. It remains to be determined whether specific microbial species may influence the selection of this immunoglobulin repertoire or if this interaction is restricted to an interaction with innate immune receptors.

The host uses pattern recognition receptors, such as toll-like receptors (TLRs), to recognize the commensal microbiota and maintain intestinal homeostasis (62). Activation of TLR signaling by intestinal tissue invading pathogens generally stimulates inflammatory responses. In contrast, commensal microbiota activation of TLR signaling promotes the production of interleukin 6 and tumor necrosis factor that protect intestinal epithelial cells against injury (62). Therefore, commensal microbial recognition by mucosal TLRs is crucial for the maintenance of intestinal homeostasis and protection of the gut from injuries. The expression of TLRs in the blood of infants (63) was downregulated with increasing age, while memory T cells, such as $CD4^+$ and $CD8^+$, increased in number (63). These changes are consistent with a decrease in innate immune responses that is balanced by an increase in adaptive immune responses with increasing age. Downregulation of innate immune responses with increasing

age has been suggested as one mechanism by which the host avoids unnecessary inflammatory responses to commensal microbiota (63). Similar results have been reported when analyzing the intestinal immune system of calves (64, 65). The expression of mucosal TLR genes was downregulated in weaned calves when compared to pre-weaned calves (65). In contrast, total leukocytes including, $CD3^+$, $CD4^+$, and $CD8^+$ T cells, increased in the jejunal and ileal mucosa of calves with increasing age (64). Moreover, a negative correlation was observed between the expression of mucosal TLRs and mucosa-attached bacteria, suggesting a possible link between the gut microbiota and the observed age-related changes in the mucosal immune responses (65). However, the mechanism by which gut microbiome colonization affects this shift of mucosal and systemic immune responses from innate to adaptive remains to be defined. There is, however, emerging evidence that microbial colonization is associated with substantial changes in the transcriptome of the bovine intestine during the first week of life (66). Transcriptome changes occurred at the level of miRNA and significant correlations were identified between the gut microbiome and these transcriptome changes.

Experiments with the mouse model have clearly demonstrated the importance of gut microbiota in the development of both innate and adaptive components of the mucosal immune system as well as development and maintenance of the intestinal epithelial barrier. Increased susceptibility to enteric infections in gnotobiotic and antibiotic treated mice may also be due to the underdeveloped mucosal immune system and epithelial barrier (54). The immunologically naïve neonatal GIT and the colonizing microbiota undergo a rapid co-evolution during early life and these interactions may be crucial in determining the susceptibility of the neonate to enteric infections. Pre-weaned ruminants are highly susceptible to a variety of viral and bacterial enteric infection within the first few weeks of life (67). Therefore, a thorough understanding of early gut microbiota and its role in regulating and directing early development of the mucosal immune system is essential to improving the health of young calves and reducing susceptibility to enteric infections.

The Commensal Microbiome and Enteric Infections in Young Ruminants

Neonatal diarrhea is the major cause of death in pre-weaned calves and accounts for >50% of calf deaths in the dairy industry (67). Establishment of the gut microbiome within the first 7 weeks of life and an association with calf health and growth (neonatal diarrhea, pneumonia, and weight gain) was recently reported (47). Bacterial diversity was lower in calves with pneumonia and neonatal diarrhea when compared to healthy calves (47), suggesting a possible link between gut microbiota and host health. The authors speculate that antibiotic treatment may have been one factor influencing the gut microbiome in pneumonic calves. Furthermore, colonization by enteric pathogens may be responsible for the observed dysbiosis in gut microbiota during neonatal diarrhea (47). Increased fecal bacteria diversity was also associated with increased weight gain in healthy calves, while a high abundance of *Faecalibacterium* during the first week of life was associated with a lower incidence of diarrhea in calves after the fourth week of life (47). Thus, it is difficult to determine if changes in the fecal microbiome were a consequence of prior disease and associated therapeutic interventions or if colonization of the GIT by specific commensal bacteria had a beneficial effect in terms of disease resistance.

Uyeno and colleagues (46) also reported a high abundance of *Faecalibacterium* in the feces of 1-week-old calves and their abundance was higher in the large intestine compared to the small intestine of 3-week-old calves (21). *Faecalibacterium prausnitzii*, one of the main butyrate producers in the large intestine, displayed a negative association with calf diarrhea incidences (47), suggesting the high prevalence of this species during early life may decrease susceptibility to enteric infections. *F. prausnitzii* also plays a pivotal role in maintaining intestinal homeostasis by promoting anti-inflammatory responses and has been shown to decrease in prevalence in patients with inflammatory bowel disease (68). Inflammatory bowel disease was also associated with a reduced prevalence of *Bifidobacterium* (68), suggesting that these two bacterial groups may have important roles in maintaining intestinal homeostasis and preventing enteric infections. Thus, it will be important to further explore the potential role of such beneficial bacteria in the early gut development and their capacity to promote host health.

Poor management of colostrum feeding in newborn calves is one of the main triggers of neonatal calf diarrhea. Feeding calves with highly contaminated (bacteria > 106 CFU/ml, coliform > 103 CFU/ml) and of low quality (IgG < 50 mg/ml) colostrum (69), poor surveillance of calves born at night, and relying on dams to feed colostrum (70) are some of the major risk factors currently contributing to poor neonatal calf health in the North American dairy industry. Although the importance of timed feeding of high quality colostrum for passive transfer of immunity has been well studied (71), the influence of colostrum on gut microbial establishment and susceptibility to enteric infection in young ruminants is not clearly understood. A recent study revealed that feeding colostrum within 1 h postpartum facilitated bacterial colonization of the small intestine within the first 12 h postpartum. Calves-fed colostrum achieved bacterial numbers similar to older calves [10^{10} 16S rRNA gene copy/g of intestinal sample (49)], but significantly fewer bacteria were observed in the intestine of calves deprived of colostrum (52). Furthermore, when comparing to colostrum-deprived calves at 12 h postpartum, there was a significant increase in the prevalence of *Bifidobacterium* and a decreased prevalence of *E. coli* in the mucosa-attached communities of calves fed either heat-treated or fresh colostrum (52). Changes in the abundance of mucosa-attached *Bifidobacterium* and *E. coli* populations were most pronounced when calves were fed heat-treated colostrum versus fresh colostrum (52). Heat treatment (60°C, 60 min) decreases the density of total bacteria including pathogens present in colostrum, which has been suggested to decrease neonatal diarrhea in calves (71). The results from Malmuthuge and colleagues (52), however, suggest that timed feeding of high quality colostrum has a direct

effect on bacterial colonization of the bovine small intestine, in particular the mucosa-attached community that is in close contact with the host mucosal immune system. Establishing a bacterial population dominated by beneficial bacteria may suppress colonization of enteropathogens (72) immediately postpartum and provide protection against enteric infections in young ruminants with a naïve immune system. Further investigations are necessary to also understand how a *Bifidobacterium*-dominated early gut microbiome may influence host performances (weight gain, resistance to enteric infections) within the first few weeks and identify the mechanisms by which the commensal microbiome alter both enteric health and general physiology.

Manipulation of the Early Gut Microbiome to Improve Health and Production

Manipulation of gut microbiota by feeding microbes, probiotics, or prebiotics has been widely studied in livestock animals as a strategy to improve production and health through altering rumen fermentation and preventing pathogen colonization (24, 42). Direct-fed microbials have been shown to decrease rumen acidosis in cattle, increase milk production in cows, and decrease fecal shedding of *E. coli* in calves (73). These direct-fed microbials may prevent enteropathogen colonization of the gut by either competing for nutrients, space in the gut environment, or producing antimicrobial substances (73). *Megasphaera elsdenii* modifies ruminal fermentation and decreases ruminal acidosis by utilizing lactic acid produced in the rumen (73). However, most of these outcomes are limited to a relatively short interval following feeding (24) or are effective only in pre-weaned calves (39), suggesting that these manipulations are either temporary or need to be instituted within a defined developmental period. Moreover, it is essential to know how the autochthonous gut microbial population responds to these dietary manipulations and how their compositional changes influence overall gut metabolic and immune functions. It may also be important to determine if developing probiotics or direct-fed microbials, based on *Faecalibacterium* and *Bifidobacterium* that have already been linked to calf health, provides a more effective or long-lasting effect. The establishment of host-specific bacteria is crucial for the development of mucosal immune system, especially for the differentiation and proliferation of T cell populations (57). Thus, there would be substantial value in both isolating and testing bacteria within the same host species that might provide the basis for the developing microbial manipulation techniques.

Conclusion

Interactions between host and gut microbiota have been explored extensively in humans and mice but these investigations are still in their infancy in ruminants (**Figure 1**). However, the studies reviewed to date are generating promising results, describing GIT microbial composition (**Figure 2**) and functions in greater depth and identifying factors that significantly influence microbial establishment. It is also notable that recent results are based primarily on nucleic acid sequencing, which may be limited by sampling location, the type of sample collected, extraction methods, sequencing depth, and the analysis pipeline used. In addition, the taxonomic and functional identification of the rumen/gut microbiome is dependent on existing databases and identified organisms and functions are remaining unclassified at lower taxonomic levels and at the level of protein coding genes. Single cell genome sequencing and more comprehensive databases for the ruminant gut microbiome are vital to understanding their role in host development.

A substantial step forward in being able to explain the role of the gut microbiome in host physiology would be to understand the metabolic capacity of the early microbiome. Metabolic functions of the rumen microbiota appear to be highly redundant, which may be essential to ensure optimum fermentation of ingested substrates. Therefore, isolation of metabolically active rumen microbiota may be important to further our understanding of their roles in monocultures and mixed populations. This information will provide the basis for future strategies designed to manipulate the microbiome and improve both production and health.

Finally, there is a substantial need to develop ruminant animal models that can be used to investigate the effects of controlled changes in the gut microbiome on both host mucosal immunity and host metabolism. The rearing of gnotobiotic calves is limited by large technical and financial barriers and to date studies have been limited to changes in diet or the feeding of pre- or probiotics and subsequent sampling of rumen or fecal microflora. The challenge is to develop animal models that allow us to ask questions regarding microbiome changes within specific regions of the GIT and to analyze local effects on mucosal immune and barrier function. The use of a surgically isolated intestinal segment model in fetal lambs (61) provided an elegant model system to create a localized gnotobiotic environment in the GIT of a developmentally normal animal. A similar model system was developed in newborn calves to study the effects of a persistent enteric bacterial infection (74). Thus, it should now be possible to manipulate local exposure to the microbiome and analyze the effects on neonatal mucosal immune system and barrier development. A critical question to be addressed is whether dysbiosis of the microbiome during colonization of the newborn GIT has long-term effects, both locally in the GIT and systemically, that impacts the health and production of animals. If long-term effects are observed, then it will be important to determine if restoration of the complex microbiome, or specific bacterial species, can effectively reverse the effects of early microbial dysbiosis.

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Development of the Chick Microbiome: How Early Exposure Influences Future Microbial Diversity

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The concept of improving animal health through improved gut health has existed in food animal production for decades; however, only recently have we had the tools to identify microbes in the intestine associated with improved performance. Currently, little is known about how the avian microbiome develops or the factors that affect its composition. To begin to address this knowledge gap, the present study assessed the development of the cecal microbiome in chicks from hatch to 28 days of age with and without a live *Salmonella* vaccine and/or probiotic supplement; both are products intended to promote gut health. The microbiome of growing chicks develops rapidly from days 1–3, and the microbiome is primarily *Enterobacteriaceae,* but *Firmicutes* increase in abundance and taxonomic diversity starting around day 7. As the microbiome continues to develop, the influence of the treatments becomes stronger. Predicted metagenomic content suggests that, functionally, treatment may stimulate more differences at day 14, despite the strong taxonomic differences at day 28. These results demonstrate that these live microbial treatments do impact the development of the bacterial taxa found in the growing chicks; however, additional experiments are needed to understand the biochemical and functional consequences of these alterations.

Keywords: chicken, microbiome development, *Salmonella*, probiotic, gut development

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INTRODUCTION

Increasing evidence in multiple species demonstrate the impact gut microbes have on intestinal function, digestion, host metabolism, and immune function (1, 2). While the food animal industry has employed various methods to control and augment the bacteria in the gut for decades, this has been done with little understanding of the complexity of the microbial populations and their association with animal health. The advent of microbiome analysis will allow for better use of these

Abbreviations: ANOSIM, analysis of similarity; DC, diluent-control; DP, diluent-probiotic; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; MANOVA, multivariate analysis of variance; OTU, operational taxonomic unit; PERMANOVA, Permutational MANOVA; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; PCoA, principal coordinate analysis; QIIME, quantitative insights into microbial ecology; ST, *Salmonella* Typhimurium; SIMPER, similarity percentage analysis; STAMP, statistical analysis of metagenomics profiles; VC, vaccine-control; VP, vaccine-probiotic.

products and the rational design of new therapies to promote animal health and performance. An estimated $585 million/year is spent globally on interventions to manage disease in food animals (3); many of these diseases are intestinal in nature (4), and the indirect costs of these intestinal diseases are far greater. The application of modern nucleotide sequencing and associated bioinformatics techniques to the avian gastrointestinal microbiome will lead to breakthroughs in our understanding of digestive processes, host metabolic regulation, immune function, and intestinal dysfunction and pathology. Collectively, increased understanding of the host–microbiome relationship, and the development of techniques to improve these interactions, could reduce the prevalence of food-borne pathogens. In order to effectively apply modern microbial ecology research techniques and elucidate the manner in which the avian intestinal microbiome interacts with the host genome, it is imperative to develop a comprehensive understanding of how the avian microbiome develops under different physiological states and management practices.

There is a dearth of information available on the development and definition of a normal avian gut microbiome. Recent investigations have begun to identify species commonly seen in adult chickens, but little is known about the intermediate and developing community (5–7). Furthermore, there is a paucity of information of the effects of treatments that target the gut environment on the development of the intestinal microbiome of chickens. This impairs our ability to understand how these gut-targeted treatments interact with each other and the host, and how they might affect gut activity and health. A better understanding of these interactions will allow for the rational use of bacterial groups to promote specific host responses.

The goal of this study was to understand the ontogeny of the chicken intestinal microbiome, and how commonly used live bacterial treatments influence this dynamic microbial community. Specifically, we included two live bacterial products currently used in the industry that are intended to improve animal health through manipulation of the host microbiota. We used a live attenuated *Salmonella enterica,* serovar Typhimurium vaccine (Salmune®, CEVA Biomune), and a probiotic feed supplement comprised of *Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium,* and *Bifidobacterium bifidum*(PrimaLac®, Star Labs). We hypothesized that the species richness of the microbiome would increase rapidly, and the addition of live bacterial treatments would alter the development of microbial diversity and the composition of the microbiome. The results from this study demonstrated exposing developing chickens to individual or combined bacterial regimens leads to treatment-specific microbial populations. These populations continue to diverge with age, even in animals receiving only a one-time dose of the *Salmonella* Typhimurium (ST) vaccine at day of hatch. Predicted metagenomic content in these populations suggest changes in potential microbial metabolic activity and microbe-derived signaling molecules; however, these changes were less numerous than the taxonomic changes seen in the same populations.

MATERIALS AND METHODS

Animals and Treatments

Two hundred one-day-old female commercial white leghorn laying type chickens (W-36, Hy-line International) were assigned to one of four treatments (50 chicks/treatment) in a 2 × 2 factorial design. The four groups were designated as follows: Diluent-Control (DC); Diluent-Probiotic (DP); Vaccine-Control (VC); and Vaccine-Probiotic (VP). Animals received either a one-time dose of a live, attenuated ST spray vaccine (Salmune®, Ceva Biomune, Lenexa, KS; Vaccine group) or a sham vaccination consisting of the vaccine diluent, water (Diluent group). The vaccine and diluent spray were administered as recommended by the manufacturer. These treatment groups were further divided into two dietary groups; one group (Control) was fed a standard corn–soybean starter diet (Table S1 in Supplementary Material) and the probiotic group was fed an identical starter diet supplemented with 0.1% (w/w) of the probiotic PrimaLac® (*L. acidophilus, L. casei, E. faecium, B. bifidum*; Star Labs Inc., Clarksdale, MO, USA; Probiotic group). Probiotic pre-mix was added to the probiotic groups' feed prior to the experiment and animals in all groups were fed *ad libitum* for 4 weeks.

Animals in all groups were housed in 934-1-WP isolators (L. H. Leathers Inc., Athens GA) climate-controlled HEPA-filtered isolation units. The animals were maintained and euthanized under an approved protocol from the North Carolina State University Institutional Animal Care and Use Committee (OLAW #A3331-01).

Sample Collection

Six chickens from each treatment group were euthanized (CO_2 followed by cervical dislocation) on days 0, 1, 3, 7, 14, and 28 and the contents of one cecal lobe collected and maintained on ice. At early timepoints, some chicks yielded minimal or no cecal digesta; these are noted in Table S2 in Supplementary Material. The cecal samples were weighed and diluted with 600 µl of 30% glycerol in PBS for storage at −80°C.

DNA Isolation and 16S Sequencing

DNA was isolated from each cecal sample using the MO BIO Power Soil kit (MO BIO, Carlsbad, CA, USA) with the following modifications: a 10-min, 65°C incubation step was added and samples were then homogenized for 45 s at 5100 RPM using garnet bead-containing tubes and a Precellys 24 homogenizer (Precellys, Montigny-le-Bretonneux, France).

DNA recovered from the extraction process was quantified using a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and 10 ng from each sample were aliquoted into 96-well plates in a random order. Some animals contained only small amounts of cecal digesta, particularly at days 0–3, resulting in very small amounts of DNA for some samples. DNA from these samples was included in the sequencing process, despite the possibility of poor quality sequencing (Table S2 in Supplementary Material). MiSeq library preparation and 151 × 151 paired-end sequencing (Illumina, San Diego, CA, USA) were performed by the Argonne National Laboratory Institute for Genomics and Systems Biology Next

Generation Sequencing Core using a protocol and primers recommended and previously described by the Earth Microbiome project and others. Primers used spanned the V4 region of the 16S rRNA gene (515F: GTG**Y**CAGCMGCCGCGGTAA, 806R: GGACTACHVGGGTWTCTAAT) (8). This primer set is commonly used to evaluate the microbiome community across a variety of fields, and is well validated in several models, including the chicken (8–11). Studies estimating microbial composition using V4 sequence information report diversity measurements comparable to those obtained with full-length 16S sequences (12).

Sequence Data Analysis

The unpaired raw sequencing reads were paired and filtered using EA-Utils (13). Paired reads were processed using the QIIME suite of tools (v 1.8.0) (14); barcode matching and quality filtering were conducted prior to picking operational taxonomic units (OTUs). The 16S sequencing process did not yield equal sequence coverage for all samples, and some samples had very low sequence coverage. Samples with low sequence coverage or consistently poor quality were excluded from analysis. Additionally, some ceca from early time points contained little to no recoverable digesta. Consequently, a small number of samples from different time points were removed at this stage (Table S2 in Supplementary Material). OTUs were picked using an open-reference protocol. Briefly, sequences were grouped into OTUs based on 97% sequence identity using uclust and the Greengenes reference database (15, 16). OTUs that failed to match to the database were reclustered, resampled, and re-compared to the database; in this way, new reference sequences are compared to the database in order to minimize the number of excluded sequences. Finally, OTUs that failed to align to any sequences in the reference database are *de novo* clustered. Representative sequences from each OTU were picked and assigned taxonomy using the uclust consensus taxonomy assigner. During this process, sequences with high identity (>97%) were grouped into the same OTU, and are reported at the lowest level of taxonomic identification common to all sequences (17, 18). Sequence coverage was normalized across samples in each analysis. Taxonomic assignments, and alpha and beta diversity metrics were generated using QIIME and Primer-E (v6.1.16; Primer-E LTD, Ivybridge, UK). Principal coordinate analysis (PCoA) plots used in this study were generated in Primer-E using the Bray–Curtis distance metric (19).

Permutational multivariate analysis of variance (PERMANOVA) was conducted using the PERMANOVA+ add-on to Primer-E. Main and pair-wise tests were conducted using up to 1000 permutations of residuals under a reduced model. Similarity percentage analysis (SIMPER) of taxonomic groups between treatment groups and times was made in Primer-E using Bray–Curtis distances. Analysis of similarity (ANOSIM) tests were conducted using Primer-E. Tests were conducted using up to 1000 permutations and the Spearman rank correlation method. A global test statistic (R) was generated for each treatment; the rank similarities between and within treatments were calculated and compared. The global R statistic is a measure of the strength of a treatment group's association with microbiome composition, with 1 being the strongest association and 0 being no association.

Metagenomic inferences from the 16S amplicon data were made using the QIIME suite of tools (14, 15, 17, 18), PICRUSt (20), and KEGG (21); statistics and visualization of functional data were depicted using STAMP (22). Closed-reference OTU-picking protocols were used to identify 16S sequences belonging to annotated genomes. Briefly, sequences were grouped into OTUs based on 97% sequence identity using uclust and the Greengenes reference database. Representative sequences from each OTU were picked and assigned taxonomy using the uclust consensus taxonomy assigner. PICRUSt and KEGG were used to generate a list of functional genes predicted to be present in the sample and to organize these genes into gene pathways. Using STAMP, heatmaps were generated displaying differences in gene-group abundance at each time point. In order to minimize the number of treatment-based differences that may not be biologically relevant, analysis was limited to those differences with an effect size greater than 0.7 as calculated by STAMP (eta-squared method) (22). Storey's FDR correction was applied to all comparisons between treatments (23). Nearest neighbor hierarchical clustering was used to group each sample according to abundance of gene groups in question.

RESULTS

Microbiome Composition and Complexity Change Rapidly with Age

16S rRNA sequence analysis of the microbiome from the ceca of untreated animals (DC) demonstrated a microbiome with low diversity in days 0 and 1, dominated by *Enterobacteriaceae* and to a lesser extent *Enterococcus* (**Figure 1**). The number of OTUs detected in the microbiome increased significantly ($P < 0.05$) by day 3 (data not shown). This increase in bacterial richness starts with *Ruminococcaceae* groups during the first week of life and continues with other *Firmicutes*. By day 14, and extending through day 28, *Ruminococcus* and other *Firmicutes* outnumber *Enterobacteriaceae* (**Figure 1**).

Age More Influential in Microbiome Development than Treatment

Principal coordinate analysis of samples across all time points and treatment groups reveals that the effect of animal age on community composition was larger than that of bacterial treatment (**Figure 2A**). The ANOSIM-generated global test statistics for time ($R = 0.67$) and the treatments (Vaccine $R = 0.361$, Probiotic $R = 0.317$) demonstrate the relative impact of each on the community. At time points 0–7, the samples show large within time point variability. At day 28, within time point variability is decreased and samples are tightly clustered in the PCoA plot (**Figure 2A**). Community analysis of cecal samples across time points and treatment groups show that Gram-negative bacteria (*Proteobacteria*) dominate at early time points, while Gram-positive *Firmicutes*, especially *Clostridia* taxa, become more prominent with age (**Figure 2B**).

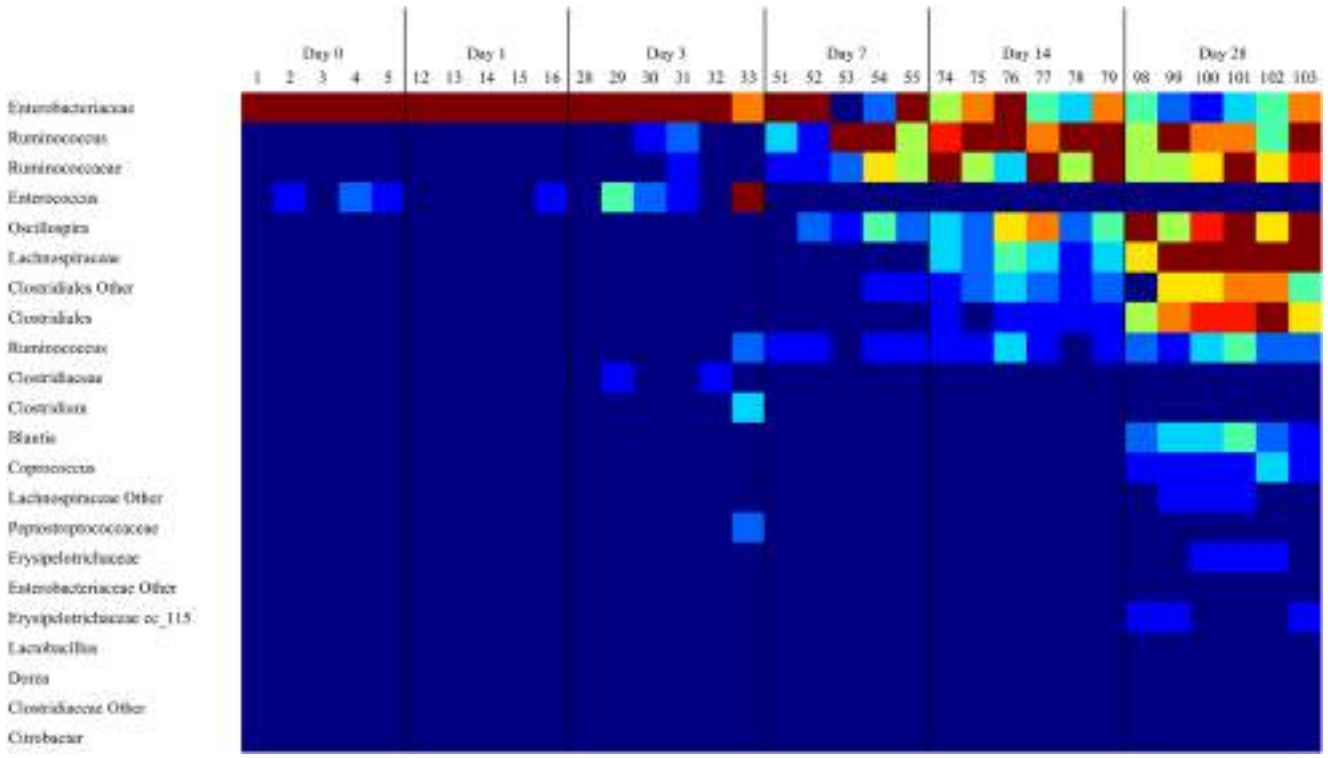

FIGURE 1 | As the cecal microbiome develops, the dominant taxa shift from Gram-negative to Gram-positive bacteria. A heatmap of taxonomic groups present in untreated (DC) samples over time was generated with Qiime. The composition of the microbiome in DC animals was evaluated to identify trends in the development of the normal microbiome over time. There is a consistent decrease in the proportion of *Enterobacteriaceae* and *Enterococcus* over time, and an increase in levels of *Clostridiales* groups like *Ruminococcus* and *Oscillospira*. Sequence coverage was normalized to 16,260 reads/sample.

Treatments Alter Microbial Composition and Rate of Development

Analyses of microbial populations were conducted within each time point (days 1, 7, 14, and 28) to assess the impact of treatment on composition and richness of the microbiome independent of age. No differences in microbial composition were detected at day 1, but significant differences in cecal microbiome composition were observed among all four treatment groups by day 7 (**Figure 3A**). A PERMANOVA showed that all four treatment groups are distinct in composition at days 7, 14, and 28 ($P < 0.05$). A comparison of taxonomic richness (alpha diversity) among treatment groups at days 1, 7, 14, and 28 was made using rarefaction plots. The treatment groups show similar levels of unique taxa at day 1; however, at days 7 and 14, probiotic groups tend to have fewer unique taxa ($P < 0.1$ at day 7, $P < 0.05$ at day 14). Interestingly, there was no significant difference in alpha diversity at day 28 (**Figure 3B**).

Treatment with Live Bacteria Affects Abundance of Taxa not Associated With Treatment

Similarity percentage analysis conducted between treatment groups at days 14 and 28 indicates that the differences between treatment groups can largely be attributed to changes in the most abundant order, *Clostridiales*, including *Lachnospiraceae* and *Ruminococcaceae* genera (**Tables 1** and **2**). MANOVA was used to identify differentially abundant taxa between treatment groups, with an FDR correction made to account for multiple comparisons. At day 14, DC animals harbored a significantly higher proportion of *Enterobacteriaceae* (**Table 1**) with 16% *Enterobacteriaceae* as compared to 3–9% in the other treatments. *Lactobacillus* was significantly increased in the DP group relative to DC. At day 14, 83% of significantly different taxa were *Firmicutes*, and 63% were *Clostridia*.

Analysis of the taxonomic groups represented in each treatment at day 28 indicate that most significant changes occur in the *Firmicutes* phylum, including differences in the abundance of *Ruminococcaceae, Lachnospiraceae,* and *Peptostreptococcaceae* (**Table 2**). *Lactobacillus* is also increased in the DP group relative to DC. Eighty-one percent of all significantly different taxa at day 28 were in the Order *Clostridiales*.

Treatment-Induced Changes in Microbiome Diversity Lead to Predicted Changes in Abundance of Functional Gene Families

Estimates were made of the functional changes that may occur in the cecal microbiome following treatment using

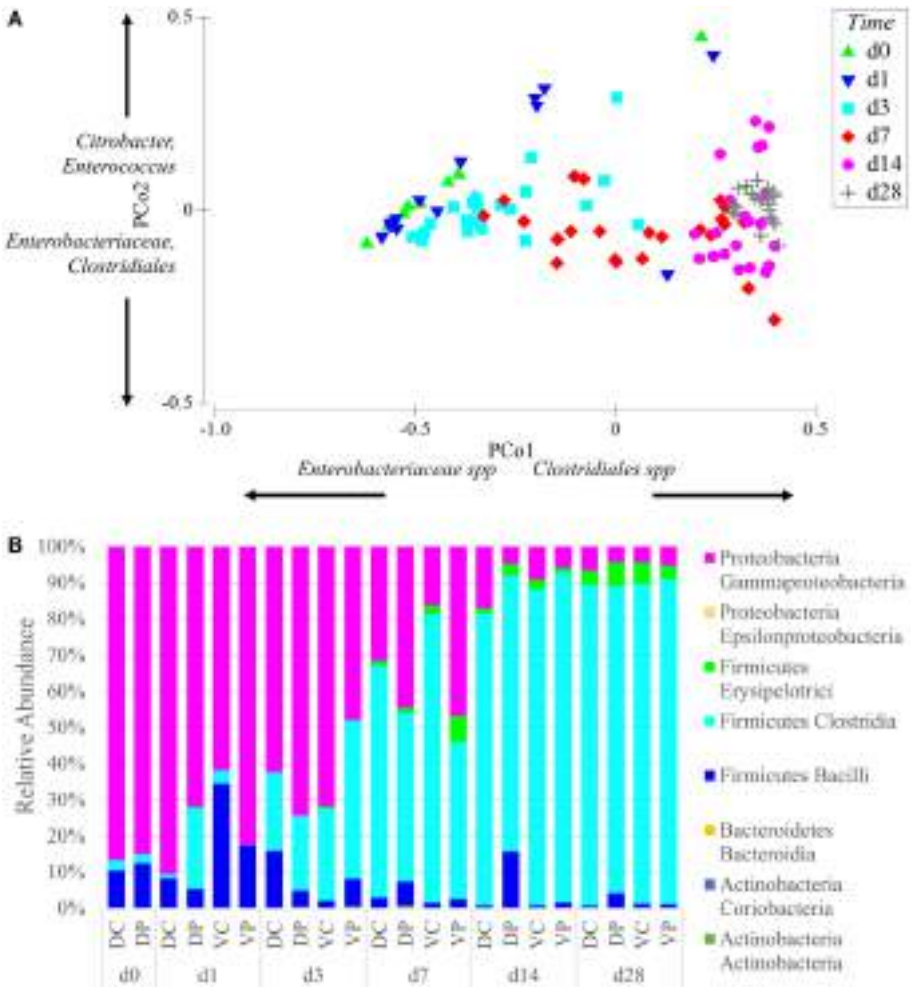

FIGURE 2 | Age is the dominant factor in the composition of the microbiome. (A) Principal coordinate analysis of samples was conducted using Primer-E and samples were labeled based on age. Samples are clustered on two axes based on a multi-dimensional analysis of their sequence diversity and abundance. As the animals age, their microbiome increases in complexity but decreases in variability between samples, even between treatment groups. The effect of age was stronger than the effect of vaccine or probiotic. Differences between all time points were significant at permutational *P*-value <0.05, with the exception of day 0 vs. days 1 and 3. Coordinate loading for each principal coordinate shows the primary taxonomic groups contributing to each axis. Each data point represents a sample in the appropriate time point, and samples from all treatment groups are included in the analysis. **(B)** The phylum and class of sequences with an average relative abundance of 1% or greater are displayed by time point and treatment. All treatment groups started with high levels of *Gammaproteobacteria* that shifted with age into a *Firmicutes*-dominated community with large numbers of *Clostridia.* Taxonomy assignments were generated with QIIME, and PCoA plots were generated with Primer-E. Sequence coverage was normalized to 16,260 reads/sample.

closed-reference OTU-picking and PICRUSt. Gene groups targeted for statistical analysis had an FDR-corrected $P < 0.01$, and an effect size of 0.7 or higher. At day 14, samples cluster primarily by probiotic treatment, and the VP group is most distinct from other treatments. The combination of ST and probiotic treatments increases the expected proportion of genes related to environment-sensing; two-component systems, bacterial motility, chemotaxis, and flagellar component assembly genes were predicted to increase. DC, DP, and VC groups have relatively higher abundance of genes related to amino acid metabolism, DNA repair and replication, and translation (**Figure 4A**). The differences between DC, DP, and VC groups were minor, but the DP group displayed the lowest expected abundance of two-component system and bacterial motility genes.

Fewer gene groups met the inclusion criteria at day 28, and the total relative abundance of included gene groups was lower than that at day 14. At day 28, DP and VP treatment groups display higher predicted levels of genes related to one carbon metabolism, terpenoid synthesis, and translation proteins (**Figure 4B**). DC and DP groups had higher proportions of fatty acid metabolism, drug metabolism, and signal transduction gene pathways.

Relative abundance tables of taxa and predicted gene groups were used to generate area charts of between-treatment changes

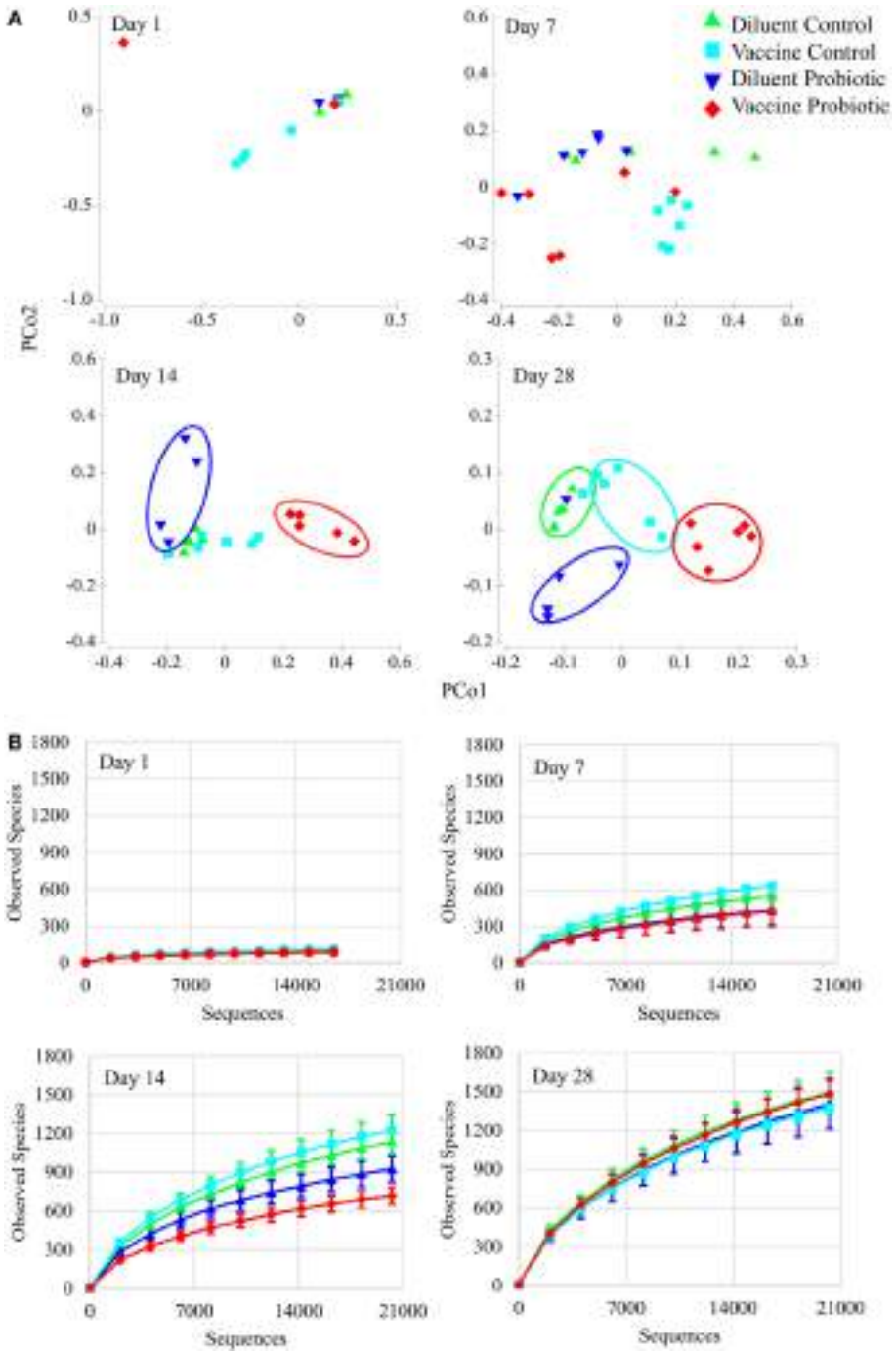

FIGURE 3 | Principal coordinate analysis and rarefaction analyses demonstrate the impact of the treatments over time. (A) Principal coordinate analysis generated with Primer-E demonstrates the effect of treatments at 1, 7, 14, and 28 days of age. There are no significant treatment differences at day 1. By day 7, treatment groups are statistically different based on PERMANOVA tests. Treatment groups cluster visually at days 14 and 28. All treatment groups at days 7, 14, and 28 were different at permutational $P < 0.05$. **(B)** Rarefaction of observed species (unique OTUs) at individual time points was conducted using QIIME, and demonstrate the rapid development of taxonomic diversity. Treatment groups show similar diversity at Day 1. At day 7, DP and VP tend to have lower diversity than VC ($P = 0.078$ and 0.054, respectively). At day 14, DP and VP diversity is significantly lower than DC and VC diversity ($P < 0.05$). By day 28, community diversity is similar between treatments. Sequence coverage was normalized for each time point individually: day 1 (16,577 reads/sample), day 7 (16,668 reads/sample), day 14 (20,263 reads/sample), and day 28 (20,263 reads/sample).

TABLE 1 | Similarity percentage analysis (SIMPER) of treatment groups at day 14[a].

Phylum	Class	Order	Family	Genus	Average abundance (%)		% Contribution to dissimilarity[b]
					DC	DP	
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	1	16*	19.4
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae[c]		16	3*	15.65
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	25	26	15.27
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		19	14*	9.01
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	11	15	8.58
					DC	VC	
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		8	16*	15.4
Firmicutes	Clostridia	Clostridiales			4	12*	14.57
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		19	13*	13.61
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	25	24	13.28
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		16	9*	13.12
					DC	VP	
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		8	39*	25.58
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	25	2*	18.69
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		19	3*	13.46
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		16	5*	9.07
Firmicutes	Clostridia	Clostridiales			4	14*	8.95

[a]*Top 5 taxa shown for each comparison.*
[b]*Percent contribution to total dissimilarity between treatment groups under comparison.*
[c]*If a sequence matches more than one possible taxon, classification stops at the next highest level.*
**Indicates significance at $P \leq 0.05$.*

TABLE 2 | Similarity percentage analysis (SIMPER) of treatment groups at day 28[a].

Phylum	Class	Order	Family	Genus	Average abundance (%)		% Contribution to dissimilarity[b]
					DC	DP	
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus[c]	12	19	16.65
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		11	18*	12.94
Firmicutes	Clostridia	Clostridiales	Other	Other[d]	11	5*	9.57
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	12	8*	9.22
Firmicutes	Clostridia	Erysipelotrichales	Erysipelotrichaceae		2	5	7.73
					DC	VC	
Firmicutes	Clostridia	Clostridiales			12	21*	15.41
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		15	10*	13.49
Firmicutes	Clostridia	Clostridiales	Other	Other	11	4*	12.26
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	5	9	7.53
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	12	12	7.45
					DC	VP	
Firmicutes	Clostridia	Clostridiales			12	36*	32.27
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		15	5*	14.03
Firmicutes	Clostridia	Clostridiales	Other	Other	11	1*	13.05
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		11	16*	6.85
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	12	11	5.11

[a]*Top 5 taxa shown for each comparison.*
[b]*Percent contribution to total dissimilarity between treatment groups under comparison.*
[c]*If a sequence matches more than one possible taxon, classification stops at the next highest level.*
[d]*"Other" indicates the sequence in question has not been assigned to a taxonomic group at that level.*
**Indicates significance at $P \leq 0.05$.*

in taxa and gene groups. Vaccine and Probiotic groups differ from the DC group taxonomically at both days 14 and 28. Gene-group abundance shows less treatment-specific variability (**Figure 5**).

DISCUSSION

Little is known about the development of the microbiome in young birds, and how it is affected by different stimuli (7). The

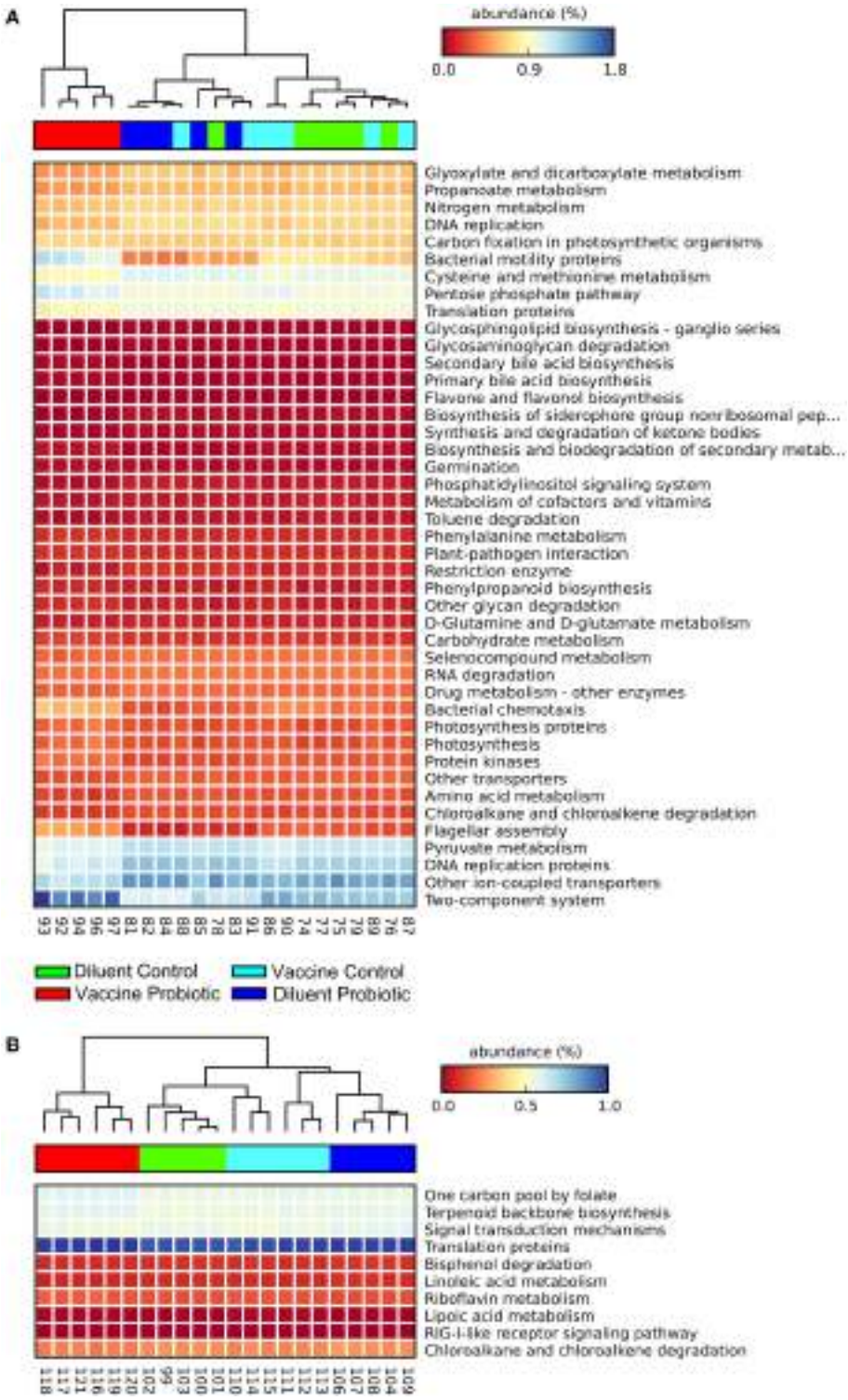

FIGURE 4 | At both days 14 and 28, the VP group shows the greatest divergence in predicted metagenomic content. PICRUSt was used to generate a list of genes inferred to be present in the samples, their relative abundance, and the gene pathways with which they are associated. A heatmap was generated with STAMP. Samples were clustered using a nearest neighbor metric, and pathways were colored based on their percent abundance relative to all measured genes. All listed gene groups are significantly different between treatment groups with an effect size >0.70 and FDR-corrected $P < 0.01$. The combination of vaccine and probiotic treatments stimulates changes in several gene groups and pathways at day 14 **(A)**, namely increases in chemotaxis, two-component system, flagellar assembly, and bacterial motility. Decreases in the VP group include metabolic processes, such as amino acid metabolism, DNA replication, and protein translation. **(B)** There are fewer significantly different pathways at day 28 and changes are largely related to cell metabolism.

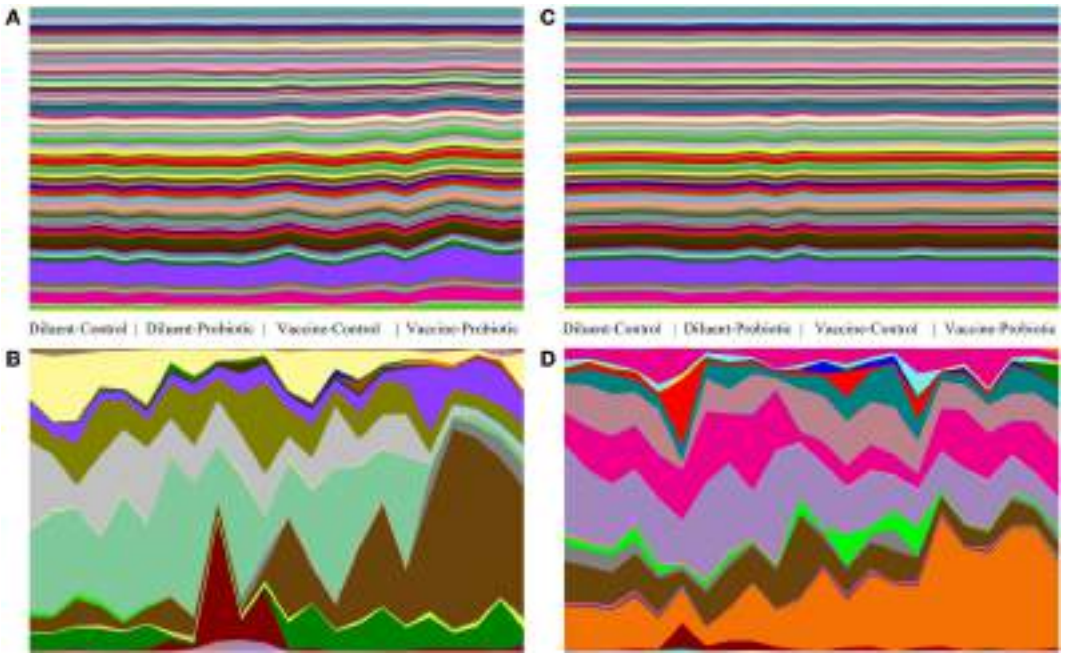

FIGURE 5 | Comparison of taxonomic and gene-group abundance trends at days 14 and 28. Relative abundance of taxonomic groups and predicted abundance of functional gene groups demonstrate the stability of gene-group abundances relative to changes in taxonomic groups. Relative abundance tables for taxa and gene group were assembled and used to generate area charts of all samples at days 14 and 28. The relative abundance of every identified taxonomic or functional gene group is shown for each sample; **(A)** predicted gene groups at day 14, **(B)** taxonomic groups at day 14, **(C)** predicted gene groups at day 28, **(D)** taxonomic groups at day 28. At day 14, there are clear taxonomic differences between treatments **(A)**, and smaller changes in the abundance of a few gene groups **(B)**. At day 28, taxonomic changes **(D)** are accompanied by few visible changes in gene-group abundance **(C)**.

goal of this study was to characterize the healthy developing microbiome in chickens and understand how commonly used bacterial treatments intended to improve or maintain health would affect this process. This is important in the food animal industry as there are numerous feed additives intended to improve animal health, either directly or indirectly through improving gut health. However, the mechanisms by which these amendments work are poorly understood. Most claim to enhance health and performance via manipulation of the host intestinal microbiome, but the mechanism of action has been studied in very few of these products (24, 25).

In the present study, we administered two commonly used live bacterial treatments applied in poultry production to enhance intestinal health and function. According to the manufacturer, the live ST vaccine used here is intended to prevent colonization of the gut and internal organs by multiple types of *Salmonella*, including Heidelberg, Typhimurium, Hadar, Kentucky, and Enteritidis (26). Similarly, the probiotic used here is intended to maintain healthy microbiota balance in the gut (27). We investigated to what extent these health-promoting treatments affect the microbiome of young chicks.

We found that the post-hatch intestinal microbiome has low diversity dominated by Gram-negative bacteria, particularly *Enterobacteriaceae,* which includes *Salmonella, Klebsiella, Proteus,* and *Escherichia coli.* During the first week of life, there is a shift to a much more diverse community comprised of a wide variety of Gram-positive bacteria, mainly within the *Clostridiales* group, resulting in a correspondingly smaller proportion of Gram-negative bacteria (**Figure 1**). The proportion of Gram-negative bacteria in the cecum at day 28 is <6%, and it is almost entirely *Enterobacteriaceae.*

Data from the present study suggest a microbiome more affected by age than treatment (**Figure 2A**). Irrespective of treatment, all groups show a sharp decline in *Enterobacteriaceae* with age, including the vaccinated groups, where levels of *Enterobacteriaceae* would be expected to increase following *Salmonella* (member of the *Enterobacteriaceae* family) vaccination. Nor does addition of a *Firmicutes*-based probiotic product stimulate more rapid conversion to a *Firmicutes*-dominated microbiome (**Figure 2B**) in probiotic-fed animals. Day-old birds begin with a gut colonized by few bacterial species at a concentration several orders of magnitude lower than mature animals (28, 29), so it is likely that the primary driver of age-dependent increase in complexity is bacteria colonizing a previously empty niche. However, diet can also play a major role in the composition of the microbiome and exerts an influence on the developing and mature gut (30, 31). Studies by Sergeant et al. characterizing the microbiome of wheat-fed chickens reported *Megamonas* and *Negativicutes* as more abundant in their adult birds, while the *Firmicutes* most commonly seen in this trial, *Lachnospiraceae* and *Ruminococcaceae,* were less abundant (32). The effect of gut development on the intestinal microbiome is more difficult to

quantify; though studies of germ free and gnotobiotic mice clearly demonstrate that the microbiome is essential to the development of a fully functioning gut (33, 34), whether the developmental stage of the gut is a variable influencing the development of the microbiome is less clear.

Despite the strong relationship between age and composition of the microbiome, the bacterial treatments included in this study did affect the microbiome. PCoA of the four treatment groups at days 1, 7, 14, and 28 illustrate the impact of both vaccination and probiotic supplementation on the microbiome (**Figure 3A**). Despite the fact that the vaccine is only administered on day 0, global R statistics demonstrate that the impact of ST on the composition of the microbiome is on par with that of the continuously fed probiotic at days 14 (vaccine = 0.802, probiotic = 0.882) and 28 (vaccine = 0.697, probiotic = 0.705). The magnitude of the effect of the one-time ST inoculation is nearly as great as that of the continuously fed probiotic despite low levels of the ST-containing taxonomic group *Enterobacteriaceae* after day 7, suggesting early colonizers influence the relative abundance of the microbiome despite being transient themselves. While little is known about the long-term effects of early microbiome perturbation, some studies support this idea (35, 36).

To understand the impact of treatment at the taxonomic level, SIMPER was conducted to identify species contributing to differences between treated and untreated animals. Most of the differences between DC and treated animals at days 14 and 28 involve *Lachnospiraceae, Ruminococcaceae,* and other *Clostridiales* (**Tables 1** and **2**). Though abundance of *Lactobacillus* in the DP and VP groups is higher at day 14 ($P < 0.05$), the magnitude of the increase in VP over DC is not great enough for *Lactobacillus* to be a major source of dissimilarity. There is little microbiological evidence that the bacterial products applied in this study interfere with each other; levels of *Lactobacillus* are not significantly lower in the VP group than the DP group, and VP animals remain ST-positive at day 28 (data not shown). There are signs of treatment interaction; however, ST vaccination decreases abundance of the group *Clostridiales Other,* and the combination of probiotic and vaccine results in the strongest difference from the DP group; 11% reduced to 1% of the identified bacteria, perhaps indicating a synergy between the two treatments, which makes the cecum a more hostile environment for this group of bacteria.

Changes in taxonomic diversity are the most used indicator to infer changes in microbiological activity, but it is becoming apparent that many of the functions of a normal microbiome can be carried out by a number of microbial groups (37, 38). Therefore, understanding how treatments affect taxonomic abundance may not provide us with a complete understanding of how they impact healthy and diseased guts, or develop therapies that target the predominant cause of gut dysbiosis; a change in function. In its entirety, the chicken gut is estimated to be colonized by as many as 10^{13} microbes, and they have a combined genetic potential far in excess of the ~20,000 genes identified in the chicken genome (39, 40). PICRUSt uses the 16S rRNA genes obtained during sequencing to infer the presence of functional genes known or predicted to be associated with those 16S sequences. At day 14, there were predicted increases in the VP group in genes related to motility, flagellar assembly, chemotaxis, and two-component system. By contrast, VP microbiomes displayed lower abundance of many protein and energy metabolism genes, as well as genes related to DNA replication and protein translation (**Figure 4A**). Supporting the taxonomic data suggesting that the microbiome is still equilibrating at day 14 (**Figures 2** and **3**), the functional changes at day 28 are both fewer and less dramatic (**Figure 5**). The VP group exhibits the most variation of the four treatments, and suggests changes in a few cellular metabolism pathways. Interestingly, the effect of probiotic supplementation and its interaction with the vaccine appears to stimulate more functional changes than the vaccine group alone. At days 14 and 28, the DP and VP groups were more likely to have either the highest or lowest levels of any given gene group.

A possible contributor to the lack of more dramatic functional diversity between treatment groups at day 28 could be limitations inherent to this technique and its application to avian microbial communities. One of these is its reliance on sequenced and annotated genomes. Though comparisons between PICRUSt results and metagenomics data from the same samples have shown that the predictive value of PICRUSt analysis is very good (20, 41), 16S genes without a confident phylogenetic assignment cannot be used as marker genes. Because of this, about 15% of the 16S sequences were filtered out at day 14 and over 20% at day 28. This number of unknown or uncharacterized sequences may be higher in the avian microbiome than in the human or murine microbiome, as the databases used in this process were all developed based on mammalian microbiota; chicken-specific microbes that may be important in this system could be excluded from analysis because they are not part of the 16s and/or KEGG databases. However, this analytical technique has been successfully used on avian microbiomes in the past (6). While the difference in excluded taxa between time points is not large, it is possible that the bacteria excluded from analysis are active in the community; evaluation of those taxa excluded from PICRUSt analysis indicates that some are differentially abundant between treatment groups (Tables S3 and S4 in Supplementary Material). These bacteria could play a significant role in the activity of the microbiome. Bacteria falling under the *Clostridiales Other* group were consistently higher in the DC group relative to other treatments, and could represent an unmeasured source of functional differences between treatment groups. However, their metagenomic contribution to the community cannot be known without further characterization of their genome.

The relative lack of functional gene differences at day 28 could also be an indication that despite continuing taxonomic differences, the microbiome in each treatment is converging toward a similar metabolic pattern. Conservation of function across a variety of microbial profiles has been described in other studies, and extreme dysregulation of the microbiome may be required before severe or protracted functional changes occur (37). **Figure 5** illustrates this concept; while the bacterial treatments applied in this study affect both the taxonomic and inferred metagenomic composition of the microbiome, even statistically significant changes in function gene content are minor when compared to the taxonomic changes seen in the same animals.

In the present study, the chickens were all free of visible disease or stress, and it is possible their gut microbiota were functioning in their optimal range with or without treatment. It is also important to note that these birds were not given a pathogen challenge or other stressor of any kind. The addition of vaccinations or probiotics to a chicken with a dysbiotic gut microbiome might yield more significant functional changes. Recent studies demonstrated that exposure of mice to antibiotics at an early age can have a deleterious effect on the diversity of the microbiome for several months following treatment (42). This study showed no such effect from probiotic or vaccination. The value of select dietary treatments and management practices in poultry production may be their ability to increase the speed at which a disturbed or stunted microbiome is able to return to a normal functional state.

In conclusion, one-time oral inoculation with a live ST strain and daily ingestion of a probiotic feed supplement both alter the microbiome of growing chicks. These differences persisted throughout the study, and are centered on changes in the abundance of core microbes present in all treatment groups. The results of this trial suggest that common bacterial treatments, such as probiotics and bacterial vaccines, affect the taxonomic composition of the microbiome, but only have transient or small effects on the function and activity of the microbiome under non-stressed growth conditions. By contrast, as has been seen in other studies (7), age played a major role in the composition and richness of the bacterial community. Major shifts from day of hatch to day 14 centered on the early dominance of *Enterobacteriaceae*, followed by a transition to *Firmicutes*-dominated ceca. Future studies will focus on understanding the functional and phylogenetic parameters of a normal developing microbiome, and to evaluate the effect of treatments like these on that normal range of microbial profiles.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fvets.2016.00002

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Spatial and Temporal Changes in the Broiler Chicken Cecal and Fecal Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression

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To better understand the ecology of the poultry gastrointestinal (GI) microbiome and its interactions with the host, we compared GI bacterial communities by sample type (fecal or cecal), time (1, 3, and 6 weeks posthatch), and experimental pen (1, 2, 3, or 4), and measured cecal mRNA transcription of the cytokines IL18, IL1β, and IL6, IL10, and TGF-β4. The microbiome was characterized by sequencing of 16S rRNA gene amplicons, and cytokine gene expression was measured by a panel of quantitative-PCR assays targeting mRNAs. Significant differences were observed in the microbiome by GI location (fecal versus cecal) and bird age as determined by permutational MANOVA and UniFrac phylogenetic hypothesis tests. At 1-week posthatch, bacterial genera significantly over-represented in fecal versus cecal samples included *Gallibacterium* and *Lactobacillus*, while the genus *Bacteroides* was significantly more abundant in the cecum. By 6-week posthatch, *Clostridium* and *Caloramator* (also a Clostridiales) sequence types had increased significantly in the cecum and *Lactobacillus* remained over-represented in fecal samples. In the ceca, the relative abundance of sequences classified as *Clostridium* increased by ca. 10-fold each sampling period from 0.1% at 1 week to 1% at 3 week and 18% at 6 week. Increasing community complexity through time were observed in increased taxonomic richness and diversity. IL18 and IL1β significantly ($p < 0.05$, pairwise *t*-tests) increased to maximum mean expression levels 1.5 fold greater at week 3 than 1, while IL6 significantly decreased to 0.8- and 0.5-fold expression at 3- and 6-week posthatch, respectively relative to week 1. Transcription of pro-inflammatory cytokines was generally negatively correlated with the relative abundance of various members of the phylum Firmicutes and positively correlated with Proteobacteria. Correlations of the microbiome with specific cytokine mRNA transcription highlight the importance of the GI microbiome for bird health and productivity and may be a successful high-throughput strategy to identify bacterial taxa with specific immune-modulatory properties.

Keywords: microbiome and immune system, pro-inflammatory mediators, cytokines, cecum, succession

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INTRODUCTION

Poultry are naturally adapted to hosting a complex gastrointestinal (GI) microbial community with hundreds of bacterial species and up to 10^{11} CFU per gram of gut contents (1). Benefits conferred by this microbial community (the GI microbiome) include promoting beneficial development of the intestinal mucus layer, epithelial monolayer, and lamina propria (2, 3), excluding pathogenic taxa (4), breaking down polysaccharides (5, 6), providing energy as amino acids and short chain fatty acids (7, 8), and promoting proper development and homeostasis of the immune system (9).

However, until relatively recently, many important aspects of the basic ecology of the poultry GI microbiome have remained hidden in a sort of black box due to technical limitations. With the use of high-throughput sequencing, we have begun to open this black box with important insights into the taxonomic (10–16) and genomic (6, 17–19) composition of the poultry GI microbiome as summarized in several recent reviews (9, 20–22). From this growing body of knowledge, an important common finding has emerged detailing highly significant successional changes in the GI microbiome as birds mature. For example, in the chicken ceca, taxonomic richness and diversity typically increase from day of hatch to market age of commercial broilers at 6 weeks as a community develops comprised almost exclusively of bacteria belonging to the phylum Firmicutes (15). Enough data are now available to also compare communities sampled from different anatomical regions of the GI tract. For example, relative to cecal communities, fecal samples typically contain higher relative proportions and absolute abundance of bacteria belonging to the Enterobacteriales and Lactobacillales (9, 16, 20–22). Proper understanding of temporal and spatial changes in the chicken GI microbiome is critically important for designing probiotic supplements, monitoring gut health, and choosing sample types to assess feed additive effects or pathogen shedding.

The establishment of a normal microbiota constitutes a key component of gut health, through colonization resistance mechanisms, and has implications for proper development of the gut and full maturation of the mucosal immune system (9, 23). The communication between the microbiota and the immune system is principally mediated by interaction between microbes and pattern recognition receptors (PRRs) expressed by the intestinal epithelium and various local antigen-presenting cells, resulting in activation or modulation of both innate and adaptive immune responses (23, 24). The composition of the GI microbiota is known to affect many host functions including nutrient utilization, gut epithelium feeding, and the development and activity of the gut immune system (25). The interaction between the immune system of the gut and commensal microbiota in chickens starts immediately after hatching and leads to a low-level of inflammation characterized by an increased cytokine and chemokine expression as well as a number of immune-associated proteins (24, 26). As a result, there is an infiltration of heterophils and lymphocytes into the lamina propria or the gut epithelium and normalization of the gut immune system (27, 28). However, to date, there has been no attempt to show an association between the development of specific commensals in the chicken gut with either the development of an efficient mucosal immune response or the development of immune homeostasis. The studies described here are the first attempt to bring insights into interactions between the commensal microbiota and the expression of regulatory cytokines in the chicken cecum over time by identifying specific taxa significantly correlated with cytokine gene expression.

In this work, we combine high-throughput sequencing of broad-range 16S rRNA gene amplicons with quantitative-PCR of cytokine gene expression to document differences in the GI microbiome according to sample type (fecal versus cecal) in the maturing bird and examine correlations between specific taxa and measures of cytokine gene expression. To our knowledge, paired cecal and fecal samples from individual birds have not been compared with modern sequencing and phylogenetic methods nor have specific bacterial taxonomic groups been correlated with cytokine mRNA transcription in local tissue in developing broilers.

MATERIALS AND METHODS

Experimental Design

At hatch, non-vaccinated broiler chicks with identical genetic backgrounds were obtained from a commercial breeder and placed into four floor pens. The birds were fed a balanced, unmedicated corn, soybean meal-based starter (0–14 days), grower (15–30 days), and finisher (31–42 days) diet. At each of three time points, fecal samples were collected from a total of 20 birds (five from each of the four pens) that were then euthanized and intestinal samples collected via necropsy. Intestinal mucosal and luminal samples were collected from the cecum. Fecal contents and intestinal samples were stored aseptically at −20°C. Time points sampled followed changes in diet from starter to grower feed, and grower to finisher feed. The experiment concluded at day 42. These samples are referred to as weeks 1, 3, and 6.

Experiments were conducted according to the regulations established by the U.S. Department of Agriculture Animal Care and Use Committee (ACUC # 2015003). Chicks were placed in floor pens containing clean wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal-based chick starter diet *ad libitum* that met or exceeded the levels of critical nutrients recommended by the National Research Council (29). *Salmonella* was not detected in the feed or from the paper tray liners using standard analytical procedures (30).

Sample Collection for mRNA

Chickens from each experimental group were euthanized at weeks 1, 3, and 6. A 25-mg piece of tissue was removed from the cecal tonsils and was washed in PBS, placed in a 2-ml microcentrifuge tube with 1 ml of RNAlater (Qiagen, Inc., Valencia, CA, USA), and stored at −20°C until processed.

RNA Isolation

Cecal tissues (25 mg) were removed from RNAlater and transferred to pre-filled 2-ml tube containing Triple-Pure™ 1.5-mm zirconium beads. RLT lysis buffer (600 µl) from the RNeasy mini kit (Qiagen) was added, and the tissue was homogenized for 1–2 min at 4,000 rpm in a Bead Bug microtube homogenizer (Benchmark

Scientific, Inc., Edison, NJ, USA). Total RNA was extracted from the homogenized lysates according to the manufacturer's instructions, eluted with 50 µl RNase-free water, and stored at −80°C until qRT-PCR analyses were performed. RNA was quantified and the quality evaluated using a spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

Quantitative Real-Time PCR

Primer and probe sets for the cytokines and 28S rRNA were designed using the Primer Express Software program (Applied Biosystems, Foster City, CA, USA) as previously described and validated (31–33) and listed in **Table 1**. The qRT-PCR was performed using the TaqMan fast universal PCR master mix and one-step RT-PCR master mix reagents (Applied Biosystems). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast real-time PCR system as described previously (25, 26) with the following cycle profile: one cycle of 48°C for 30 min and 95°C for 20 s and 40 cycles of 95°C for 3 s and 60°C for 30 s. Quantification was based on the increased fluorescence detected by the 7500 Fast sequence detection system due to hydrolysis of the target-specific probes by the 5′-nuclease activity of the r*Tth* DNA polymerase during PCR amplification. Normalization was carried out using 28S rRNA as a normalizer gene. To correct for differences in RNA levels between samples within the experiment, the correction factor for each sample was calculated by dividing the mean threshold cycle (*CT*) value for 28S rRNA-specific product for each sample by the overall mean *CT* value for the 28S rRNA-specific product from all samples. The corrected cytokine mean was calculated as follows: average of each replicate × cytokine slope/28S slope × 28S correction factor. The data shown are corrected 40 C_t values.

TABLE 1 | Real-time quantitative RT-PCR probes and primers for pro- and anti-inflammatory cytokines.

RNA target		Probe/primer sequence	Accession number[a]
28S	Probe	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	F[b]	5′-GGCGAAGCCAGAGGAAACT-3′	
	R[c]	5′-GACGACCGATTGCACGTC-3′	
IL-1β	Probe	5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′	AJ245728
	F	5′-GCTCTACATGTCGTGTGTGATGAG-3′	
	R	5′-TGTCGATGTCCCGCATGA-3′	
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′	
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′	
IL-18	Probe	5′-(FAM)-CCGCGCCTTCAAGCAGGGATG-(TAMRA)-3′	AJ416937
	F	5′-AGGTGAAATCTGGCAGTGGAAT-3′	
	R	5′-ACCTGGACGCTGAATGCAA-3′	
IL-10	Probe	5′ (FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′	AJ621735
	F	5′-CATGCTGCTGGGCCTGAA-3′	
	R	5′-CGTCTCCTTGATCTGCTTGATG-3′	
TGF-β4	Probe	5′-(FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)-3′	M31160
	F	5′-AGGATCTGCAGTGGAAGTGGAT-3′	
	R	5′-CCCCGGGGTTGTGTGTTGGT-3′	

[a]*Genomic DNA sequence.*
[b]*Forward.*
[c]*Reverse.*

16S rRNA Sequencing and Data Analysis

DNA was extracted from cecal samples using the MoBio UltraClean Soil DNA extraction kit and DNA quality and concentration checked by spectrophotometry (NanoDrop Products, Wilmington, DE, USA). PCR and pyrosequencing of the V1–V3 regions of 16S rRNA genes were performed using tagged amplicon methods with Roche 454 Titanium chemistry at Research and Testing Laboratory (Lubbock, TX, USA) as previously described (15, 34, 35). Following sequencing, sequences were de-multiplexed and preprocessed with the Galaxy toolkit (36) and custom Perl, R, and shell scripts (37); additional quality controls according to standard protocols (38) were completed by trimming tag sequences, screening for presence of the forward PCR primer sequence, and removing sequences with any ambiguous base calls. Based on expected amplicon sizes and frequency distributions of sequence lengths in v115 of the Silva reference database, sequences were further limited to a range of 325–425 bp. Putative chimeric sequences were identified with usearch (39) and ChimeraSlayer in mothur (40).

Taxonomic classifications of sequences were performed in two ways. First with the RDP naive Bayesian classifier (41) v2.6 and second with usearch with the global alignment option (39) using the EMBL taxonomy from v115 of the Silva project curated seed database (42). To assess phylotype richness (number of taxa) and diversity [number of taxa weighted by relative abundance per the Shannon diversity index (43)] independent of taxonomic classifications, sequences, which passed all the screens described above were grouped into similarity clusters (operational taxonomic units; OTUs) using similarity cutoffs of 90, 95, and 97% with uclust (39). The output from usearch provided the inputs for our own customized analysis pipeline to parse the clustering results and produce graphical and statistical summaries of the data for the desired sampling units using perl and R (44) as previously described (35, 37). Clustering of communities was performed using the CCA function of the vegan package (45) in R based on OTU and taxonomic classifications.

The relative effects of GI location (fecal versus cecal samples) and time (number of days posthatch) versus experimental treatment (and their interactive effects) on microbial communities was determined by a permutational multivariate analysis of variance (MANOVA) using the adonis function of the vegan package in R. Either OTU or taxonomic classifications of sequences from each bird were used to partition sums of squared deviations from centroids in a distance matrix to determine how variation was explained by experimental treatments or uncontrolled covariates (46). Unifrac (47) implemented in mother (40) was used to compare the phylogenetic distribution of sequences for each bird by comparing phylogenetic branch lengths shared or unique to each sample type of the experimentally derived tree

to a null distribution of samples randomly shuffled within the same tree.

To compare cytokine gene expression among and between time points, ANOVA and *post hoc* pairwise *t*-tests were performed. To search for taxa with significant positive or negative correlations with cytokine gene expression, slices of the dataset were taken to generate Pearson correlation coefficients and linear regression models for the relative abundance of each taxon versus cytokine expression values for a given bird at a given time point. All phyla and genera were compared against each cytokine expression profile for each time point; cutoffs of Pearson correlation coefficients >0.4 and r^2 values >0.3 were chosen based on empirical testing.

RESULTS

Spatial Differences in Microbiome

Significant differences were observed in the microbiome depending on sampling location (fecal versus cecal) and bird age (1, 3, or 6 weeks of age) using a variety of metrics. First, we used a variety of taxonomic classifications (e.g., phylum or genus-level classifications with the Silva or RDP taxonomy) or taxonomic-independent classifications (binning sequences into sequence-similarity groups or operational taxonomic units; OTUs) to partition variance of distance matrices by sample location and bird age. For all classification approaches, both sampling location and bird age (and their interactive effects) were highly significant explanatory variables (**Table 2**).

Next, to further test the hypothesis that different sets of bacteria are found in fecal versus cecal samples, we compared the phylogenetic distribution of sequences for each bird using the unifrac statistic (47) as described in the Section "Materials and Methods." Beginning at 1 week of age, the phylogenetic distributions of sequences from fecal versus cecal samples were highly significantly different (**Figure 1**). Of the 20 birds sampled at 1 week of age, 18 birds had sufficient sequence data from both fecal and cecal samples to make this phylogenetic comparison

TABLE 2 | Permutational ANOVA results partitioning effects of bird age and sample type (cecal or fecal) on microbial community composition as calculated at a 95% OTU cutoff as described in the text.

	Degrees of freedom	Sums of squares	Mean squares	*F*	Pr (>*F*)
Age	2	8.53	4.26	20.91	<0.0001
Sample type	1	3.14	3.14	15.39	<0.0001
Age:type	2	1.40	0.70	3.45	<0.0001
Residuals	110	22.43	0.20		
Total	115	35.51			

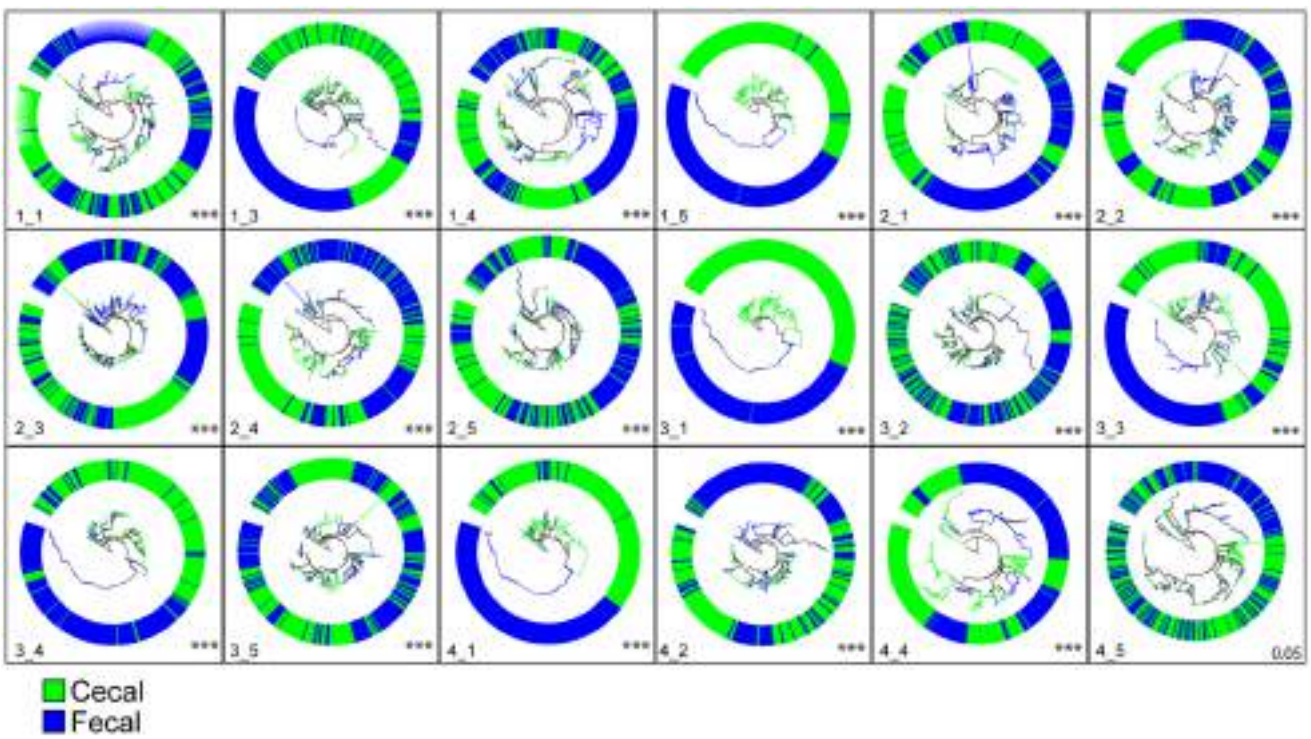

FIGURE 1 | Phylogenetic clustering of cecal versus fecal bacterial communities from birds at 1 week of age (n = 18). Each circle represents a phylogenetic tree of cecal and fecal samples taken from a single bird. For each bird, 250 sequences were randomly sampled from each sample type, phylogenies constructed in ARB, and unique versus shared branch lengths compared using Unifrac as described in the methods section. All comparisons were highly significant ($p < 0.0001$; indicative of phylogenetic clustering) except for bird 4_5 shown in the lower right ($p = 0.054$). Results for weeks 3 and 6 showed similar results. Comparisons of genera significantly over-represented in fecal samples relative to cecal samples showed *Lactobacillus* and *Gallibacterium* were the most abundant while the genera *Bacteroides, Pseudoflavonifractor, Oscillibacter, Flavonifractor, and Subdoligranulum* were significantly more abundant in the ceca than in feces.

and only one bird had marginally ($p = 0.05$) different communities in fecal versus cecal samples while all other comparisons were highly significant ($p < 0.0001$; **Figure 1**). For each of the two other time points (3 and 6 weeks of age), the results were essentially identical with only one non-significant difference ($p = 0.09$) for one 6-week-old bird (data not shown). Bacteria inhabiting the ceca are clearly very different than those collected from fecal droppings excreted through the cloaca.

Several genera were identified with significantly different representations in fecal versus cecal samples using metastats (data not shown). At 1 week posthatch, two bacterial genera were significantly over-represented in fecal samples relative to cecal samples, *Lactobacillus* and *Gallibacterium*, present at 15- and 5-fold greater relative abundance respectively. In the ceca, the genera *Bacteroides, Pseudoflavonifractor, Oscillibacter, Flavonifractor, and Subdoligranulum* (the latter four all in the Clostridiales family) were significantly more abundant (2.5- to 3.5-fold) than in fecal samples. By 6-week posthatch, *Clostridium* and *Caloramator* (also a Clostridiales) sequence types had increased significantly in the cecum and *Lactobacillus* remained over-represented in fecal samples.

Temporal Changes in Microbiome

Next, to assess how the microbial communities in the ceca and feces change through time during the 6 weeks of growth to market age, we first clustered sequences with an ordination approach (correspondence analysis; cca) as described in Section "Materials and Methods." Because of the significant differences in the cecal versus fecal communities shown above, we performed these analyses separately for each sample type. For the cecal communities, the samples were clearly clustered according to bird age (**Figure 2A**) while the communities in the fecal samples were more variable with age-related differences less obvious (**Figure 2B**). Permutational ANOVA of the distance matrices used for these ordinations showed that bird age was a significant explanatory variable for the variance of both cecal and fecal communities while experimental pen had non-significant effects (**Tables 3** and **4**).

At a phylum level, clear changes could be seen in the microbial communities as the birds aged (**Figure 3**). At 1 week of age, *Bacteroides* were common in the ceca, ranging from 5 to 40% relative abundance (**Figure 3A**). In the feces, *Bacteroides* were less common and abundant with only 6/19 birds having >10% relative abundance of *Bacteroides* (**Figure 3B**). More than half of the birds had at least 10% Proteobacteria, with a maximum exceeding 80% in one bird (**Figure 3B**). By 3 weeks of age, the same two birds had >20% *Bacteroides* in the cecal and fecal communities, but in all other samples, Firmicutes exceeded 80% relative abundance (**Figure 3**).

Significant changes through time for both cecal and fecal communities were also observed in richness and diversity indices (**Figure 4**). At a 95% OTU level (roughly equivalent to a genus-level classification) there was a significant increase in both richness and diversity in 6-week-old birds compared to

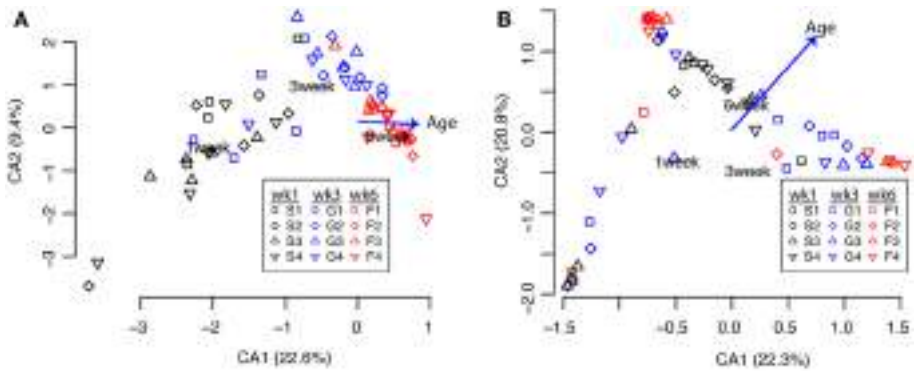

FIGURE 2 | Clustering of cecal [(A), $n = 59$] and fecal [(B), $n = 57$] communities by bird age (weeks 1, 3, 6) or pen (1, 2, 3, 4). Each point represents the community from a single bird using 95% OTU classifications as described in the text. Clustering and environmental fitting of bird age was performed with the cca function in R; labels indicate the centroids of each bird age with vectors indicating the direction and magnitude of influence of the bird age relative to the axes.

TABLE 3 | Permutational ANOVA results partitioning effects of bird age and experimental pen on microbial community composition as calculated at a 95% OTU cutoff as described in the text for cecal samples.

	Degrees of freedom	Sums of squares	Mean squares	*F*	Pr (>*F*)
Age	2	6.38	3.19	29.51	0.0001
Pen	3	0.50	0.17	1.53	0.0963
Age:pen	6	1.04	0.17	1.60	0.0410
Residuals	47	5.08	0.11		
Total	58	13.00			

TABLE 4 | Permutational ANOVA results partitioning effects of bird age and experimental pen on microbial community composition as calculated at a 95% OTU cutoff as described in the text for fecal samples.

	Degrees of freedom	Sums of squares	Mean squares	*F*	Pr (>*F*)
Age	2	3.31	1.66	5.95	0.001
Pen	3	1.02	0.34	1.23	0.204
Age:pen	6	2.12	0.35	1.27	0.124
Residuals	45	12.54	0.27		
Total	56	19.00			

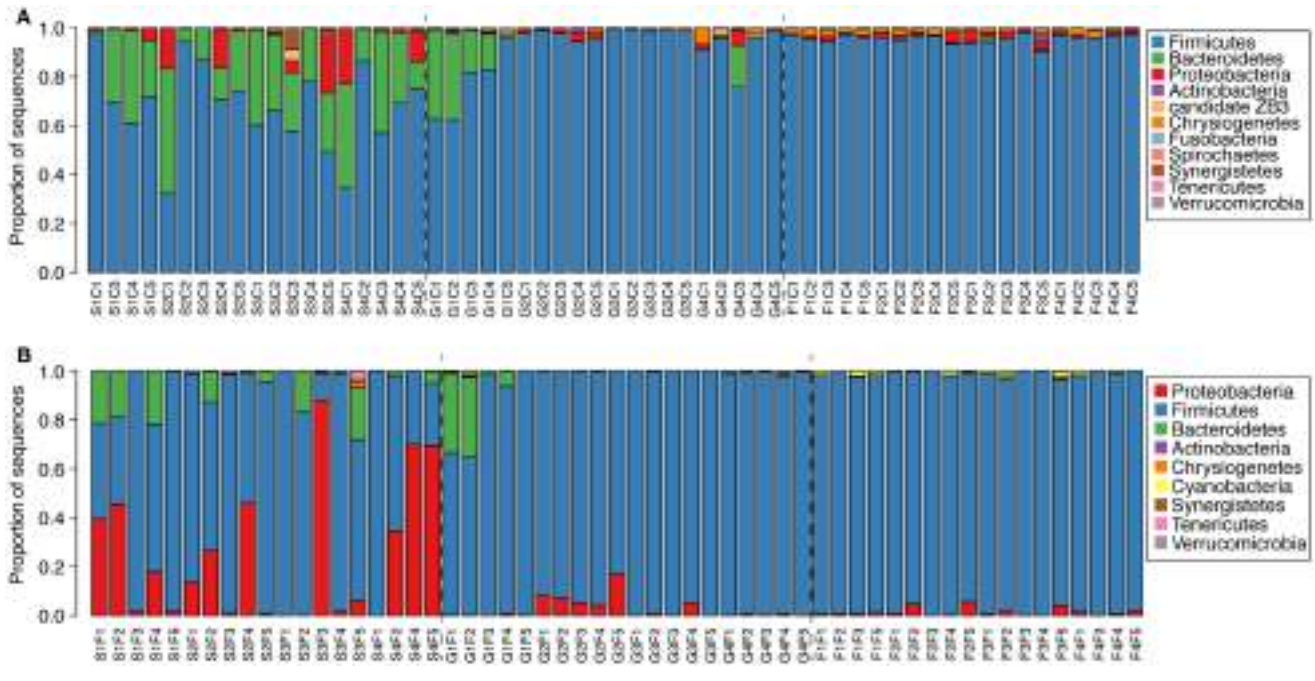

FIGURE 3 | **Phylogenetic classification of sequences from cecal [(A), *n* = 59] and fecal [(B), *n* = 57] samples from each bird at the phylum level**. Classifications were performed with the RDP taxonomy and naïve Bayesian classifier as described in the text.

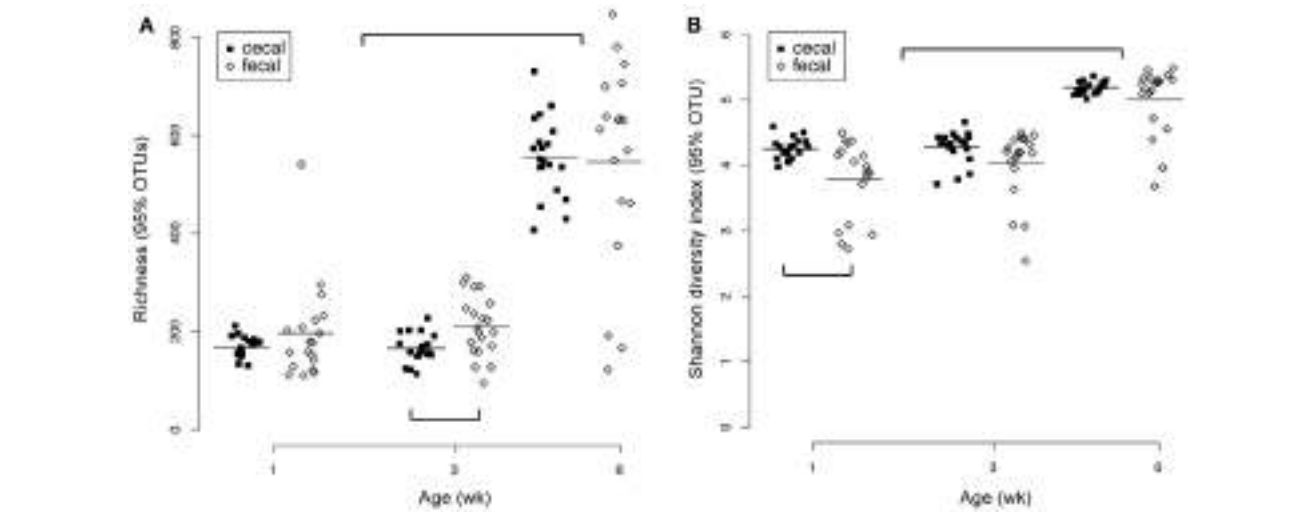

FIGURE 4 | **Richness (A) and diversity (B) measures for cecal (*n* = 59) and fecal (*n* = 57) samples**. Both metrics were calculated from 95% OTU cutoffs as described in the text. Significant differences (pairwise *t*-tests) were observed between cecal and fecal richness at 3 weeks and cecal versus fecal diversity at 1 week. Both richness and diversity were significantly higher at week 6 than weeks 3 or 1.

3- or 1-week-old birds (**Figure 4**). At each age, fecal and cecal samples had generally comparable richness and diversity (despite some significant differences in cecal richness at 3 weeks and fecal diversity at 1 week). Interestingly, inter-bird variability for both richness and diversity metrics was greater for fecal than cecal samples (**Figure 4**).

Temporal Changes in Cytokine Expression

Expression of the pro-inflammatory cytokine IL1β increased significantly from weeks 1 to 3 and then decreased significantly from weeks 3 to 6 (**Figure 5A**). IL6 expression was highest at week 1 and decreased significantly thereafter at weeks 3 and 6 (**Figure 5B**). The expression pattern of the Th1 cytokine IL18 was

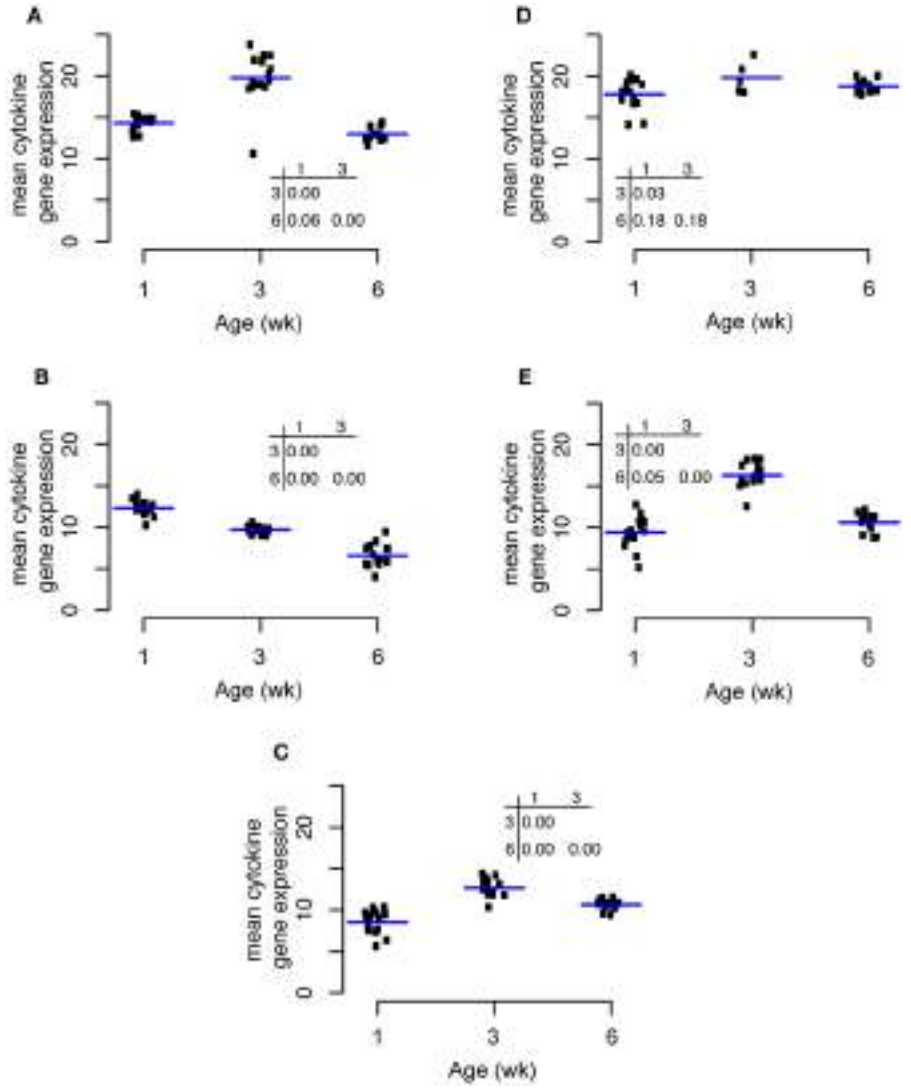

FIGURE 5 | Changes in cytokine expression through time for IL1β (A), IL6(B), IL18(C), TGF-β4 (D), and IL10 (E).

similar to that of IL1β with an increase from weeks 1 to 3 followed by a significant decrease from weeks 3 to 6 (**Figure 5C**). TGF-β4 expression was almost unchanged through the experiment with a small increase from weeks 1 to 3 (**Figure 5D**). Changes in IL10 expression through time were qualitatively similar to IL18 and IL1β with a maximum at week 3 (**Figure 5E**).

Correlations of Specific Taxa with Cytokine Expression

To search for correlations between specific taxonomic groups and expression of the five cytokines we measured, we first considered taxa at the phylum level. A data mining approach to the microbiome and cytokine data sets as described in the Section "Materials and Methods" revealed several correlations at this level (**Figure 6**). Because of the significant changes in community structure that occurred through time, each time point was considered separately. The relative abundance of Proteobacteria was positively correlated with the expression of IL1β, IL6, and IL18 at 6 weeks of age (**Figures 6A,B,D**). Firmicute relative abundance was negatively correlated with IL6 expression at 6 weeks (**Figure 6B**), IL18 expression at 1 week (**Figure 6C**), and TGF-β4 expression at 1 week (**Figure 6E**). Firmicute relative abundance was positively correlated with IL10 expression at week 3 (**Figure 6F**). The relative abundance of Bacteroidetes was positively correlated with TGF-β4 expression at 1 week (**Figure 6E**) and negatively correlated with IL10 expression at week 3 (**Figure 6F**).

At the genus level, all taxa that passed our correlation screens belonged to the Clostridiales family within the phylum Firmicutes. *Faecalibacterium* was negatively correlated with IL1β, IL18, TGF-β4, and IL10 at 1 week of age (**Figure 7**). The genus *Clostridium* was negatively correlated with IL1β and IL6 at week 6 (**Figure 7**). *Ruminococcus* was positively correlated with IL1β and IL6 expression at week 6

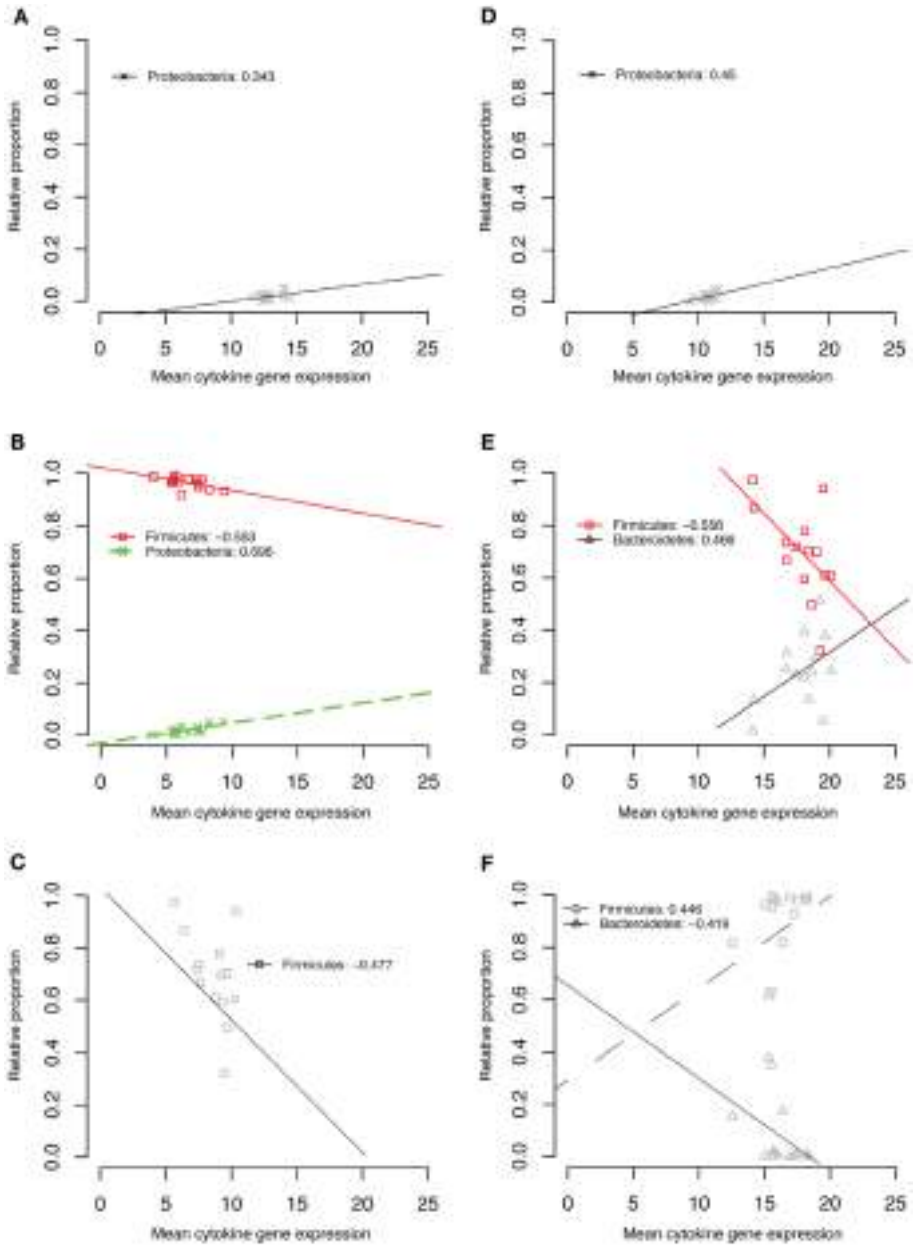

FIGURE 6 | IL1β week 6 (A), IL6 week 6 (B), IL18 week 1 (C), IL18 week 6 (D), TGF-β4 week 1 (E), IL10 week 3 (F).

(**Figure 7**). *Calorameter* was also negatively correlated with IL6 at week 6 (**Figure 7C**) and positively correlated with TGF-β4 expression at week 6 (**Figure 7F**). The genus *Butyricicoccus* was positively correlated with IL10 expression at both 1 and 3 weeks (**Figures 7G,H**).

DISCUSSION

The differences documented here between fecal and cecal samples and changes in both sample types as birds mature provide important data about the community composition of each sample type at specific points in the maturation of commercial broiler chickens. Our results highlight the importance of comparing communities using multiple levels of phylogenetic resolution. For example, the significant increase in richness and diversity at 6 weeks at a 95% OTU level was not apparent in the phylum-level classifications that were almost exclusively Firmicutes after week 1. Interestingly, the increase in richness and diversity between weeks 3 and 6 must therefore reflect diversification within the Firmicutes. Over 115 genera present at week 6 were absent in week 3, but these were all present only at very low abundance – only one genus [*Allisonella*, a Firmicute known to produce histamine from using histidine as a sole energy source (48)] comprised >0.5% average relative abundance in the

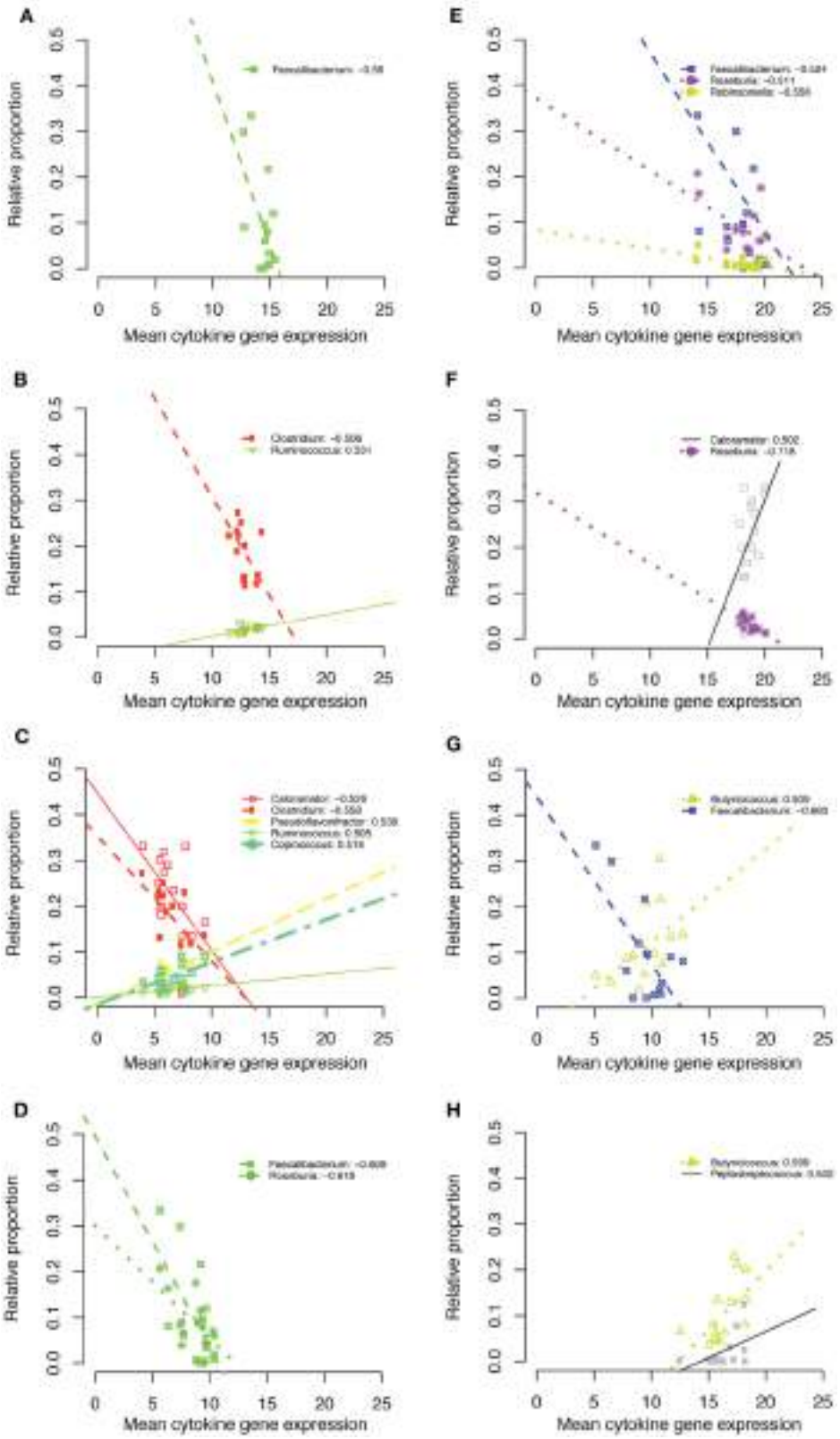

FIGURE 7 | IL1β week 1 (A), IL1β week 6 (B), IL6 week 6 (C), IL18 week 1 (D), TGF-β4 week 1 (E), TGF-β4 week 6 (F), IL10 week 1 (G), IL10 week 3 (H).

week 6 birds. Genera significantly more abundant at week 3 versus 1 were *Caloramator*, *Peptostreptococcus*, *Clostridium*, *Butyrivibrio*, *Faecalibacterium*, and *Oscillibacter*. The relative abundance of these genera increased from 2- to 127-fold between weeks 1 and 3.

This latter genus, *Oscillibacter* was the most abundant member of the week 6 community (42% average relative abundance). Interestingly, *Oscillibacter* belongs to Clostridium cluster IV that produces valerate as an end product of fermentation and has been identified as a "healthy biomarker" in a study of human patients with Crohn's disease (49) but also significantly associated with diet-induced obesity (50). It is now well established that various Firmicutes such as *Faecalibacterium* and *Subdoligranulum* are numerically abundant and proportionally dominant in the chicken cecum (51).

Phylogenetic comparisons of sequences between paired cecal and fecal samples from individual birds illustrated the significant differences between these two sample types. While specialization of microbial communities associated with anatomical region and physiological function of the chicken GI tract has long been noted (52), the data shown here give important new details about the magnitude and nature of these phylogenetic differences. As an anatomical chamber gated by the ileocecal valve, the cecum harbors a distinct and relatively homogeneous microbial community mediating anaerobic fermentations of cellulose and other substrates. In contrast, the material we collected as fecal droppings is by nature more variable after transit through the colorectum, reflecting the different environments of the GI tract, likely in different ways for each dropping. For example, the mixing of nitrogenous liquid waste with feces in the urodeum prior to excretion almost certainly influences the microbial community via changes in pH, etc. The differences in microbial community composition between fecal and cecal samples we observed within individual birds has important implications for food safety, animal health and nutrition or related research – collecting only one sample type will not give a representative picture of the GI tract and may miss pathogens or mischaracterize effects of a treatment on the community.

Correlations of the relative abundance of bacterial taxa with cytokine gene expression revealed some important associations. In all cases, Proteobacteria were correlated with a pro-inflammatory response, most strongly with IL6 expression at 6 weeks of age. Many human and animal pathogens such as *E. coli*, *Shigella*, *Salmonella*, and *Klebsiella* are Proteobacteria with well-established pro-inflammatory mechanisms. In our data, no genera within the Proteobacteria were significantly correlated with cytokine expression, but the most abundant genera within the group of Proteobacteria positively correlated with IL6 expression were sequences classified as *Escherichia/Shigella*, *Parasutterella*, and *Vampirovibrio*. This latter genus has an uncertain taxonomic classification and has recently been proposed as a Cyanobacterium with an *Agrobacterium tumefaciens*-like conjugative type IV secretion system (53). Many of our sequence reads classified as *Vampirovibrio* by the RDP classifier were designated by the Silva taxonomy as *Brevundimonas*, an organism not known to be pathogenic but resistant to fluoroquinolones (54).

Inverse relationships between Firmicute relative abundance and expression of pro-inflammatory cytokines (e.g., IL6, IL18) suggest a potential for inflammatory modulation by certain Firmicute taxa. In particular, the genus *Faecalibacterium* was inversely correlated with the expression of the classical pro-inflammatory cytokine IL1β and IL18. This genus has been noted repeatedly in human microbiome studies – for example, reductions in *F. prausnitzii* have been linked to Crohn's Disease, perhaps due to metabolites secreted by the bacterium blocking NF-Kβ activation and IL8 production (55). Several other Firmicute genera such as *Caloramator* were negatively correlated with pro-inflammatory (IL6) and positively correlated with anti-inflammatory (TGF-β4) cytokine expression, consistent with a growing body of evidence demonstrating positive influences of Firmicutes on gut health. However, it is important to keep in mind the diversity represented within a single bacterial phylum, as several Firmicute genera were positively correlated with expression of pro-inflammatory cytokines (**Figure 7**).

Harnessing the ability of the microbiome to affect host immunity would be an important immunotherapeutic alternative to antibiotic strategies currently used in poultry to improve performance and exclude pathogens. The work presented here is the first to try to identify commensals in poultry that are associated with immunomodulatory effects as has been previously done in mammalian systems (56–61). Further research is needed to ascertain whether the commensal taxa identified in this study as associated with cytokine signaling are actually immunomodulatory. However, the possibility to use organisms that are members of the commensal microbiota as immunomodulators is intriguing.

Though our data do not reveal mechanisms by which the taxa we identified may interact with the cecal cytokine signaling pathways, the "data mining" approach presented here may be particularly useful as a first step in screening complex communities for taxa with desirable (and undesirable) immunomodulatory properties. This may be particularly useful when testing the effects of feed additives or designing probiotic formulations.

In future studies, we anticipate high-throughput sequencing and associated bioinformatics approaches will continue to provide new insights into the structure and function of chicken GI microbial communities. The approach we took here was based on sequencing of 16S rRNA genes, but metagenomic studies of gene content (17, 18, 62) and transcriptomic studies of microbial gene expression will continue to offer additional insights into genetic potential and activity. We anticipate these approaches will become standard tools for assessing the impact of feed additives or probiotics on the chicken GI microbiome and host responses.

AUTHOR CONTRIBUTIONS

BO analyzed data and wrote the ms, MK designed experiments, analyzed data, and wrote the ms.

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Temporal Relationships Exist Between Cecum, Ileum, and Litter Bacterial Microbiomes in a Commercial Turkey Flock, and Subtherapeutic Penicillin Treatment Impacts Ileum Bacterial Community Establishment

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Gut health is paramount for commercial poultry production, and improved methods to assess gut health are critically needed to better understand how the avian gastrointestinal tract matures over time. One important aspect of gut health is the totality of bacterial populations inhabiting different sites of the avian gastrointestinal tract, and associations of these populations with the poultry farm environment, since these bacteria are thought to drive metabolism and prime the developing host immune system. In this study, a single flock of commercial turkeys was followed over the course of 12 weeks to examine bacterial microbiome inhabiting the ceca, ileum, and corresponding poultry litter. Furthermore, the effects of low-dose, growth-promoting penicillin treatment (50 g/ton) in feed on the ileum bacterial microbiome were also examined during the early brood period. The cecum and ileum bacterial communities of turkeys were distinct, yet shifted in parallel to one another over time during bird maturation. Corresponding poultry litter was also distinct yet more closely represented the ileal bacterial populations than cecal bacterial populations, and also changed parallel to ileum bacterial populations over time. Penicillin applied at low dose in feed significantly enhanced early weight gain in commercial poults, and this correlated with predictable shifts in the ileum bacterial populations in control versus treatment groups. Overall, this study identified the dynamics of the turkey gastrointestinal microbiome during development, correlations between bacterial populations in the gastrointestinal tract and the litter environment, and the impact of low-dose penicillin on modulation of bacterial communities in the ileum. Such modulations provide a target for alternatives to low-dose antibiotics.

Keywords: poultry, avian, Turkey, bacteria, penicillin, microbiome, antibiotic, growth promoter

INTRODUCTION

Turkey meat is one of the leanest meat sources of protein available, and its production is a multibillion dollar per year U.S. industry (1). Nearly 250 million turkeys are grown each year, making the U.S. the world's largest producer of turkeys and the biggest exporter of turkey products. The U.S. produces 7.5 billion pounds of turkey meat per year, and this number is steadily increasing.

The gastrointestinal health of an animal is key to its successful growth and development. For many years, gut health and development in U.S. commercial poultry has been routinely managed through the use of low-dose levels of antibiotics in feed to prevent diseases, improve overall flock consistency, and increase final body weights (2). Even with the use of low-dose antibiotics, gut health issues still occur. For example, turkey flocks are still plagued by a condition known as "Light Turkey Syndrome," or LTS (3, 4). LTS has not been attributed to any known pathogen or management practice (3), yet some farms yield market weights 1–3 pounds below the national average, using the same source of poults as farms achieving these weight goals. Higher doses of some antibiotics can alleviate these problems, but they present their own set of problems related to the development of antibiotic resistant bacterial pathogens that threaten both human and animal health. With an ongoing movement to withdraw the use of low-dose antibiotics on poultry farms, alternatives to antibiotics are greatly needed to sustain health and performance in commercial turkey flocks.

In the avian intestinal bacterial community, it is well established that great differences exist from a spatial (proximal to distal) standpoint (5). The chicken ileum and ceca alone are thought to harbor at least 10^8 and 10^{11} organisms per gram of digesta, respectively (6), and this density is achieved within days after hatch (6). However, the taxonomic composition of these microbes changes rapidly during the first week of development. Early studies involving denaturing gradient gel electrophoresis (DGGE) revealed that the chicken cecal microbiome, while quite diverse, is dominated by a small subset of conserved bacterial species in mature birds (7, 8). In general, the cecal microbiome is dominated by Clostridiales, and the small intestine is dominated by Lactobacillales (9). However, the avian microbiome is highly dependent on bird age, and there is great diversity at the bacterial species level (10). Most microbiome-based studies in both chickens and turkeys have focused on the microbiome related to carriage of pathogens (11–13). Because of a primary emphasis on pathogens in the more distal portions of the intestinal tract, fewer studies have examined the ileum microbiome. Pioneering work by Lu et al. examined the ileal and cecal bacterial communities of the chicken during bird development (14). They found that the broiler ileum was dominated by Lactobacillaceae, whereas the cecum was dominated by Clostridiaceae.

A number of studies have sought to examine the effects of antibiotic growth promoters (AGPs) on the intestinal microbiome. For example, virginiamycin and other AGPs applied in broilers were shown to exert the greatest modulatory effect on the proximal small intestinal microbiome correlating with increased average daily weight gain, as compared to the distal intestine and ceca (9, 15). Several AGPs [avilamycin, bacitracin methylene disalicylate (BMD), and enramycin] applied to broilers housed in floor pens resulted in improvements in growth performance, grossly correlated with changes to the intestinal microbiota (16). An interesting result validated through multiple studies is that AGP treatment decreased bacterial diversity in the avian ileum and decreased *Lactobacillus* populations (17–19). AGPs also appear to decrease bird-to-bird variations in weight and performance (20).

Penicillin G procaine has been shown to enhance weight gain and feed efficiency in commercial poultry (2). However, the underlying mechanisms by which administration of penicillin in feed is effective have not been fully examined. The purpose of this study was to define the baseline correlations between the bacterial populations inhabiting the turkey cecum, ileum, and surrounding litter environment and to assess the impact of penicillin in feed on the ileum bacterial microbiome of turkeys during the early brood period when the turkey gastrointestinal microbiome is most dynamic (10).

MATERIALS AND METHODS

Study Design

All studies were performed on commercial turkeys; therefore, ethical standards for commercial turkey production were followed by the company performing the study. Animals were euthanized using methods approved by the American Veterinary Medical Association. Two trials were performed at a commercial turkey research facility in Willmar, Minnesota. The barn was divided into 24 pens, with 16 pens on one half of the barn each housing 1,500 turkey poults, and 8 pens on the other half of the barn each housing 3,000 birds. Feed was mixed and supplied using a Feedlogic robot (Feedlogic Corporation, Willmar, MN, USA). In the first trial, Hybrid Converter female poults (Willmar Poultry Company, Willmar, MN, USA) were placed at day-of-hatch into a pen housing 3,000 birds. At days 7, 14, 21, 28, 35, 42, 56, 70, and 84, five birds were randomly selected and euthanized. For this trial, birds were moved to a commercial grow-out facility at 5 weeks and were separated from other commercial birds by fencing within the grow-out barn. They were subsequently raised under standard commercial turkey management practices. The following measurements were taken from each bird sampled: total body weight, intestinal weight, intestinal length, and cecal score. Cecal score was recorded throughout the study by a single person blinded to the experimental groups. The scoring system ranged from 0 to 3, based upon consistency, color, and gas present in the cecal contents. A score of 0 indicated pasty and dark brown cecal content; a score of 1 indicated changes in consistency toward watery but still dark brown content; a score of 2 indicated changes in color and consistency of the cecal contents toward watery and yellow color; and a score of 3 indicated watery, gassy, and yellow-colored cecal content. Both ceca and the ileum from euthanized poults were aseptically collected intact, homogenized, and frozen at −20°C for future processing. Grab litter samples of representative bedding from each group were aseptically collected in whirl-pak bags at the same time points. Each litter sample collected was composed of a pool of five samples collected randomly from dry areas in the barn not including fecal or cecal droppings.

In the second trial, four pens were used, including two replicates each of control and treatment groups. Birds were immediately placed on standard feed containing 50 g/ton of BMD for control birds, and 50 g/ton BMD plus 50 g/ton penicillin G procaine for treatment birds. At days 7, 14, and 21, five birds per pen were randomly selected and euthanized. The following measurements were taken: total body weight, intestinal weight, intestinal length, and cecal score. Ilea from euthanized poults were aseptically collected intact, homogenized, and frozen at −20°C for future processing.

Sample Processing and Sequencing

DNA was extracted using a bead-beating procedure and the QIAmp® DNA Stool Kit (Qiagen, Valencia, CA, USA) as previously described (21). PCR was used to amplify the V3 hypervariable region of the 16S rRNA gene using primers containing Illumina barcoding and sequencing primers, as well as sample-specific barcodes on the reverse primers, as previously described (22). The PCR conditions used were an initial denaturation step at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR product was excised from a 1.5% gel and purified using the QIAquick Gel Extraction Kit following manufacturer's instructions (Qiagen). Sample DNA quality and quantity were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA-1000 lab chip. Sequencing was performed at the University of Minnesota Genomics Center using Illumina MiSeq paired-end 2 × 250 bp technology.

Data Analyses

Following sequencing, sorting by barcode was performed to generate fastq files for each sample. Paired-end reads were assembled and quality screened using Pandaseq, using a threshold quality cut-off value of 0.6 and eliminating any assembled reads with ambiguous base calls (23). Proximal and distal primers were trimmed from the sequence reads. Random subsets of 20,000 high-quality reads per sample for Trial #1 and 40,000 reads per sample for Trial #2 were selected using the sub.sample approach in Mothur (24). In total, 45 cecum samples, 45 ileum samples, and 16 pooled litter samples were analyzed from Trial #1 (2.12 million reads), and 30 ileum samples were analyzed from Trial #2 (1.2 million reads). A *de novo* operational taxonomic unit (OTU) picking approach was used in QIIME (25) using uclust (26) independently for each dataset (Trial #1 and Trial #2). OTUs containing <25 sequences were removed to eliminate possible spurious OTUs due to sequencing error. Potential chimeras were removed using ChimeraSlayer (27). QIIME was used for assessments of alpha diversity, beta diversity using Unifrac (28), and phylogenetic classifications using the RDP database (29). Differential abundances of OTUs and other phylogenetic classifications were identified using METASTATS (30). Construction of heatmaps was performed using the R statistical software (31). Statistical analyses for differences in community structure were performed using distance matrices analyzed via the AMOVA command in Mothur (24). A paired two-sample *t*-test was used to statistically compare total body weights, intestinal weights, intestinal lengths, and cecal scores at each time point during trial #2.

The data from this project is freely available at the Data Repository for the University of Minnesota (DRUM) at the following link: http://hdl.handle.net/11299/174930.

RESULTS

Trial #1: Relationships Between Cecum, Ileum, and Litter Bacterial Microbiomes During Flock Succession

In Trial #1, 38,861 OTUs were identified using open reference OTU picking. Our goal was to assess the dominant bacterial populations and avoid spurious OTU calling due to sequencing error. After removing OTUs with <25 total sequencing reads, 1,101 OTUs remained in the dataset. Using the greengenes database, OTUs were classified at the bacterial class level (**Figure 1**). In cecal samples, Clostridia was the dominant class (>70% of the population) throughout trial 1 with lower levels of Bacteroidia (5–20% of the population) appearing at 28 days and beyond. Levels of Gammaproteobacteria remained low throughout, typically <1% of the total population. In contrast, Bacilli was the dominant bacterial class in ileum samples throughout trial 1 (50–90% of the population), with levels of Clostridia increasing (0–75% of the population) throughout the study. Gammaproteobacteria were high (up to 50% of the population) in ileum samples at day 7, then decreased substantially in the subsequent weeks. Actinobacteria also increased in abundance in ileum samples (5–30% of the population) at day 28 and beyond. Litter samples were distinct from both ceca and ileum in terms of bacterial class composition, but more closely represented ileum samples than ceca samples. Notably, Gammaproteobacteria and Actinobacteria were of substantially higher relative abundance in litter samples at day 7 and days 21–56, respectively.

Using an OTU-based approach, bacterial species richness was highest in ceca samples throughout the study, followed by litter and ileum samples (**Figure 2**). Species richness increased in samples through 35 days, when the birds were moved to a commercial grow-out barn. At day 42, following movement to the grow-out barn and change in feed to reduced protein composition, bacterial species richness decreased in the ceca and litter samples but remained the same in ileum samples. At day 56, bacterial species richness in these samples increased to pre-movement levels or greater. Ileum and ceca samples continued to increase in species richness through day 84. Using a non-parametric two-sample *t*-test with Bonferroni correction, alpha diversity measurements were significantly different ($P = 0.003$) comparing ileum versus cecum samples ($P = 0.003$) and litter versus cecum samples ($P = 0.003$), but not ileum versus litter samples.

Community-level similarities in the bacterial microbiome of samples were compared using principal coordinates analysis (PCoA; **Figure 3**). Samples were stratified primarily by age of flock, and also separated by sample type. Using AMOVA based upon distance matrix, bacterial communities from all three sample types were distinct ($P < 0.001$). Upon visualization of the PCoA plots, litter and ileum samples had some overlap, while both of these sample types were clearly distinct from cecal samples. However, all sample types shifted similarly over time on the plot, indicating that bird age has a predominant impact on the barn bacterial microbiome irrespective of sample type.

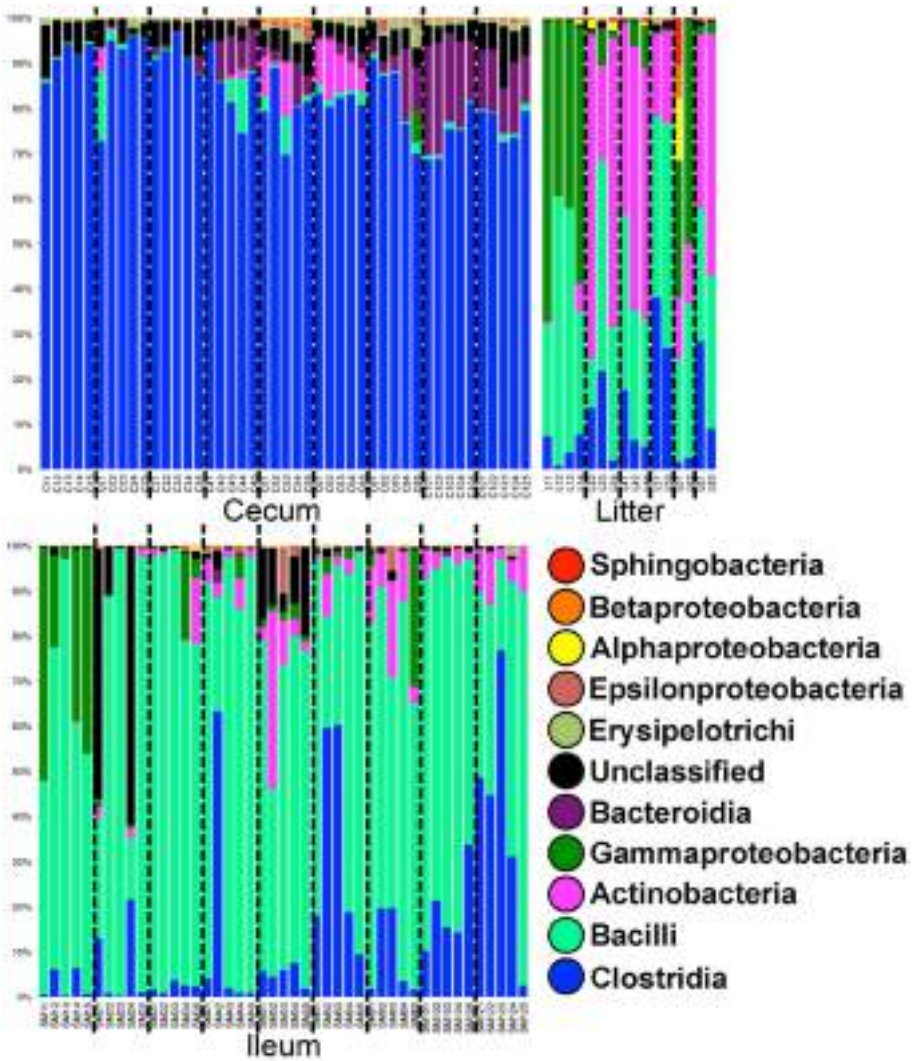

FIGURE 1 | Class-level taxonomic compositions of bacterial microbiome samples in Trial #1. For each sample type (ileum = SM, litter = L, and cecum = C) individual samples are depicted by age (weeks 1–12) followed by bird number (1–5). Black dashed lines divide samples by age.

Samples were also analyzed at the OTU level for specific subsets of OTUs representing sample types and age (Figure S1 in Supplementary Material; **Figure 4**). It was clear from this analysis that there were shared subsets of OTUs present across all samples, unique subsets that were defining of a particular sample type(s), and OTUs that were dependent on flock age. For example, numerous OTUs were identified belonging to the phylum Firmicutes and class Clostridia that were dominant in cecal samples, and present in ileum and litter samples but at much lower abundance. Similarly, OTUs belonging to phylum Firmicutes and class Bacilli were dominant in ileum and litter samples but present in cecal samples at much lower abundance. OTUs classified at the genus level such as *Brachybacterium*, *Brevibacterium*, *Staphylococcus*, *Corynebacterium*, *Jeotgalicoccus*, and *Weissella* were found in ileum and litter samples, but were absent from cecal samples. An OTU that we had previously identified as Candidatus division Arthromitus (10) was identified in ileum samples but not found in litter or cecal samples. Finally, some OTUs displayed a temporal trend and were found more prominently in later-aged samples, such as those classified as *Lactobacillus aviarius*, *Lactobacillus johnsonii*, Clostridium group XI, *Megamonas*, and *Lactobacillus ingluviei*. OTU-based clustering of the sample confirmed what was observed with PCoA-based analysis, with cecum samples clearly separating from ileum/litter samples which contained considerable overlap in bacterial microbiome composition (Figure S2 in Supplementary Material).

Trial #2: Effects of Low-Dose Penicillin Treatment on the Ileum Bacterial Microbiome

The effect of low-dose penicillin (50 g/ton) in feed on commercial turkey poults was examined in a second trial. Total bird weights were significantly higher at days 14 and 21 in the penicillin-treated

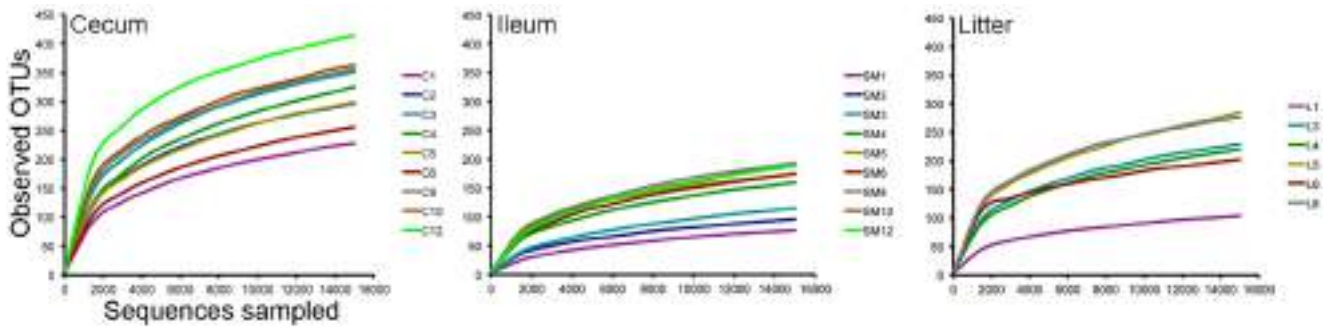

FIGURE 2 | Rarefaction curves of cecum, ileum, and litter samples. Legends refer to sample type (C = cecum, SM = ileum, L = litter) and weeks of age (1–12) for each sample type.

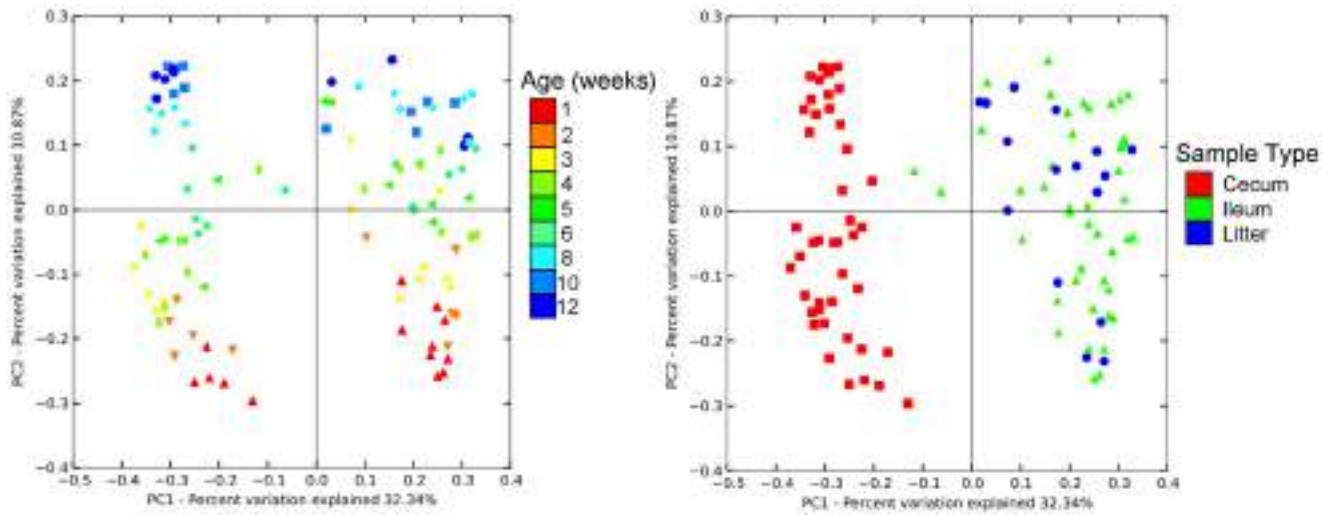

FIGURE 3 | Principal coordinates analysis (PCoA) of individual samples from turkey cecum, ileum, and litter within a single flock. Left image depicts PCoA by flock age, and right image depicts PCoA by sample type.

groups compared to control groups (**Table 1**; $P < 0.05$). Intestinal weights and lengths were also significantly higher in penicillin-treated groups at day 21 ($P < 0.05$), but cecal score was unaffected (**Table 2**).

Analysis of the ileum bacterial microbiome was performed on five individual birds per treatment group and time point to determine if there were shifts in the microbiome associated with penicillin treatment. In total, 7,857 OTUs were identified from these samples using open reference OTU picking. After removing OTUs with <25 total sequencing reads, 786 OTUs remained in the dataset. OTUs were examined by treatment group and flock age (**Figure 5**), and hierarchical clustering suggested that penicillin treatment had effects on the ileum bacterial microbiome at weeks 2 and 3. AMOVA also revealed significantly different community structures in the control versus penicillin-treated groups at days 14 ($P = 0.003$) and 21 ($P < 0.001$). Using METASTATS comparison, OTUs that were of significantly higher abundance ($P < 0.05$) at days 14 and 21 in penicillin-treated groups included those classified as *L. aviarius*, *L. johnsonii*, *Streptococcus* sp., and several other *Lactobacillus* spp. that were unclassified beyond genus level (Data Sheet S1 in Supplementary Material). A PCoA plot confirmed bacterial community differences between control and treatment groups, with day 14 and day 21 samples shifted on the plot in the penicillin-treated groups (**Figure 6**). Shannon diversity and species richness were also assessed, and at days 7 and 14, the penicillin-treated groups were significantly increased compared to the control groups ($P < 0.05$) (Figure S3 in Supplementary Material).

DISCUSSION

Recent bans on the use of AGPs in the European Union (EU) broiler industry have been associated with numerous production-associated problems, including decreased feed

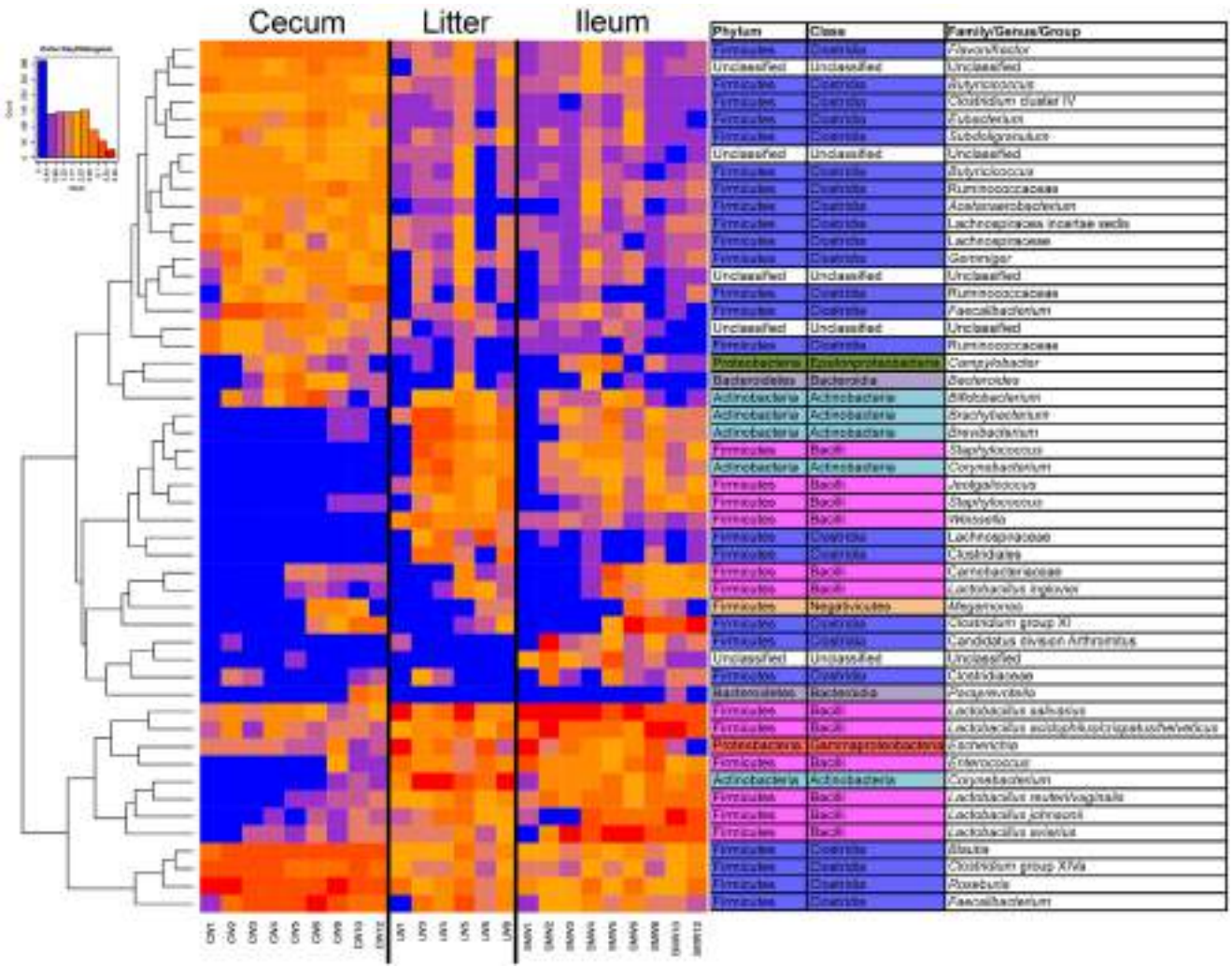

FIGURE 4 | Heatmap depicting abundance of the top 50 OTUs by overall abundance across samples, averaged by age/sample. Heatmap was constructed using normalized $\log_{10}$ abundance of each OTU in each sample type. To the right of the heatmap is a table depicting classification of each OTU using RDP database assignment or best-hit classification where appropriate. "W" depicts the age of sample in weeks.

TABLE 1 | Total body weights of turkeys treated with and without 50 g/ton of penicillin in feed.

	Day 7 weight (g)		Day 14 weight (g)		Day 21 weight (g)	
	Control	Penicillin	Control	Penicillin	Control	Penicillin
Average weight replicate 1	148.4	156.4	338	390.8[a]	665.8	779.8[a]
Average weight replicate 2	141.6	152.4	302.6	378.4[a]	664.4	736.8[a]
Standard deviation overall	21.6	18.0	54.1	44.5	111.2	59.4

[a]*Significantly different from control group ($P < 0.05$) using Student's t-test.*

efficiency, watery feces, and disease, among other conditions (32). Considering this, and that supplementation of antibiotics in poultry feed is a highly controversial issue (33), it is apparent that alternatives with similar modes of action are greatly needed. In order to identify these alternatives, we need to also understand the mechanisms by which antibiotic usage results in accelerated weight gain.

The purpose of Trial 1 was to examine the relationship between bacterial populations in the ileum, cecum, and litter within one flock over time. Age was a key factor in population shifts across all sample types, which has also been previously observed in turkey cecum and ileum studies (10, 21). Several OTUs were found predominantly in later time points across samples, including several classified *Lactobacillus* spp (*L. aviarius*, *L. johnsonii*, and

TABLE 2 | Intestinal measurements and cecal scores of turkeys with and without 50 g/ton of penicillin in feed.

		Cecal score	Intestinal length (cm)	Intestinal weight (g)
Day 7	Control	1.5 ± 0.8	93.0 ± 6.4	18.2 ± 2.2
	Penicillin	1.5 ± 0.4	95.1 ± 6.4	16.5 ± 2.1
Day 14	Control	2.4 ± 0.5	122.3 ± 9.9	35.2 ± 5.5
	Penicillin	2.0 ± 0.7	127.4 ± 6.2	37.1 ± 3.7
Day 21	Control	1.6 ± 0.6	148.1 ± 7.4	52.0 ± 6.4
	Penicillin	1.8 ± 0.5	158.8 ± 10.8[a]	65.9 ± 11.9[a]

[a]*Significantly different from control group ($P < 0.05$) using Student's t-test.*

L. ingluviei), which have been previously identified as key marker species of turkey ileum microbiome succession (10). In addition, *Clostridium* group XI was another OTU increasing in abundance with age of the flock; this diverse group includes *Eubacterium* and *Peptostreptococcus* spp, as well as *Clostridium bartlettii* (34), which may be another indicator of gut microbiome succession (10), in part due to the high ability of this species to ferment aromatic amino acids in the gut (35).

Sample type appeared to be another dominant factor affecting bacterial populations, with clear distinctions between the ileum and cecum populations; the litter samples were also distinct, but

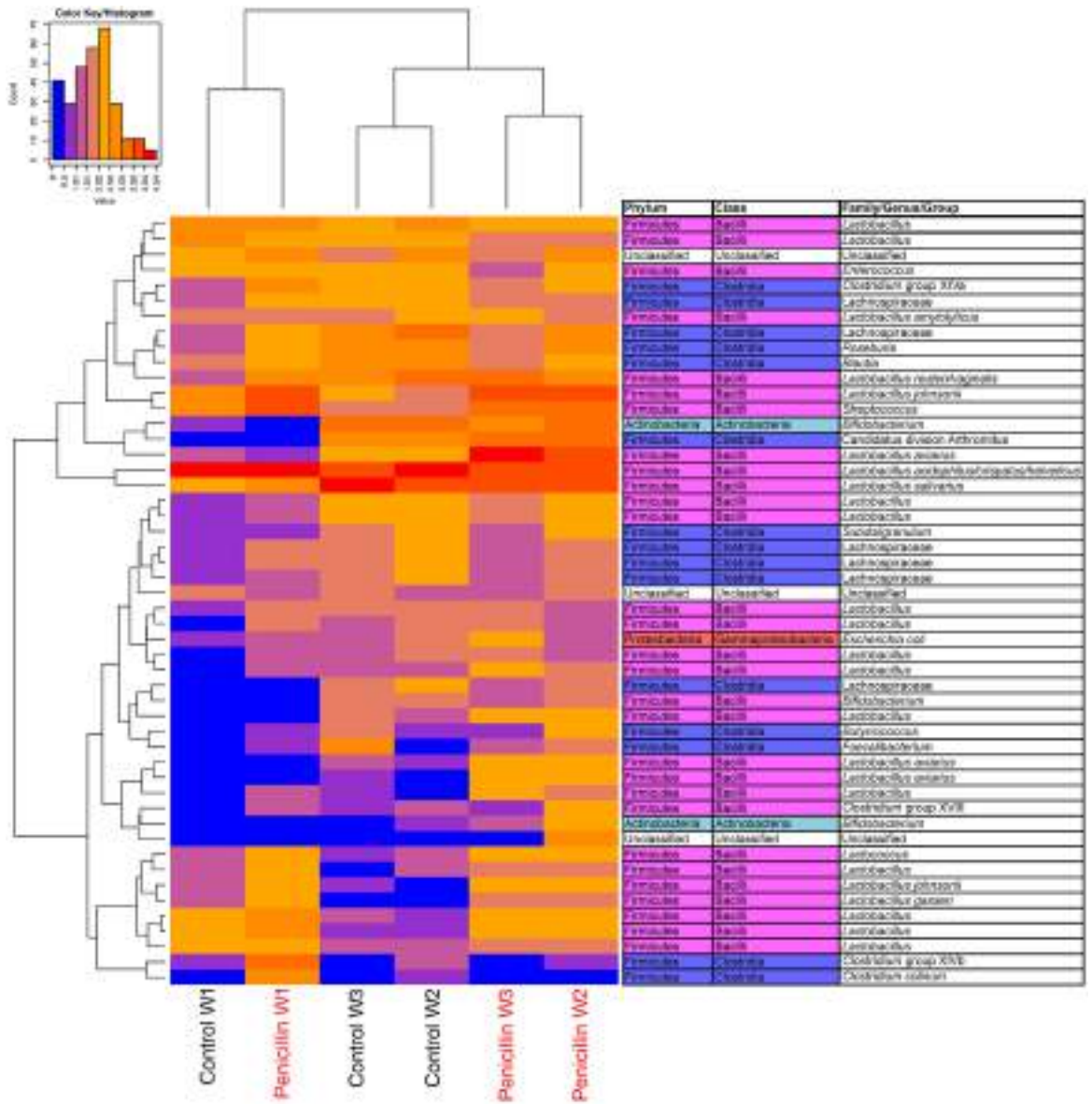

FIGURE 5 | Heatmap depicting abundance of the top 50 OTUs by overall abundance across samples, averaged by age/sample. Heatmap was constructed using normalized $\log_{10}$ abundance of each OTU in each sample type. To the right of the heatmap is a table depicting classification of each OTU using RDP database assignment or best-hit classification where appropriate. "W" depicts the age of sample in weeks. Control = birds receiving standard feed with BMD, penicillin = birds receiving BMD plus penicillin in feed.

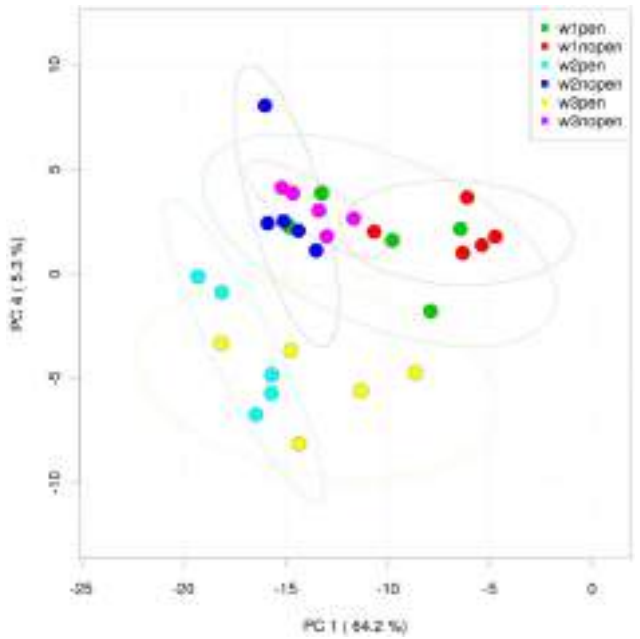

FIGURE 6 | Principal coordinates analysis (PCoA) of individual ileum samples from penicillin-treated versus control groups. "W" = flock age in weeks; "pen" = penicillin-treated samples; "nopen" = control samples.

more closely represented the ileum. The differences between the cecum samples and ileum and litter samples were largely within the Firmicutes phylum; more specifically, Clostridia OTUs were enriched in the cecal samples, whereas Bacilli were more abundant in the ileum and litter. Several identified OTUs were present only in ileum and litter samples, including *Brachybacterium*, which was first detected in poultry litter (36), and *Brevibacterium*, a soil bacterium that has been used as an indicator organism for poultry waste contamination in the environment (37). Other OTUs identified only in the ileum and litter were *Staphylococcus*, *Corynebacterium*, *Jeotgalicoccus*, the latter of which is a lactic acid bacterium also found in the air in poultry houses (38), and *Weissella*, a genus reported in high abundance in healthy birds (39). A recently published study found that cecal content populations reflect cecal drop, whereas fecal drop populations were dissimilar to cecal populations, further suggesting that ileum populations would be more closely related to litter than to cecal community structure (40). However, another study found that the fecal microbiome represents a large portion of the cecal diversity, though it was not a good quantitative measure (41). The birds in the present study were placed on clean litter for the brood period (first 5 weeks), suggesting that the ileum microbiome influenced the litter microbiome; however, before week 6 the birds were moved into a commercial finisher barn setting, in which the bedding was mostly reused with just a thin layer of clean litter, which could have resulted in the litter microbiome influencing ileum populations from weeks 6 to 12. The reasons for litter more closely reflecting ileum are likely due to less frequent cecal discharge and a litter growth environment that would better support facultative anaerobes compared to strict anaerobes.

One OTU that was found only in the ileum samples was classified as Candidatus division Arthromitus, a segmented filamentous bacteria (SFB) previously reported to play a potential role in gut health in turkeys and other animals (10, 42). Little is known about this bacterium, as it has only recently been sequenced (43) and grown *in vitro* (44). SFBs are thought to be host-specific as they have reduced genomes and rely heavily on host metabolic functions, and very little is known about the turkey-specific strains; however, these bacteria have been reported to play a role in early innate immune system development in mice as well (45). Overall, while temporal succession of bacterial populations was observed in this flock similar to previous studies (10), differences in sample type were more prominent. In addition, our results corroborate findings that poultry fecal droppings or litter samples are a better predictor of ileal rather than cecal bacterial composition (40).

It is a well-known fact that antibiotics are commonly used as a feed additive in poultry operations as a way to enhance the growth of birds to reach market weights faster (46). In previous studies, supplementation of subtherapeutic levels of several antibiotics, such as penicillin, to poultry broiler feeds have been associated with increases in weight gain (9). Through our study, we have also shown that antibiotic usage, specifically penicillin combined with BMD, results in significantly increased weight gain in turkey broilers up to at least 3 weeks. A limitation of the study was that BMD was used in the control groups, thus the sole effects of penicillin on the microbiome were not identified in this study. However, the scenario used in these experiments reflects commonly applied practices in the turkey industry, so it is more relevant as a real-life application.

While limited information currently exists, hypotheses aimed at explaining mode of action of AGPs have been proposed, including shifts in microbiome composition in gastrointestinal tract act to improve feed efficiency (32). In our study, the relative increase in weight gain in penicillin-supplemented broilers as compared to control groups can be temporally correlated with shifts in microbiome composition. Given our findings, we believe mining the microbiome is a means for finding potential replacements for AGPs, such as by identifying specific bacterial taxa responsible for improving feed efficiency, which can be used as probiotics.

Of the probiotics that have been investigated as growth promoters in poultry, many have included *Lactobacillus* spp. (47). In our study, relative abundances of *Lactobacillus* spp., including *L. johnsonii* and *L. aviarius*, were higher in penicillin-supplemented groups as compared to control groups. In terms of growth promotion, *Lactobacillus* spp. have been associated with both beneficial and detrimental effects. One mechanism whereby lactobacilli have been shown to decrease weight gain in a pig model is through the production of bile salt hydrolase (BSH) (48). On the contrary, *Lactobacillus* spp. has been demonstrated in broiler chicks to antagonize pathogenic bacteria, thus resulting in weight gain (47). *Lactobacillus* spp., specifically *L. johnsonii*, has been shown to possess antibacterial activity against pathogenic bacteria (49), and Zulkifli et al. showed that feeding lactobacilli cultures to broiler chicks resulted in increased weight gain (50), comparable to feeding oxytetracycline. It seems plausible that supplementing poultry feeds with appropriate lactobacilli cultures could serve as an alternative method for improving feed efficiency in poultry flocks.

Few studies have investigated the ileum bacterial community structure focusing on commercial turkeys. Our data suggest that low-dose penicillin treatment has a discernable impact on ileum bacterial community structure. The initial effects during the first week of age increase bacterial diversity in the ileum, and subsequent effects apparently drive the ileal microbiome composition toward a state correlating with significant enhancements of body weight (10). Some important factors remain to be examined, such as the effects of AGP treatment on total microbial biomass and on the turkey immune system. Since penicillin treatment modulates the turkey ileal microbiome in a fashion similar to that previously observed between commercial turkey flocks with differing weight outcomes, modulating the ileal microbiome similarly using antibiotic-free approaches may provide an alternative approach by which to enhance performance and prevent disease in the commercial bird.

AUTHOR CONTRIBUTIONS

TJ and BM conceived the study. JD and BM performed the experiments. JC, HH, DK, and TJ analyzed the data. TJ, JC, JD, and SH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fvets.2015.00056

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Salmonella enterica Serovars Enteritidis Infection Alters the Indigenous Microbiota Diversity in Young Layer Chicks

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Avian gastrointestinal (GI) tracts are highly populated with a diverse array of microorganisms that share a symbiotic relationship with their hosts and contribute to the overall health and disease state of the intestinal tract. The microbiome of the young chick is easily prone to alteration in its composition by both exogenous and endogenous factors, especially during the early posthatch period. The genetic background of the host and exposure to pathogens can impact the diversity of the microbial profile that consequently contributes to the disease progression in the host. The objective of this study was to profile the composition and structure of the gut microbiota in young chickens from two genetically distinct highly inbred lines. Furthermore, the effect of the *Salmonella* Enteritidis infection on altering the composition makeup of the chicken microbiome was evaluated through the 16S rRNA gene sequencing analysis. One-day-old layer chicks were challenged with *S.* Enteritidis and the host cecal microbiota profile as well as the degree of susceptibility to *Salmonella* infection was examined at 2 and 7 days post infection. Our result indicated that host genotype had a limited effect on resistance to *S.* Enteritidis infection. Alpha diversity, beta diversity, and overall microbiota composition were analyzed for four factors: host genotype, age, treatment, and postinfection time points. *S.* Enteritidis infection in young chicks was found to significantly reduce the overall diversity of the microbiota population with expansion of *Enterobacteriaceae* family. These changes indicated that *Salmonella* colonization in the GI tract of the chickens has a direct effect on altering the natural development of the GI microbiota. The impact of *S.* Enteritidis infection on microbial communities was also more substantial in the late stage of infection. Significant inverse correlation between *Enterobacteriaceae* and *Lachnospiraceae* family in both non-infected and infected groups, suggested possible antagonistic interaction between members of these two taxa, which could potentially influences the overall microbial population in the gut. Our results also revealed that genetic difference between two lines had minimal effect on the establishment of microbiota population. Overall, this study provided preliminary insights into the contributing role of *S.* Enteritidis in influencing the overall makeup of chicken's gut microbiota.

Keywords: *Salmonella* Enteritidis, chickens, gut microbiota, 16S rRNA, MHC haplotypes

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INTRODUCTION

The avian gastrointestinal (GI) tract is home to complex and diverse bacterial populations that provide many beneficial functions to host, which includes conferring colonization resistance against the invading pathogenic microorganisms. Development of the GI microbiota in chickens occurs immediately after hatching and is influenced by both genetic and external factors like diet and environment (1). Unlike other animals, a newly hatched chick does not have acquired healthy maternal microbiota as they are housed separately from the adult hens immediately after hatch in commercial production (2). Therefore, the GI tract of newly hatched chickens is usually sterile and presents an empty ecological niche that provides easy access for the pathogen to colonize with limited restriction (2). This factor alone makes young chickens highly susceptible to enteric bacterial infections, such as *Salmonella*, which can result in different degrees of disease spectrum from a subclinical carrier state to a high mortality rate depending on the infecting bacterial serovar and host's susceptibility.

Salmonella enterica subsp. *enterica* serovar Enteritidis is a zoonotic enteric pathogen that is most frequently associated with diarrheal disease in humans while chickens serve as asymptotic carrier (3). Consumption of contaminated eggs produced by infected layer hens is one of the leading causes of *Salmonella* food poisoning in humans (4). In chickens, *S.* Enteritidis can be easily transmitted horizontally via the fecal–oral route as well as vertically via the reproductive tract, which can contaminate the egg (5). Additionally, chickens can also harbor *S.* Enteritidis asymptomatically and persist throughout their lifespan, which makes the identification of infected chickens and the eradication of the pathogen much more challenging. Young chickens can be exposed to *S.* Enteritidis through numerous external sources like contaminated feed or environment. The sterile GI tract of the newly hatched chickens also provides ample opportunities for a pathogenic organism like *S.* Enteritidis to firmly establish its own niche in the gut as early colonizer and potentially further impact the development of the gut microbiota during the disease state. Early exposure to *Salmonella* in young chick could result in two potential alternative outcomes: high mortality rate or persistence of infection in surviving chickens (6). Prolonged persistent infection with *S.* Enteritidis in the GI tract of chickens throughout their lifespan could alter the development of gut microbiota and have detrimental effect on the overall gut health of the chicken host.

The impact of genetic background on the composition of chicken gut microbiota has been mostly investigated in broilers due to the association of intestinal microbiota with performance of broiler chickens in terms of feed conversion efficiency (7–11). Studies in broiler chickens have indeed shown evidence that host genotype had significant impact on shaping the composition of the gut microbiota (7, 9, 11). Few studies had explored the relationship between the host genotype and its influence on microbiota composition in layer chickens, especially related to disease resistance. The host genetic background plays an important role in the resistance and susceptibility to *Salmonella* infection (12). Several studies have reported that many genes have been found to be associated with *Salmonella* resistance in the chicken (6, 13). One of the key candidate genes, known as major histocompatibility complex (MHC), plays an important role in disease resistance in the chicken (13–20). University of California, Davis (UCD) maintains a number of congenic layer lines differing in MHC B-complex haplotypes. A study by Cotter et al. had previously examined the association of B-complex immunity to *S.* Enteritidis using 12 congenic lines from UCD, differing in various B-complex haplotypes (13). Results from the study had suggested that chickens from UCD254 (B^{15}/B^{15}) were more susceptible to *Salmonella* infection compared to other lines in term of mortality and morbidity (13). However, underlying mechanism associated with susceptibility to *Salmonella* remains to be elucidated. As microbiota is a significant contributor to disease resistance, two highly inbred line UCD254 (B^{15}/B^{15}) and UCD077 (B^{15}/B^{16}) at UCD were used to examine MHC effect on microbial community in chicken intestinal gut.

The main objective of this study was to examine the impact of host genetic background on influencing early establishment of microbiota in combination with *S.* Enteritidis infection to determine *S.* Enteritidis-associated alteration in gut microbiota.

MATERIALS AND METHODS

Experimental Animals

Two genetically distinct, highly inbred layer chickens from line UCD077 and UCD254 were obtained on the day of hatch from UCD's poultry farm. A cloaca swab was performed to ensure all birds were *Salmonella*-free. The chickens were then transferred and housed in the temperature-controlled chambers with *ad libitum* access to water and commercial feed without antibiotic treatment. At 1 day of age, chickens were orally inoculated with 1×10^8 c.f.u of *S.* Enteritidis TN2 nalidixic acid-resistant strain (kindly provided by Dr. Andreas Baumler) or PBS for the non-infected birds. Dosage of *S.* Enteritidis was confirmed by serial dilution plating of the inoculum. A total of three replicate trials were conducted. For the duration of the trials, all non-infected chickens were housed together in the concrete floor pen with fresh, wood shaving for bedding material inside the environmental chamber. The infected group of chickens was housed separately in another chamber with the same environmental conditions as the control chamber. At 2 and 7 days postinoculation (DPI), chickens were euthanized by the carbon dioxide asphyxiation to collect spleen and cecal content for further analysis. Similarly, the organs from the same-age counterpart in non-infected group of 3 days old (3 D) and 8 days old (8 D) were also collected. All animal experiments were performed according to the guidelines approved by the Institutional Animal Care and Use Committee at the UCD.

Enumeration of Bacteria in Spleen and Cecal Content

Viable counts of *S.* Enteritidis were recovered from one of the ceca pouches by squeezing its contents into 10 ml PBS and placing immediately on ice after the collection. The second pouch of ceca was collected on ice and frozen at −20°C for the DNA extraction. The weight of the cecum content was measured prior

to spreading serial 10-fold dilutions on Xylose Lysine Tergitol-4 (XLT4) selective agar plates containing tetracycline. Similarly, half of the spleen was weighted and homogenized in 1 ml PBS by using the black rubber end of the sterile plunger from the 2 ml syringe before plating. The plates were then incubated at 37°C for 24 h. Counts of *S.* Enteritidis were log transformed and expressed as $\log_{10}$ CFU per gram of the cecal content for further statistical analysis.

DNA Extraction and PCR Amplification of 16S rRNA Gene Sequences

Approximately 150 mg of total cecal content was used for DNA isolation by Zymo fecal DNA miniprep (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's instructions. In brief, bead-beating step was performed using the Bullet Blender Storm 24 (Next Advance Inc., Averill Park, NY, USA) for 5 min at maximum speed setting. Concentration and purity of the extracted DNA was measured on the NanoDrop ND-2000C spectrophotometer (ThermoScientific Inc., USA). All extracted DNA samples were stored at −20°C until further analysis. PCR amplification was performed with F515 (5′NNNNNNNN**GT**GTGCCAGCMGCCGCGGTAA3′) and R806 (5′GGACTACHVGGGTWTCTAAT3′) primers targeting the V4 segment of the bacterial 16S rRNA gene where the forward primer was modified to contain the linker region (GT) for sequencing on the Illumina MiSeq platform and a unique 8 bp barcode sequence (*N*) for each sample (21). PCR conditions were set at initial denaturation for 94°C for 3 min; followed by 35 cycles of 94°C for 45 sec, 50°C for 1 min, 72°C for 1 min 30 s with final extension step at 72°C for 10 min. The PCR reaction contained 12.5 µl 2× GoTaq Green Master Mix (Promega, Madison, WI, USA), 9.5 µl nuclease-free water, 0.5 µl forward and reverse primers, and 2.0 µl DNA. All samples were amplified in triplicate and combined after PCR for the purification. The PCR products were inspected on a 1% agarose gel stained with SYBR safe (Life Technologies, CA, USA) and stored at −20°C. The PCR amplicons were purified with QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The pooled amplicons were then submitted to the University of California, Davis Genome Center, DNA Technology Core Facility for generating 250 paired-end reads on the Illumina MiSeq sequencing platform.

16S rRNA Sequence Data Processing

The Quantitative Insights into Microbial Ecology (QIIME) version 1.9.1 was used to analyze the sequencing data generated from three replicate trials samples (**Table 1**). Raw data were demultiplexed, and quality filtered with QIIME default settings (22). The 250-bp reads were truncated at any site of more than three sequential bases receiving a quality score <Q10 and any read containing ambiguous base calls or barcode/primer errors were discarded as were reads with <75% (of total read length) consecutive high-quality base calls. Similar sequences were clustered together into the operational taxonomic units (OTUs) at 97% identity using QIIME open reference OTU picking against the Greengenes 16S rRNA database (version 13_8) (23).

TABLE 1 | Summary of number of chickens used in each of the replicate trials for 16S rRNA gene sequencing analysis.

Trial	Line	Non-infected (3 D)	*S.* Enteritidis-infected (2 DPI)	Non-infected (8 D)	*S.* Enteritidis-infected (7 DPI)
1	UCD077	4	11	0	0
	UCD254	4	21	3	11
2	UCD077	5	8	4	5
	UCD254	5	6	5	5
3	UCD077	5	8	5	5
	UCD254	5	10	5	8

3 D, 3 days old; 8 D, 8 days old; 2 DPI, 2 days post infection; 7 DPI, 7 days post infection.

Microbiota Diversity Analysis

Both alpha and beta diversity metrics were used to analyze microbiome composition. Alpha diversity metrics analysis includes Chao1 index (richness estimate), Shannon's diversity, and Simpson's diversity index. Chao1 richness estimates the total number of species present in the community. The difference between the Shannon and Simpson indices is that the weights of abundant species are accounted differently. Both the abundance and evenness in distribution of species present in the community is included in Shannon index analysis, while only the abundance of species is considered in Simpson indices (24). Microbial community dominated by a few species is considered to exhibit low evenness, while the community where the species abundances are distributed equally within the community are considered as balance community.

Rarefaction curve was constructed based on the observed number of OTUs as function of number of sequences analyzed with QIIME to compare between non-infected and infected groups. Estimates of beta diversity were made using both unweighted UniFrac and weighted UniFrac (25) followed by principal coordinate analysis (PCoA) in QIIME to characterize the microbial population diversity. To analyze the relative abundance of the microbial members at the family level, we identified eight major family groups that adhered to two conditions: classified OTU and population density detectable at more than 2% of the total community in all samples. For OTUs that were unclassified or in low abundances (below 2%), were binned together in others/unknown category. The results from the QIIME were further analyzed with linear discriminate analysis effect size (LEfSe) (26). Then Kruskal–Wallis rank sum test was used to identify significantly differential abundance of the microbiota community between the comparison groups. Differentially distributed microbiome taxa were identified based on Ribosomal Database Project (RDP) that generated LEfSe cladograms for the each category comparison. Cladograms that had statistically significant taxonomic differences between the groups were identified. Significant alpha values of 0.05 and effect size threshold of 2 were used in the LEfSe analysis.

Statistical Analysis

Splenic and cecal bacterial burden recovered from individual chickens between comparison groups were evaluated using

unpaired *t*-test by the GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Furthermore, statistically significant differences in alpha diversity metrics were determined by performing Mann–Whitney *U* test with the Prism. Comparisons of relative abundance level of microbial members at the family level between different categories of comparisons that include treatment at different time point of experiment (non-infected at 3 and 8 D vs. *S.* Enteritidis-infected chickens at 2 and 7 DPI, respectively), days post infection (2 vs. 7 DPI), and age (3 vs. 8 D), were evaluated by performing Wilcoxon rank sum test with the JMP statistical software (version 12). To compare the relative abundance of dominant bacteria group at the family level in both non-infected and infected group, correlation coefficients and linear regression were also performed using the JMP.

RESULTS

Effect of MHC Haplotypes on the Degree of Susceptibility to *S.* Enteritidis Infection Between Two Genetically Distinct Inbred Layer Lines

To determine whether the chicken MHC haplotype difference between the two genetic lines has an effect on the resistance or susceptibility to *S.* Enteritidis infection, kinetics of *S.* Enteritidis dissemination into spleen organ was examined to characterize the phenotypic difference between the two genetically distinct inbred lines. Three replicate trials were carried out. There was significant difference in splenic bacterial load detected between the two genetic lines at 2 DPI for the trial 1 ($p < 0.0001$) and combined data of three trials ($p < 0.01$) (Figure S1 in Supplementary Material). *S.* Enteritidis-infected chickens from UCD254 showing significantly higher bacterial burden at 2 DPI than from UCD077. There was no significant difference in splenic bacterial load at 7 DPI for either three individual trials or combined data of three trials.

The intestinal colonization level in the ceca of the two lines was also evaluated. There was no significant difference between two genetic lines in both trials 1 and 2 for both time points except trial 3 at 2 DPI ($p < 0.0001$). *S.* Enteritidis colonization level in cecal was significantly higher for UCD077 than UCD254 (Figure S2 in Supplementary Material). However, combined data from all three trials was significant at 7 DPI ($p < 0.01$) with higher cecal colonization detected in UCD254 than in UCD077 (Figure S2 in Supplementary Material).

MHC Haplotype Effect on Microbiota Composition in Non-Infected and Infected Chickens

A total of 1,773,077 reads were generated from a total of 148 individuals combined from all three trials. Altogether, 15,618 different OTUs were identified from 50 non-infected chickens and 98 *S.* Enteritidis-infected chickens with 64 samples from 2 DPI and 34 samples from 7 DPI. There was no significant difference in alpha diversity metrics between two genetic lines of non-infected chickens at both 3 and 8 D (Figures S3 and S4 in Supplementary Material).

Alpha diversity metrics evaluated between two genetic lines of *S.* Enteritidis-infected chickens showed no significant difference at both days of postinfection periods (2 and 7 DPI) for all indices except for Simpson's diversity in *S.* Enteritidis-infected groups at 7 DPI ($p < 0.05$) (Figures S5 and S6 in Supplementary Material).

Developmental Differences in Cecal Microbiota Composition in Non-Infected Chickens

In general, the results above suggested that there was no significant difference in microbial composition between two genetic lines. Therefore, data from both genetic lines were combined for further analysis. The alpha diversity metrics were compared between the two age groups of non-infected chickens. Both Chao1 richness and Simpson's diversity showed no significant differences between the two age groups (**Figures 1A,B**). However, chickens at 8 days old of age had significantly more diverse microbial community structure in Shannon's index ($p < 0.01$), suggesting a more balance distribution of the species in the community in older chickens than younger birds (**Figure 1C**).

Beta diversity of the two age groups was also compared via unweighted UniFrac distance metric followed by the PCoA analysis (**Figure 2A**). Microbial composition differences between two age groups were significant ($p = 0.001$), but two clusters were not clearly separable ($r = 0.488$) (**Figure 2A**). Furthermore, weighted UniFrac distance metric was also used to compare between the two age groups followed by the PCoA analysis in considering the effect of relative abundance of microorganisms in each age group (**Figure 2B**). ANOSIM analysis showed significant difference in microbial community structure between two age groups ($p = 0.001$) and higher r value of more than 0.5 ($r = 0.5403$) indicated that separation of two groups was significant (**Figure 2B**).

Microbiota compositions between two age groups were further analyzed using the linear discriminant analysis with effect size (LEfSe). Differentially abundant phyla detected in the age groups showed that Proteobacteria phylum was most dominantly present in younger chickens (3 D), while the most abundant phylum was Firmicutes for the older chickens (8 D) (**Figure 3A**). Three differentially represented core major groups at the order level were identified. For 8-day-old chickens, overrepresentation of Clostridiales and underrepresentation of Burkholderiales and Enterobacteriales were found (**Figure 3B**). Relative abundance of microbiota composition differences at eight major families were then compared between the two age groups using a Wilcoxon rank sum test. There was significantly marked decrease in *Clostridiaceae* ($p < 0.0001$), *Peptostreptococcaceae* ($p < 0.0015$), and *Enterobacteriaceae* ($p < 0.0001$) and higher abundance of *Lachnospiraceae* ($p < 0.0001$), and *Ruminococcaceae* ($p < 0.0001$) in older chickens than in young chickens (**Figure 3C**). In addition, the correlation between different members of the gut microbiota was also assessed for eight major families. A strong inverse correlation was observed between the *Enterobacteriaceae* and *Lachnospiraceae* ($r = -0.7881$, $p < 0.0001$), which suggested potential competition between these two members of the community.

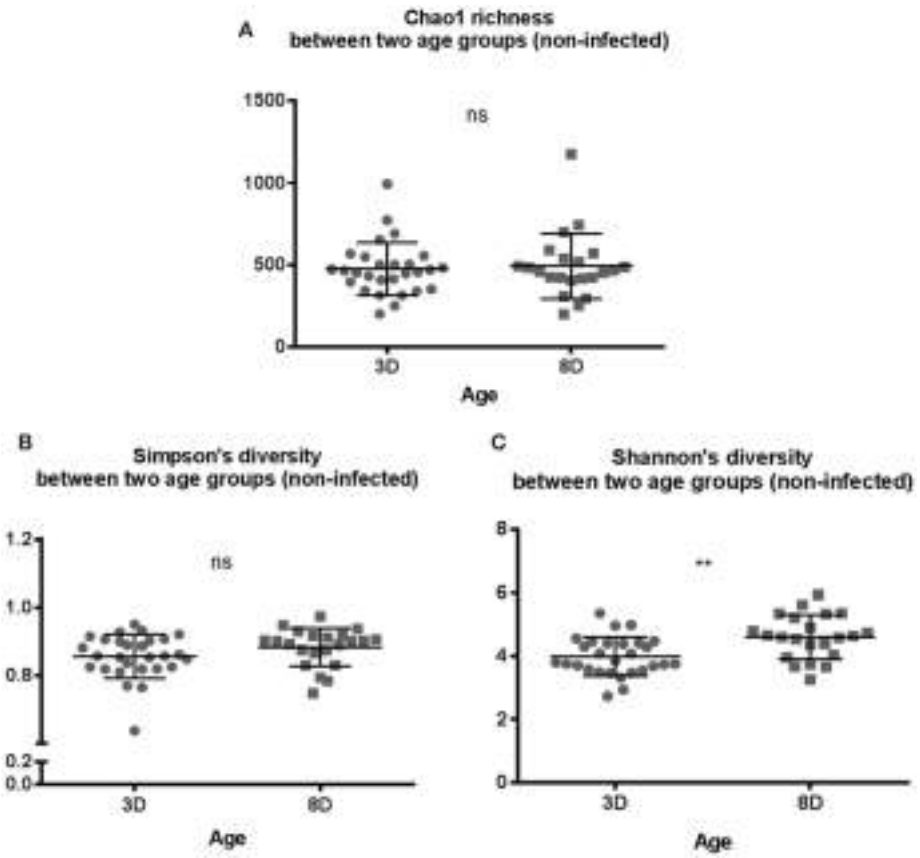

FIGURE 1 | Microbial alpha diversity between two age groups of 3 days old (3 D) and 8 days old (8 D). Alpha diversity metrics of **(A)** Chao1 richness estimate, **(B)** Simpson's diversity, and **(C)** Shannon's diversity index were analyzed. Shannon's diversity index was significantly higher for 8-days-old chicks suggesting that as number of species increases, there is more even distribution of species in the community compared to 3-days-old chicks. All three diversity metrics were evaluated using Mann–Whitney *U* test. $^{**}p$ value < 0.01 and ns = non-significant.

Developmental Differences in Cecal Microbiota Composition in Infected Chickens

To assess whether the microbiota diversity of the infected chickens differed between the two postinfection periods, both alpha and beta diversity indices were analyzed. There was no significant difference in Chao1 richness index (**Figure 4A**). However, both Simpson and Shannon indices showed highly significant difference in microbiota diversity ($p < 0.0001$) with increased diversity at 7 DPI compared to 2 DPI (**Figures 4B,C**). There was no significant difference in PCoA plot of unweighted UniFrac distance ($r = 0.035$, $p = 0.150$) (**Figure 5A**) between two postinfection time points. On the other hand, the weighted UniFrac distance analyzed with PCoA plot showed significant difference between two groups with $p = 0.001$ from ANOSIM analysis, suggesting the relative abundance of dominant taxa contributing to the differences, although the r value ($r = 0.414$) did not meet the cut-off threshold of 0.5 defined as two separated microbial community (**Figure 5B**). With LEfSe analysis, the phylum of Proteobacteria was dominated at (2 DPI) while Firmicutes phylum was found to be most abundant at 7 DPI (**Figure 6A**). At 7 DPI, a total of seven core microbiome groups at the order level were identified with enrichment of Erysipelotrichales, Clostridiales, and Lactobacillales (**Figure 6B**). Specifically at the family level, significantly ($p < 0.0001$) highly relative abundant of *Enterococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Peptostreptococcaceae* ($p = 0.0013$), and *Ruminococcaceae* ($p = 0.0025$), and lower levels of *Enterobacteriaceae* ($p < 0.0001$) were observed at 7 DPI (**Figure 6C**). These findings indicated a slow recovery of microbial diversity in the infected individuals at 7 DPI with significant reduction of the dominant *Enterobacteriaceae* family.

While assessing the microbiota profile of individual chick within the *S.* Enteritidis-infected group, a general trend pattern with an increase in *Enterobacteriaceae* accompanied by either a decrease or absence of *Lachnospiraceae* and *Ruminococcaceae* was found at the family classification level (Figures S7 and S8 in Supplementary Material). Therefore, the inverse correlations in relative abundance of *Enterobacteriaceae* with seven other major family groups were further evaluated. Significant inverse correlations ($p < 0.0001$) were found between *Enterobacteriaceae* and four other bacterial families including *Lachnospiraceae* ($r = -0.7985$), *Erysipelotrichaceae* ($r = -0.7586$), *Ruminococcaceae* ($r = -0.6569$) and *Peptostreptococcaceae* ($r = -0.6105$). Linear regression analysis revealed that the

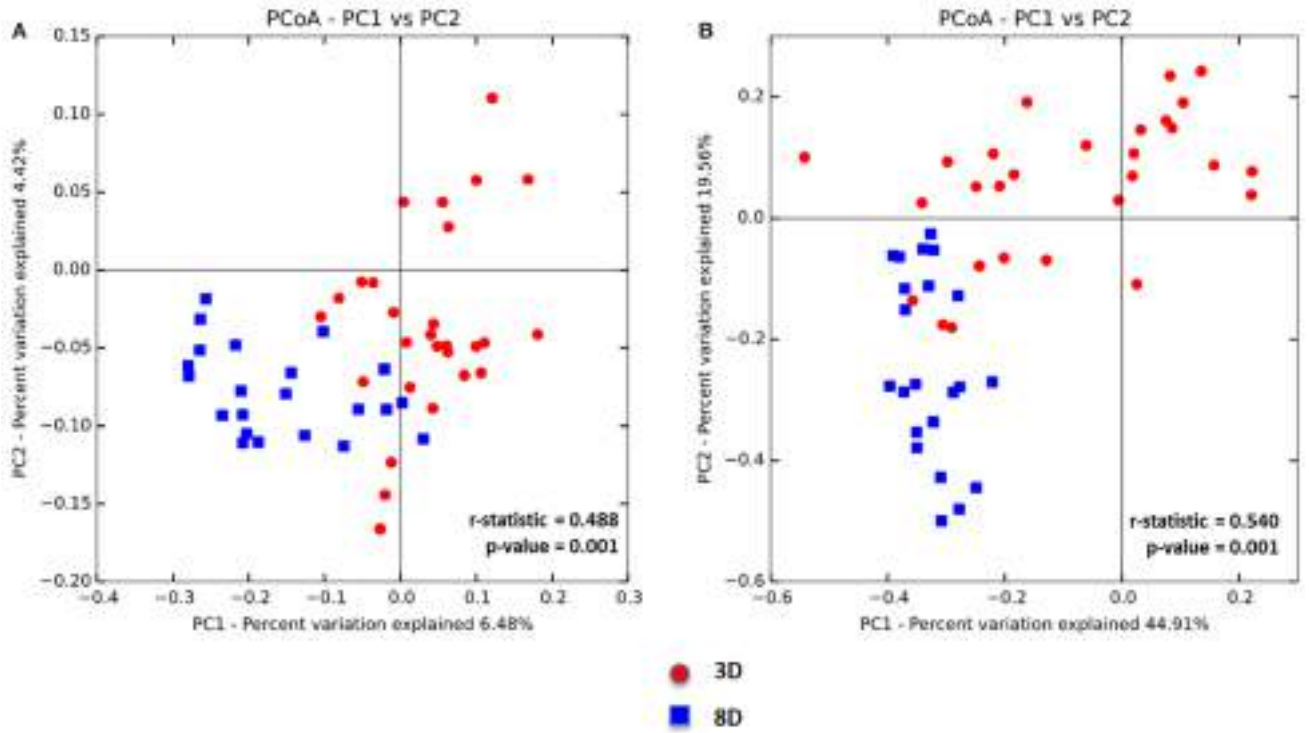

FIGURE 2 | Principal coordinate analysis (PCoA) based on (A) unweighted and (B) weighted UniFrac distances was analyzed for two age groups of non-infected chicks. ANOISM with 999 permutations was used to detect the statistical significant difference between microbial communities of different groups, where both *r* and *p* value is reported. Abbreviations: 3 D, 3 days old; 8 D, 8 days old.

population density of *Enterobacteriaceae* was negatively correlated with other family members of the community (**Figures 7A–D**).

S. Enteritidis-Associated Alteration in Chicken Cecum Microbiota Profile

Microbiota data were also analyzed to examine the effect of S. Enteritidis infection at two different postinfection times of the experiments. With *S.* Enteritidis infection, Chao1 richness showed no differences between the two groups at both postinfection time points (**Figures 8A,D**). Both Simpson and Shannon's diversity indices showed that there was significant reduction in microbiota diversity of the *S.* Enteritidis-infected chickens at 2 DPI compared to non-infected chickens ($p < 0.0001$ and $p < 0.001$, respectively) (**Figures 8B,C**). However, there was no significant difference between non-infected and *S.* Enteritidis-infected groups at 7 DPI for both indices (**Figures 8E,F**). Rarefaction curves highlighted a lower species richness of *S.* Enteritidis-infected groups at both time points compared to non-infected groups (**Figure 9**). Beta diversity was also analyzed to examine differences or similarities in cecal microbiota community composition between non-infected and *S.* Enteritidis-infected groups. PCoA plots based on unweighted UniFrac distance metric showed that there was significant separation in microbial community of non-infected and infected chickens at later postinfection time of 7 DPI ($p = 0.001$, $r = 0.618$) compared to early postinfection time at 2 DPI ($p = 0.032$, $r = 0.089$) (**Figures 10A,B**). With the PCoA plot based on weighted UniFrac distance metric where the relative abundance of OTUs were considered, there was more significant clustering pattern observed between the 8-day-old non-infected chickens and the same-age infected counterparts at 7 DPI ($p = 0.001$, $r = 0.841$) (**Figure 10D**). In contrast, the microbial communities of non-infected and infected groups at early time points (3 D vs. 2 DPI) showed no visible separation between two groups ($r = 0.398$) although the p value $= 0.001$ (**Figure 10C**).

To compare OTUs abundance between two treatment groups that were significantly different, ANOVA test was performed. Abundance of *Lachnospiraceae* family was found to significantly decrease in *S.* Enteritidis-infected group compared to the non-infected group [false discovery rate (FDR) < 0.05]. GI tract of young layer chickens were dominated by two main phyla belonging to Firmicutes and Proteobacteria. With *S.* Enteritidis infection, major phylum level shifted toward increased abundance of Proteobacteria at both time points when compared to non-infected same-age counterpart (Figures S9 and S10 in Supplementary Material). Representative of the bacterial family belonging to *Enterobacteriaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Lactobacillaceae*, and *Enterococcaceae* dominated in the cecal microbiota of the young layer chickens.

The microbiota compositions of non-infected were compared against the same-age counterparts of chickens in infected groups with LEfSe. The phyla of Actinobacteria and Proteobacteria

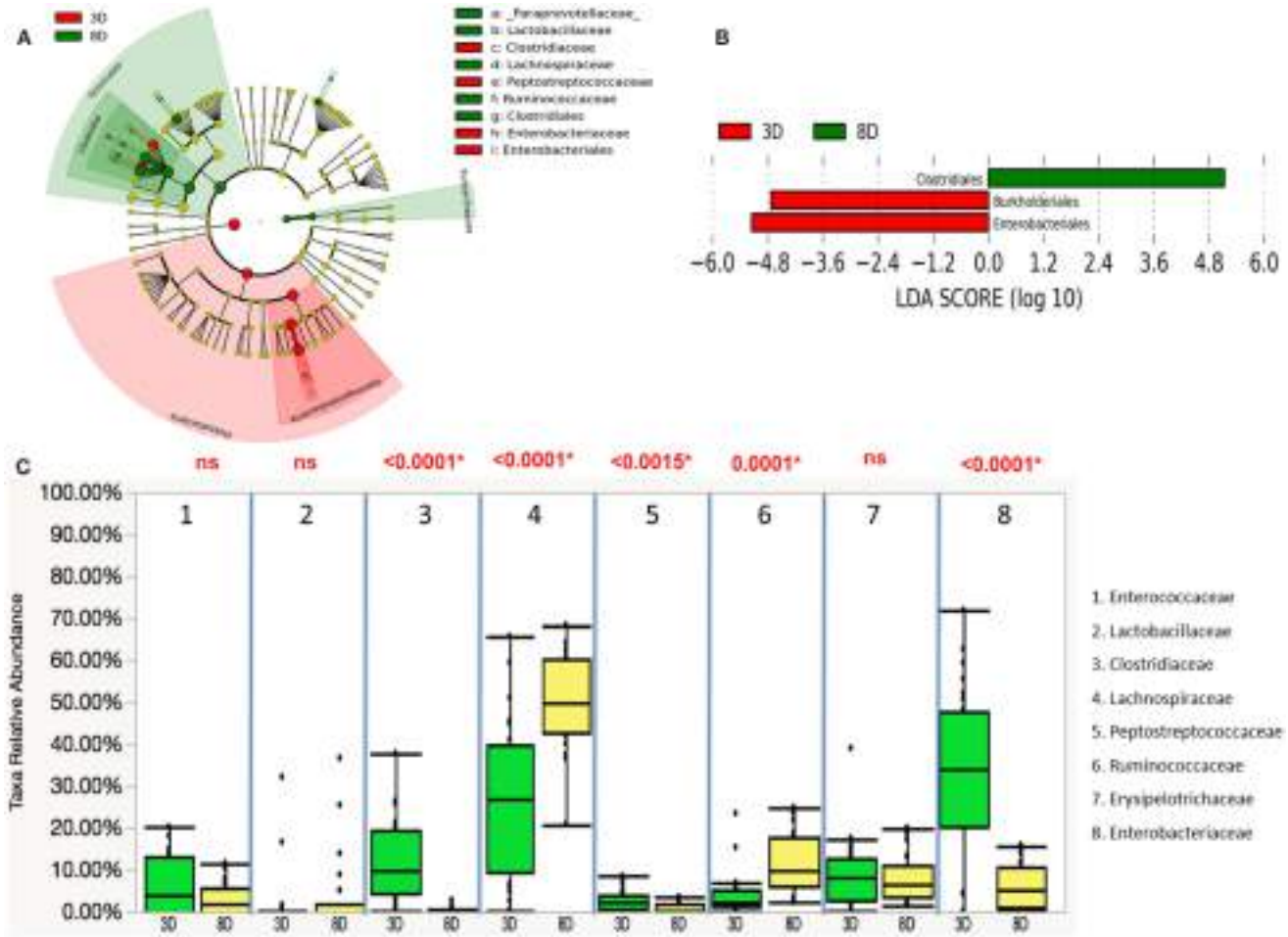

FIGURE 3 | Differential abundances of cecal microbial communities between two age groups. **(A)** Taxonomic cladogram generated from LefSe analysis showing significant difference in microbiota profile of two age groups, red represented the enriched taxa in 3 days olds' microbial community and green represented the enriched taxa in 8 days olds' microbial community. **(B)** Differently abundant taxa detected with cut-off value of linear discriminant analysis (LDA) score >2.0. Enriched taxa in 8-days-old chicks (green) were indicated with positive LDA score, while taxa enriched in 3-days-old chicks (red) have negative LDA score. **(C)** Comparison of relative abundance levels of cecal microbiota at family level in 3 and 8 days old chicks was evaluated. The boxplot shows the quartiles above and below the median with dark line at center of the box denoting median, black dots showing the outlier. The respective *p* value for each family group is reported using Wilcoxon rank sum test. Abbreviations: 3 D, 3 days old; 8 D, 8 days old.

were significantly enriched in the *S.* Enteritidis-infected groups of 2 DPI, while Firmicutes were significantly enriched in the non-infected group of 3 D (**Figure 11A**). Differentially representation of 11 groups at order level were identified with underrepresentation of four groups and enrichment of seven groups in the *S.* Enteritidis-infected group at 2 DPI (**Figure 11B**). Using a Wilcoxon rank sum test by JMP software, the relative abundance of gut microbes at the family level was compared between the *S.* Enteritidis-infected group of 2 DPI and the non-infected group of 3 D (**Figure 11C**). A marked decrease in *Enterococcaceae* ($p = 0.0092$), *Clostridiaceae* ($p < 0.0001$), *Lachnospiraceae* ($p < 0.0001$), *Peptostreptococcaceae* ($p < 0.0001$), *Ruminococcaceae* ($p = 0.0006$), and *Erysipelotrichaceae* ($p = 0.0025$) was found in the *S.* Enteritidis-infected group. On the other hand, *Enterobacteriaceae* ($p < 0.0001$) were highly abundant in the *S.* Enteritidis-infected group at 2 DPI.

Similarly, both Actinobacteria and Proteobacteria phyla were also enriched in *S.* Enteritidis-infected groups of 7 DPI, while the non-infected group at 8 D showed significant abundance in Firmicutes and Euryarchaeota phyla (**Figure 12A**). A total of six groups at order level were differentially represented with underrepresentation of two groups and overrepresentation of four groups in *S.* Enteritidis-infected group at 7 DPI (**Figure 12B**). Using Wilcoxon rank sum test, higher abundance level of *Enterococcaceae* ($p = 0.0073$), *Clostridiaceae* ($p = 0.0008$), *Peptostreptococcaceae* ($p = 0.0089$), *Erysipelotrichaceae* ($p < 0.0001$), and *Enterobacteriaceae* ($p < 0.0001$) were found in the *S.* Enteritidis-infected group than in the non-infected group at 7 DPI. In contrast, *Lachnospiraceae* ($p < 0.0001$), and *Ruminococcaceae* ($p < 0.0001$) were significantly decreased in the infected group compared to the non-infected same-age counterparts (**Figure 12C**).

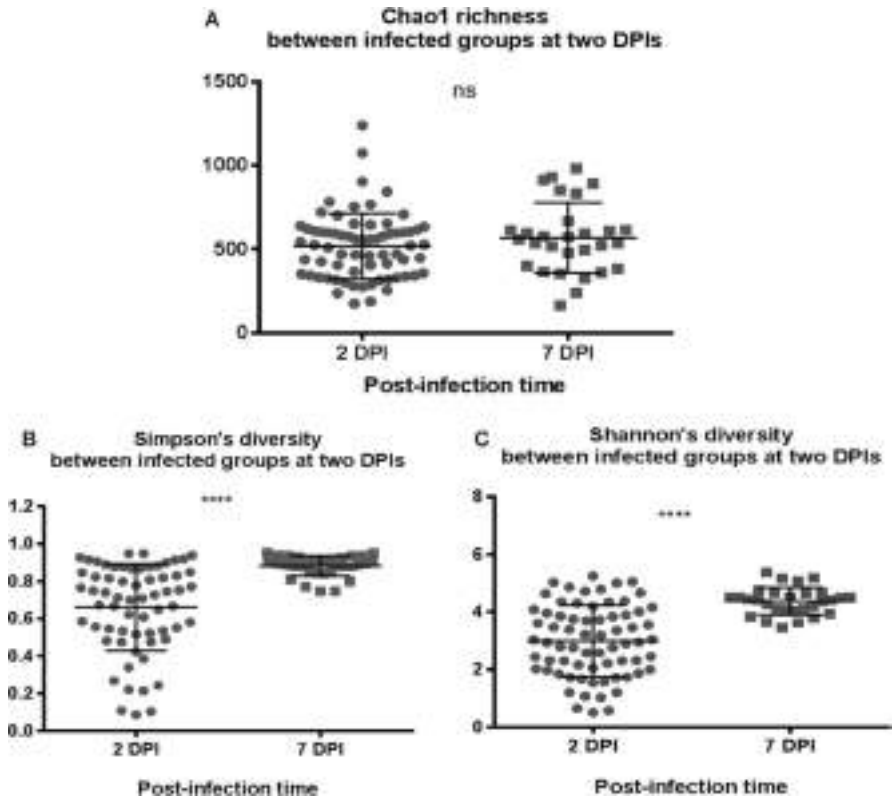

FIGURE 4 | Comparison of the diversity indices of *S.* Enteritidis-infected chicks at different time-points of post infection periods at 2 and 7 DPI. **(A)** Chao1 richness estimate, **(B)** Simpson's diversity, and **(C)** Shannon's diversity index were analyzed. **(B,C)** Simpson and Shannon's diversity showed significant difference in microbial diversity between two infected groups of chicks with increased diversity in microbiota composition at 7 DPI compared to 2 DPI. Both diversity metrics were evaluated using Mann–Whitney *U* test. ****p value < 0.0001.

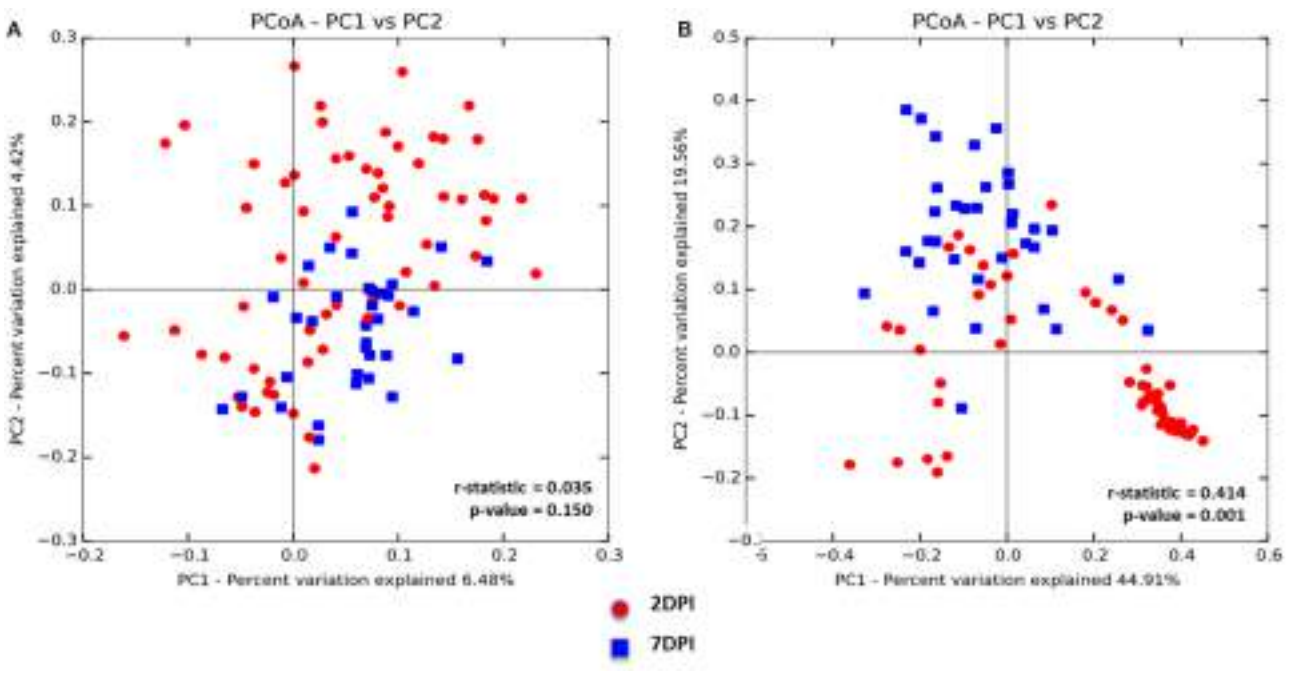

FIGURE 5 | Principal coordinate analysis (PCoA) based on (A) unweighted and (B) weighted UniFrac distances was analyzed for two age groups of infected chicks. ANOISM with 999 permutations was used to detect the statistical significant difference between microbial communities of different groups, where both *r* and *p* value is reported. Abbreviations: 2 DPI, 2 days post infection; 7 DPI, 7 days post infection.

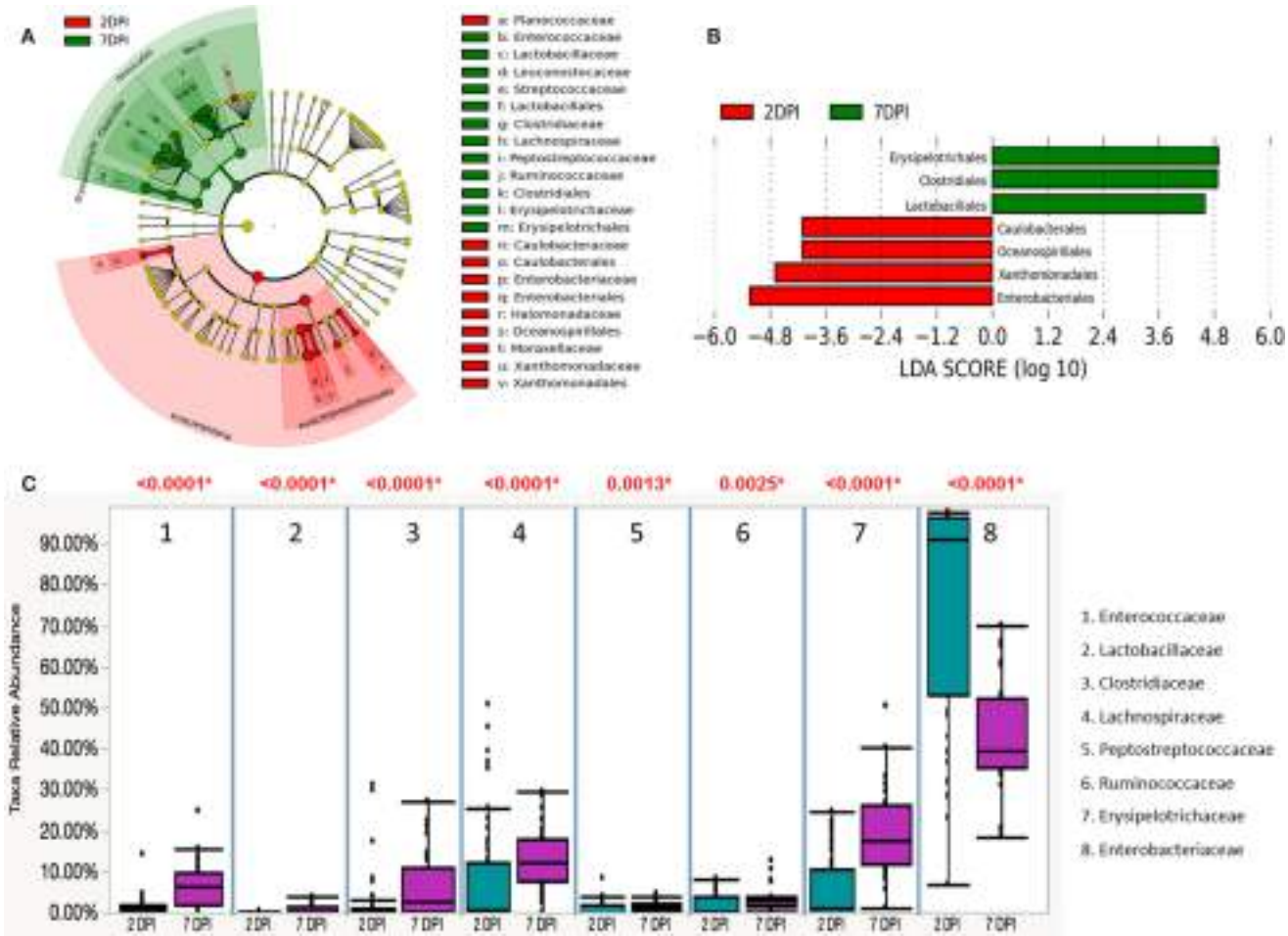

FIGURE 6 | Differential abundances of cecal microbial communities between two postinfection times of *S.* Enteritidis-infected chicks. **(A)** Taxonomic cladogram generated from LefSe analysis showing significant difference in microbiota profile of 2 DPI (red) and 7 DPI (green). **(B)** Differently abundant taxa detected with cut-off value of linear discriminant analysis (LDA) score >2.0. Enriched taxa in 2 DPI group are indicated with negative LDA score (red) and taxa enriched in 7 DPI have positive LDA score (green). **(C)** Comparison of relative abundance levels of cecal microbiota at family level in *S.* Enteritidis-infected chicks between two postinfection times was evaluated. The boxplot shows the quartiles above and below the median with dark line at center of the box denoting median, black dots showing the outlier. The respective *p* value for each family group is reported using Wilcoxon rank sum test. Abbreviations: 2 DPI; 2 days post infection, 7 DPI; 7 days post infection.

DISCUSSION

Maintenance of intestinal microbiota homeostasis is a key determinant for overall health and nutrition state of the host. Development of gut microbiota in the chick begins immediately after the hatch where a number of external factors such as environment, feed, and contact with chick handler could influence the overall microbial community structure (27). In addition, the host genotype is another important factor in affecting the composition of the gut microbiota (7, 9, 11). Early encounter with diverse enteric pathogens present in the environment during posthatch period also poses great risk for newly hatched chicks. This early host–pathogen interaction could potentially impact the further colonization of other microbes and shape the overall structure of gut microbiota. In this study, we therefore address this dominant potential of early pathogen exposure on shaping the microbiota composition by using 1-day-old chickens from two genetically distinct lines as infection model.

Chicken MHC and its association with resistance to avian disease, such as Marek's disease, infectious bronchitis virus (IBV), avian influenza virus, ectoparasite, and *Staphylococcus aureus* had been documented in several studies (14, 15, 17, 20). However, the role of MHC on the resistance to *S.* Enteritidis infection had been contradictory. Study by Cotter et al. (13) suggested an association of MHC B haplotype with resistance to *S.* Enteritidis infection in a neonatal chick infection model of 12 MHC-congenic chicken lines. On the other hand, another study by the Bumstead and Barrow (12) stated that there was no evidence to support strong association of MHC haplotypes with resistance to *Salmonella typhimurium* in newly hatched chicks. Our findings in the current study indicated that MHC haplotype had significant effects only on early stage of systemic infection of *S.* Enteritidis

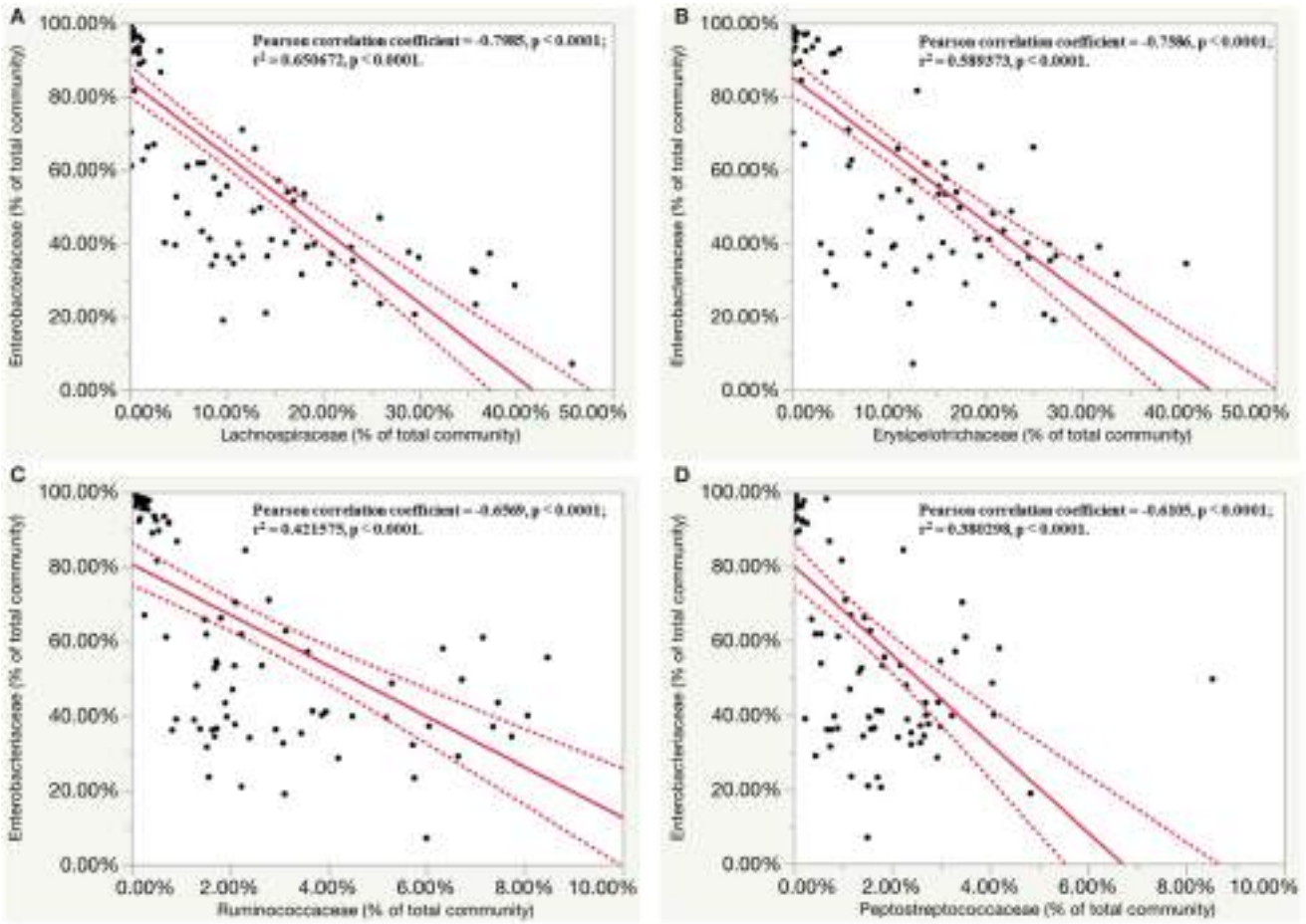

FIGURE 7 | Inverse correlation between relative abundance of members of *Enterobacteriaceae* family with other four major family groups. Dashed line indicated 95% confidence intervals. **(A)** Linear regression plots of relative abundance of bacteria (% of total community) belonging to *Enterobacteriaceae* family and *Lachnospiraceae* family in *S.* Enteritidis-infected group. **(B)** Linear regression plots of relative abundance of bacteria belonging to *Enterobacteriaceae* family and *Erysipelotrichaceae* family in *S.* Enteritidis-infected group. **(C)** Linear regression plots of relative abundance of bacteria belonging to *Enterobacteriaceae* family and *Ruminococcaceae* family in *S.* Enteritidis-infected group. **(D)** Linear regression plots of relative abundance of bacteria belonging to *Enterobacteriaceae* family and *Peptostreptococcaceae* family in *S.* Enteritidis-infected group.

(splenic bacterial burden difference at 2 DPI) and on late stage of local infection of *S.* Enteritidis (cecal bacterial burden difference at 7 DPI). However, in general, MHC genetic background had a limited effect on resistance to *S.* Enteritidis infection.

Developmental stage can have a significant impact on the microbiota composition in the GI tract. The early inoculum challenged to young chickens in this study could be a possible driving force in potentially rendering the microbiota composition of chickens of late stage. Without pathogen infection, microbiota diversity and complexity often increase with age of the chick (28, 29). Temporal changes in the chicken gut microbiota with aging could have important consequences on susceptibility to pathogen infection. The use of very young chickens as infection model is important for the current study as chickens are often exposed to *Salmonella* at very young age in natural setting. However, the immaturity of the immune system as well as non-establishment of complex microbiota in young chickens could have a significant effect on the outcome of the infection (30). Beaumont et al. (31) showed that increased resistance to *Salmonella* at adult chickens was negatively correlated to genetic resistance at the young age. Therefore, MHC haplotype effect on microbiota profile could be different by the use of different age infection model. Although it is beyond the scope of the current study, challenging at 2 weeks of age instead of 1-day-old chicks could provide additional insights of host genetic background impact on microflora composition and warranted further investigation.

In the non-infected chickens, Firmicutes followed by Proteobacteria phylum dominated the microbiota composition. Temporal fluctuation in the microbial community structure at the family level was observed as chick aged (from 3 to 8 D). *Enterobacteriaceae* family was significantly enriched in younger chickens, while *Lachnospiraceae* and *Ruminococcaceae* families were more abundant with a reduction in *Enterobacteriaceae* family in older chickens. The overall bacterial diversity in early life stage of chick host (both age groups) in the current study was low with a few members predominantly occupying the GI tract, which was in agreement with other studies (28, 29). Similar to another study, the non-infected chickens at 3 days old had high abundance of

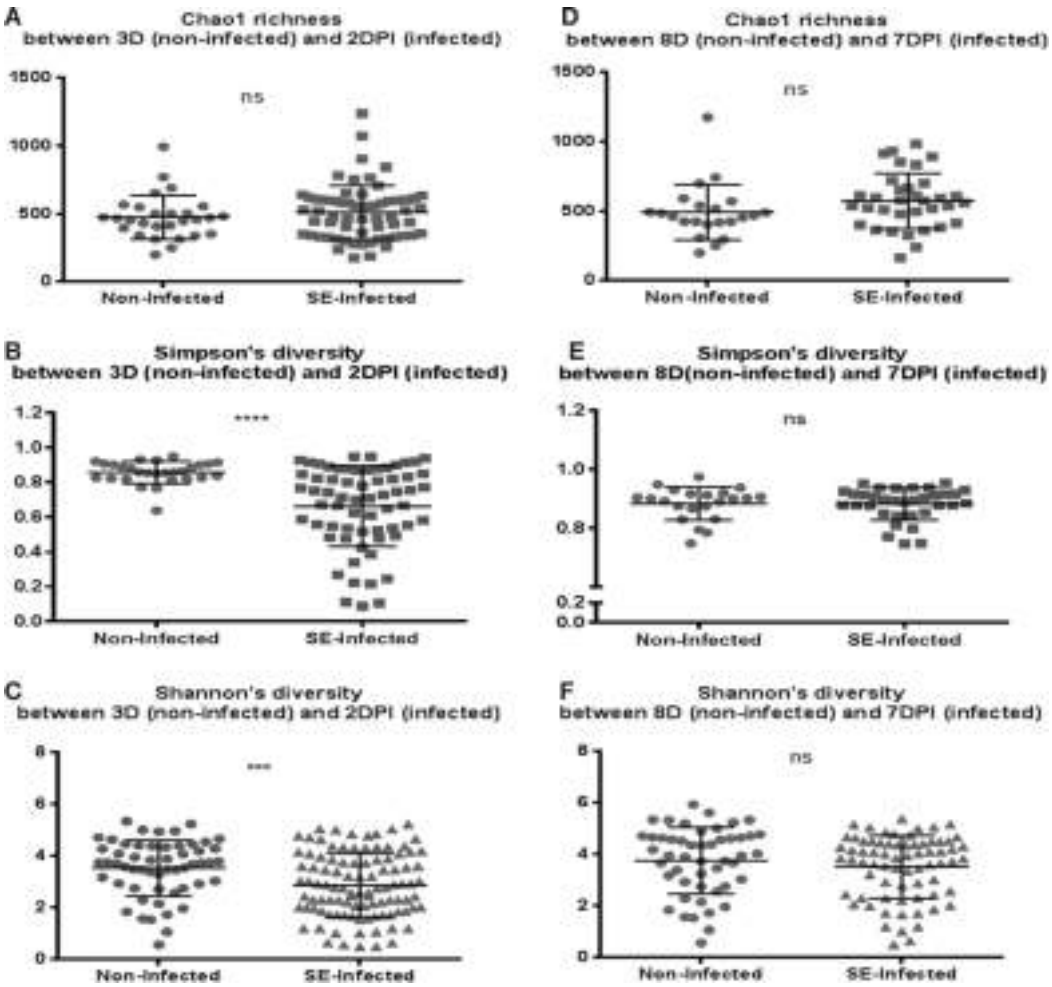

FIGURE 8 | Comparison of the diversity indices between the non-infected chicks and *S.* Enteritidis-infected chicks at 2 and 7 DPI: (A,D) Chao1 richness estimate, (B,E) Simpson's diversity, and (C,F) Shannon's diversity. At 2 DPI, there was significant reduction in microbial diversity of the *S.* Enteritidis-infected chicks compared to non-infected chicks for measurement of both Simpson and Shannon's diversity indices. Diversity metrics were evaluated using Mann–Whitney *U* test. ***p value <0.001, ****p value <0.0001, and ns = non-significant.

Enterobacteriaceae (32). Members of the *Enterobacteriaceae* family including bacterial pathogens like *Salmonella*, *Escherichia coli*, and *Shigella* are known enteric pathogens in the GI tract. Harboring high level of *Enterobacteriaceae* in young chickens could potentially increase their susceptibility to infection by related enteric pathogens. Indeed, studies in mouse model have shown that increased susceptibility to related enteric pathogen infections were observed in host whose microbiota composition was dominated by the presence of *E. coli* belonging to *Enterobacteriaceae* family (33, 34). The concept of "like will to like" was proposed by the Stecher et al. to help explain the bloom of closely related bacterial species in the GI tract that result in dysbiosis of microbiota in the disease host (33). It had been suggested that high prevalence of certain bacterial species in the microbiota community could alter the conditions within the gut that selectively confer the fitness advantage upon other related species within the same phylogenetic group (35, 36). We speculate that early colonization by members of *Enterobacteriaceae* family in GI tract of the newly hatched chickens could potentially precondition the intestinal tract of the chick host to allow easy colonization by enteric pathogens. Thus, depletion of certain bacteria taxa from *Enterobacteriaceae* family during the early posthatch period could potentially enhance the host resistance to enteric pathogen infection. This may be how growth promoters work in poultry feed as antibiotics in the growth promoters can eliminate certain members of *Enterobacteriaceae* family.

Once successful invasion by pathogen like *S.* Enteritidis is established within the niche of the GI tract, pathogen-associated alteration in microbial community structure occurred (37–39). Our results revealed that *S.* Enteritidis infection resulted in significant reduction in bacterial diversity specifically at early postinfection period. Reduction in bacterial diversity in the infected birds was partially attributed by the presence of the *Enterobacteriaceae* family that dominated the microbial community. Major phylum shift was observed in infected group at 2 DPI where there was expansion of Proteobacteria with concomitant reduction in Firmicutes phyla. This sudden shift in microbial population structure due to *S.* Enteritidis infection changed the ratio

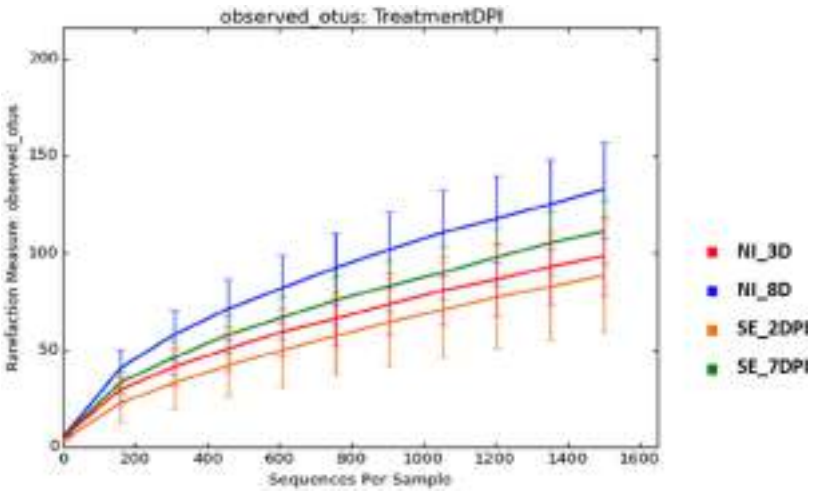

FIGURE 9 | Rarefaction curves of number of observed OTUs based on 97% sequence similarities for treatment group at different time points. Abbreviations: NI_3 D, non-infected chicks at 3 days old; NI_8 D, non-infected chicks at 8 days old; SE_2 DPI, *S.* Enteritidis-infected chicks at 2 days post infection; SE_7 DPI, *S.* Enteritidis-infected chicks at 7 days post infection.

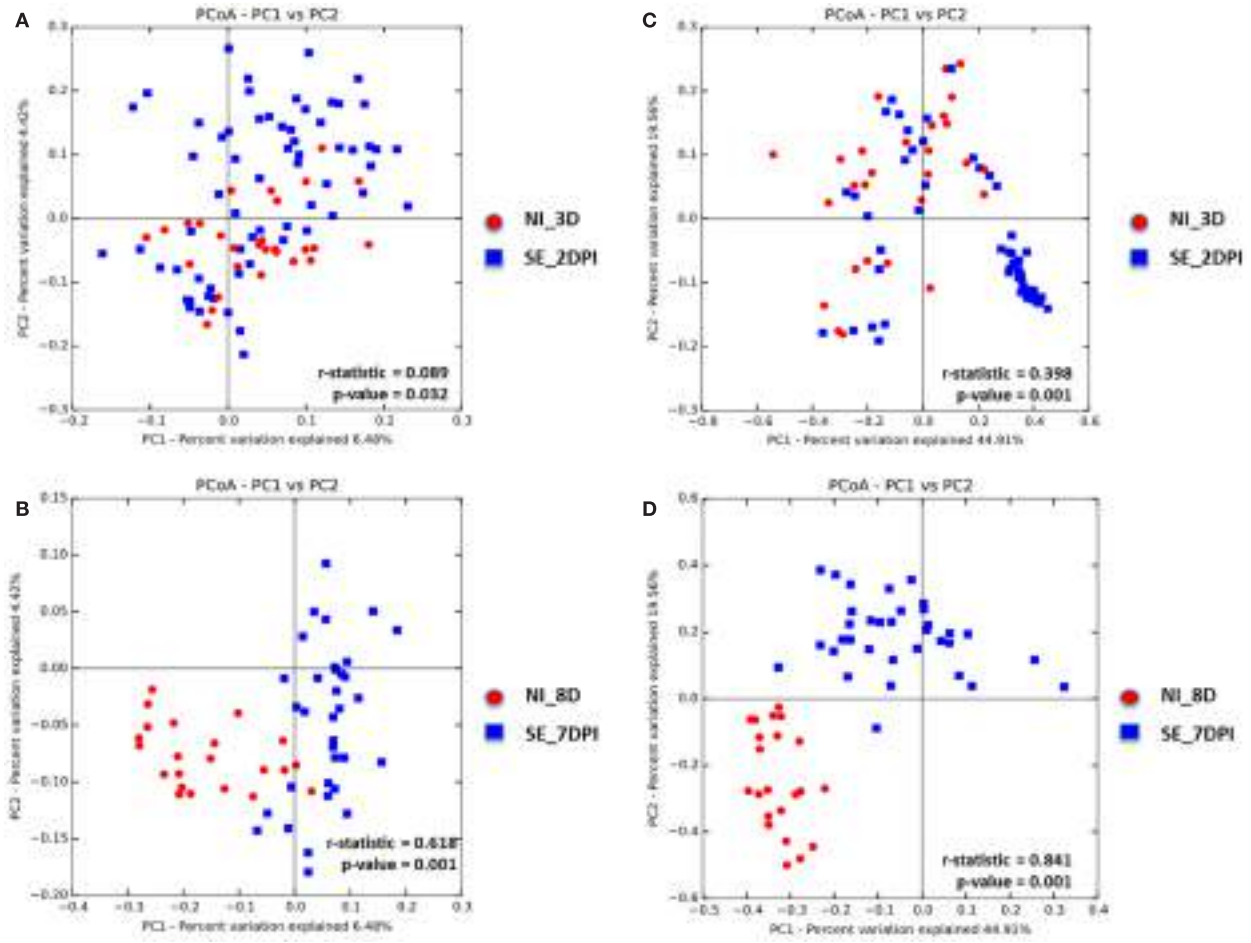

FIGURE 10 | Principal coordinate analysis (PCoA) based on (A,B) unweighted and (C,D) weighted UniFrac distances was analyzed for same-age group comparison between non-infected and infected chicks. ANOISM with 999 permutations was used to detect the statistical significant difference between microbial communities of different groups, where both *r* and *p* value is reported. Abbreviations: NI_3 D, non-infected chicks at 3 days old; SE_2 DPI, *S.* Enteritidis-infected chicks at 2 days post infection; NI_8 D, non-infected chicks at 8 days old; SE_7 DPI, *S.* Enteritidis-infected chicks at 7 days post infection.

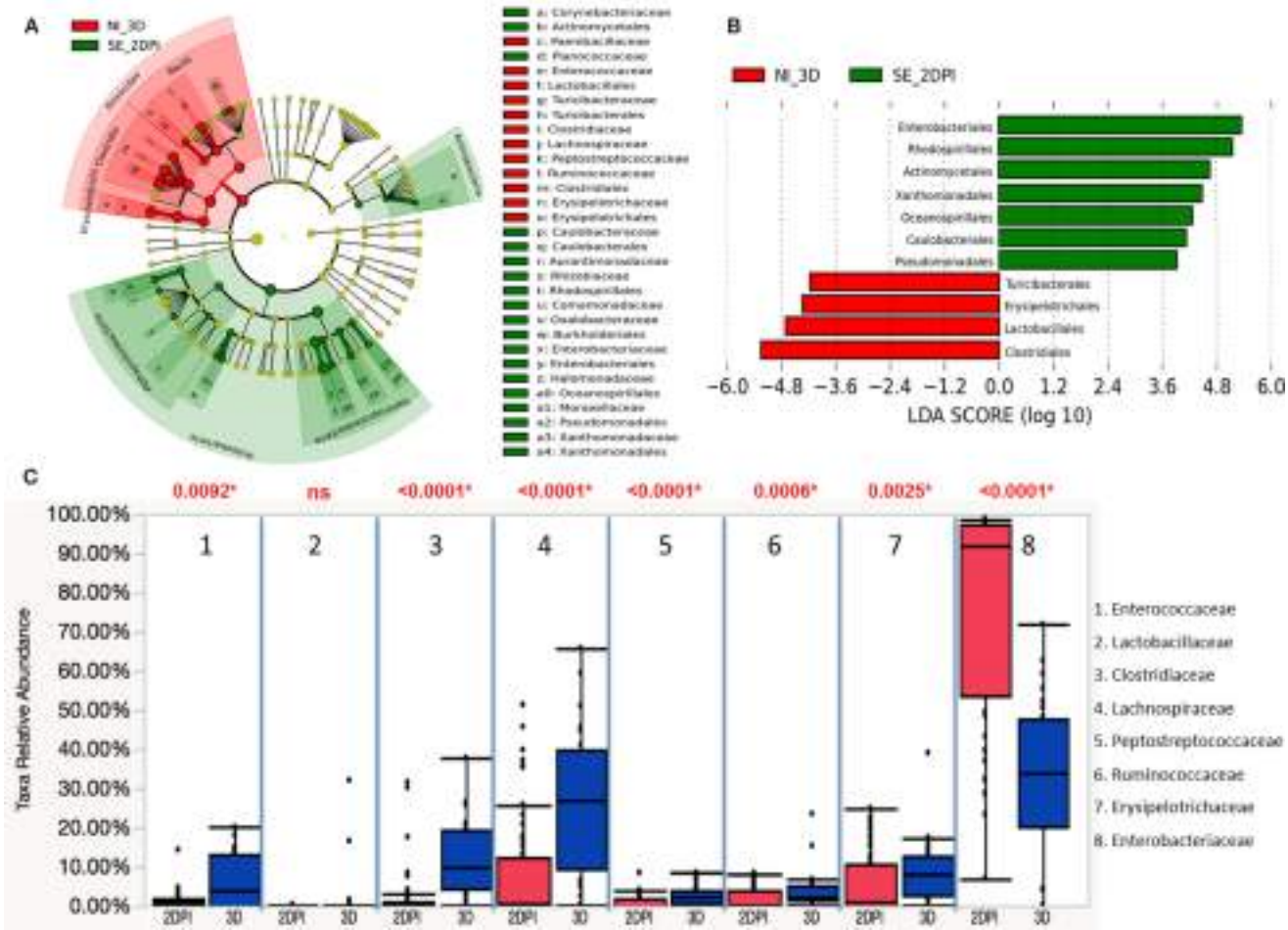

FIGURE 11 | Differential abundances of cecal microbial communities between infected and non-infected group at 2 DPI. **(A)** Taxonomic cladogram generated from LefSe analysis showing significant difference in microbiota profile of non-infected (red) and *S.* Enteritidis-infected (green) at early days post infection (2 DPI). **(B)** Differently abundant taxa detected with cut-off value of linear discriminant analysis (LDA) score >2.0. Non-infected enriched taxa are indicated with negative LDA score (red) and taxa enriched in *S.* Enteritidis-infected have positive LDA score (green). **(C)** Comparison of relative abundance levels of cecal microbiota at family level in treatment group was evaluated. The boxplot shows the quartiles above and below the median with dark line at center of the box denoting median, black dots showing the outlier. The respective *p* value for each family group is reported using Wilcoxon rank sum test. Abbreviation: NI_3 D, non-infected chicks at 3 days old; SE_2 DPI, *S.* Enteritidis-infected chicks at 2 days post infection.

of two major phyla groups, which is the hallmark indicator of intestinal microbiota dysbiosis in disease host (40, 41). *Salmonella* associated alteration of the gut microbiota could be a result of either pathogen-commensal microbiota interaction or host mucosal immune response to the pathogen or even a combination of both (38). Host-mediated inflammation response triggered by the presence of the pathogen could also change the conditions within the GI tract to favor and support the growth of specific member of the microorganisms. Studies in mouse colitis models have showed that inflammation allow facultative anaerobes like *Salmonella* or other members of the *Enterobacteriaceae* family to utilize anaerobic respiration as alternative growth pathway to gain competitive advantage over resident microbes that are mostly obligate anaerobe (34, 38, 40, 42–44). The underlying mechanism that is driving the bloom of Proteobacteria phylum in chick host following *Salmonella* infection is not yet known. Whether similar route of respiration pathway is being utilized by *Salmonella* to gain growth advantage in inflamed chicken gut is the hypothesis that we are currently investigating.

Comparisons between microbial communities of non-infected and infected groups showed that the community structure of the two groups appeared to be more similar initially at an early development stage (3 D vs. 2 DPI). However, as time progresses with *S.* Enteritidis infection, a significant difference in community structure between the two groups was apparent, with clear separation in the group's clustering pattern on PCoA plots (8 D vs. 7 DPI). This result suggests that the impact of *S.* Enteritidis infection on microbial communities was more substantial in late stage than in early stage. A study by Videnska et al. (45) found that members of the families *Lachnospiraceae* and *Ruminococcaceae* are predominantly present in the 2-week-old laying chickens, and likely play an important role in the overall development of the gut microbial community. Further analysis at the family level found that two core members of the gut microbiota belonging to

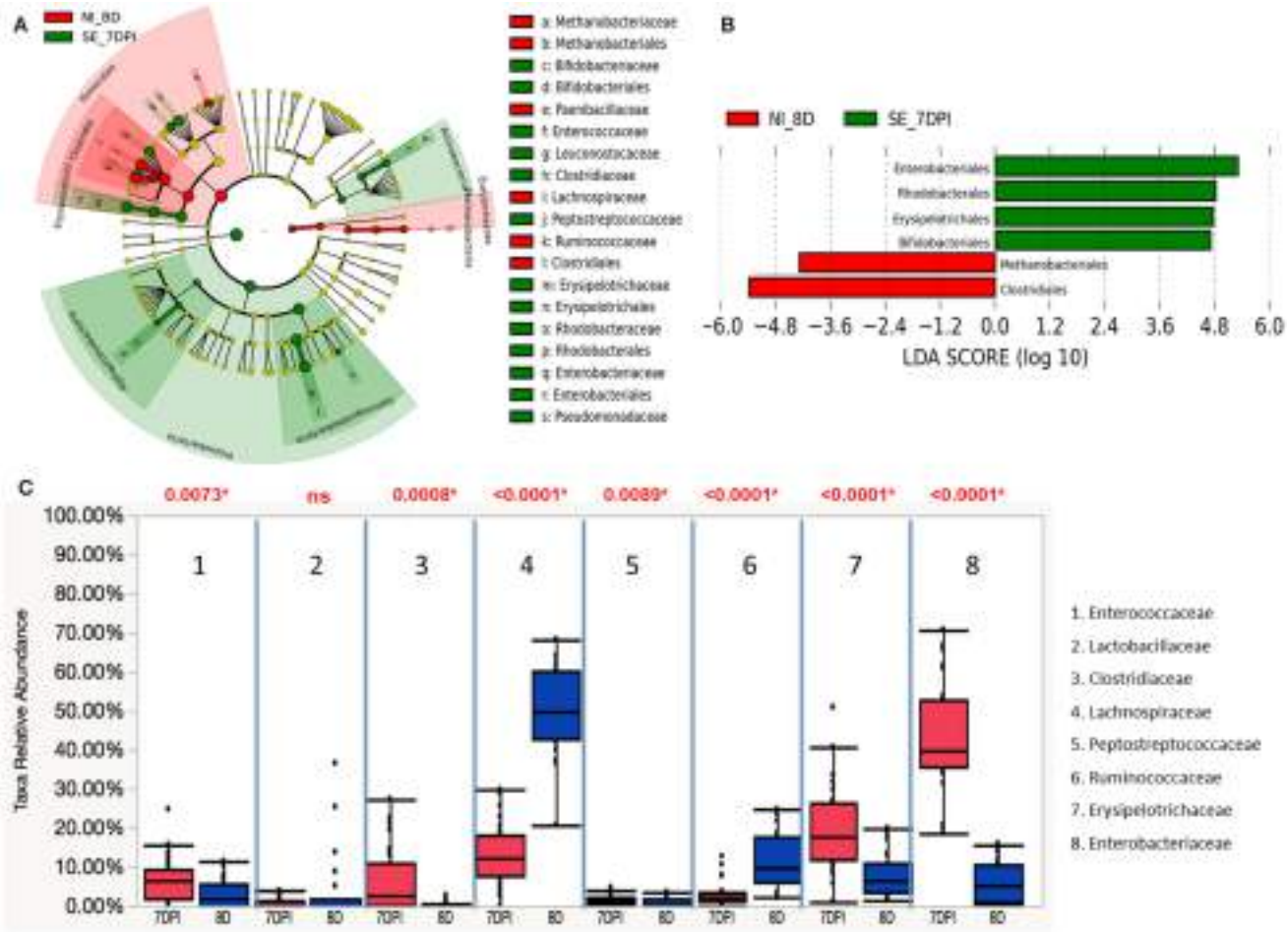

FIGURE 12 | Different abundances of cecal microbial communities between infected and non-infected group at 7 DPI. (A) Taxonomic cladogram generated from LefSe analysis showing significant difference in microbiota profile of non-infected (red) and *S.* Enteritidis-infected (green) at 7 DPI. (B) Differently abundant taxa detected with cut-off value of linear discriminant analysis (LDA) score >2.0. Non-infected enriched taxa are indicated with negative LDA score (red) and taxa enriched in *S.* Enteritidis-infected have positive LDA score (green). **(C)** Comparison of relative abundance levels of cecal microbiota at family level in treatment group was evaluated. The boxplot shows the quartiles above and below the median with dark line at center of the box denoting median, black dots showing the outlier. Abbreviation: NI_8 D, non-infected chicks at 8 days old; SE_7 DPI, *S.* Enteritidis-infected chicks at 7 days post infection.

Lachnospiraceae and *Ruminococcaceae* families were significantly reduced in the infected groups. Our findings suggested that with *S.* Enteritidis infection, selective reduction of these bacterial genera could negatively impact gut microbial diversity and development. Although long-term impact of *S.* Enteritidis infection on microbiome development in adult chickens was not possible to be evaluated in the current study, further investigation in this regard could provide important insights on it.

Interestingly, a strong inverse correlation between *Enterobacteriaceae* and *Lachnospiraceae* was observed in both the non-infected and infected birds, suggesting a possible antagonistic interaction between the two members of these taxa that could influence the prevalence of different microbial populations in the gut. In addition, the abundance of members belonging to *Lachnospiraceae* family was significantly decreased with *S.* Enteritidis infection. Contrary to our findings, Videnska et al. (37) observed only minor modification in chicken gut microbiota with no significant changes in *Lachnospiraceae* family following *S.* Enteritidis infection. The discrepancies observed between this study and our findings may be attributed to different age of infection model, samples collected on different days of postinfection and different genetic background of chickens. *Lachnospiraceae* as well as another family, *Ruminococcaceae*, that also show significant reduction in the infected group, belong to the Clostridium clusters IV and XIVa (45). Members of these groups generate butyric acid, short chain fatty acids (SCFAs) that are produced as end products of fermentation of carbohydrate by anaerobic intestinal microbes. There is complex interplay between diet, SCFAs concentration, and microbiota composition that regulate the colonization level of members of the Proteobacteria phylum (36, 46). Depending on the type of SCFAs being produced and its concentration level in the gut, it can affect different members of microbial community in a different way. Specifically for *Salmonella*, high concentration of acetate production was found to increase the invasion gene expression of *Salmonella* Pathogenicity Island 1 (SPI1) (47). High concentration level of butyric acid, on the

other hand, down-regulated the SPI1 gene expression level, which can reduce invasion capability of bacteria in the host (48). In the poultry industry, addition of butyric acid in feed has been shown to reduce both colonization and shedding of *Salmonella* in chickens (49, 50). Taken together, reduction in butyric acid producing bacteria such as *Lachnospiraceae* and *Ruminococcaceae* families with *S.* Enteritidis infection may implied that both producers and its products may have a potential protective role in providing colonization resistance against *Salmonella* infection or reducing the members of *Enterobacteriaceae* family in gut microbiota to maintain homeostasis. A novel *Salmonella* preventive strategies that implement combined approach of competitive exclusion bacteria with SCFAs should be explored to eliminate enteric pathogens and improve overall gut health of the chicken host.

In conclusion, our findings indicated that early exposure in young chickens to *Salmonella* influences and shape the overall microbiota composition. Microbial diversity was significantly reduced in *S.* Enteritidis-infected host compared to same-age non-infected group. Overall perturbation of microbiota community was found to be associated with expansion of *Enterobacteriaceae* family at early postinfection period. Decrease in butyrate producing bacteria belonging to *Lachnospiraceae* family was found to have a negative correlation with high prevalence of *Enterobacteriaceae* family, suggesting possible competitive interaction between the two bacterial taxa in the gut. Additionally, increased susceptibility to *Salmonella* infection in young chickens could be contributed by highly relative abundance of *Enterobacteriaceae* family in the gut. Predominance of this bacterial taxa could potentially confer competitive growth advantage upon its related species over resident microbiota during enteric infection via altering the environmental conditions of the GI tract of the host, which further promote the imbalance state of the young chick's gut microbiota. This study provided a preliminary insight into the contributing role of early host–pathogen interaction that influences the composition makeup of gut microbiota.

AUTHOR CONTRIBUTIONS

KM performed the experiment, analyzed the sequencing data, and drafted the manuscript. HZ designed the experiment, provided the concept of the analysis, and was involved in critical revision of the manuscript. PS and MH ran the scripts for sequencing data and help revised the manuscript. GC and HC helped with animal trials, sample collection and DNA extraction of samples. LG and EM provided primers, contributed ideas for analyzing the data and helped in revising the manuscript. All authors submitted comments, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fvets.2015.00061

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An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives

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The poultry industry has been searching for a replacement for antibiotic growth promoters in poultry feed as public concerns over the use of antibiotics and the appearance of antibiotic resistance has become more intense. An ideal replacement would be feed amendments that could eliminate pathogens and disease while retaining economic value via improvements on body weight and feed conversion ratios. Establishing a healthy gut microbiota can have a positive impact on growth and development of both body weight and the immune system of poultry while reducing pathogen invasion and disease. The addition of prebiotics to poultry feed represents one such recognized way to establish a healthy gut microbiota. Prebiotics are feed additives, mainly in the form of specific types of carbohydrates that are indigestible to the host while serving as substrates to select beneficial bacteria and altering the gut microbiota. Beneficial bacteria in the ceca easily ferment commonly studied prebiotics, producing short-chain fatty acids, while pathogenic bacteria and the host are unable to digest their molecular bonds. Prebiotic-like substances are less commonly studied, but show promise in their effects on the prevention of pathogen colonization, improvements on the immune system, and host growth. Inclusion of yeast and yeast derivatives as probiotic and prebiotic-like substances, respectively, in animal feed has demonstrated positive associations with growth performance and modification of gut morphology. This review will aim to link together how such prebiotics and prebiotic-like substances function to influence the native and beneficial microorganisms that result in a diverse and well-developed gut microbiota.

Keywords: poultry, microbiota, lactobacillus, *Bifidobacterium*, yeast

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Introduction

Poultry production in the past century has transitioned from predominantly breeding layers to breeding a mixture of both layers and broilers, based on the evolution of consumer demand (1–3). Success in the optimization of different broiler lines is due to genetics as well as optimizing diets with more precise nutritional formulations (4, 5). Comparison of individual genetic lines has revealed differing intestinal development, feed intake, and digestibility traits among other characteristics, which may impact performance (6–9). Improved diets have allowed broilers to reach their optimum body weight and feed conversion rate while minimizing mortality. Comparing poultry diets from the 1950s to those of the 1990s and 2000s illustrates the progress made (10, 11). For example, broiler

chickens raised on a typical diet in 1957 had an average weight of 1,430 g at 84 days of age, whereas broilers fed a diet from 2001 yielded an average weight of 5,520 g at the same age. The feed conversion ratio in 2001 (2.68) was also considerably better compared to 1957 (3.26) (11). The current poultry diet contains the appropriate balance of amino acids, fatty acids, major and trace minerals, energy, and protein necessary for optimum growth (12).

Supplementation of various biologics have been attempted to enhance poultry feed for maximum growth development and health. Antibiotics enhance growth and reduce pathogens and although the exact mechanisms remain unclear, numerous working hypotheses have been offered (13–17). Antibiotic incorporation into poultry feed has since been tightly restricted and/or omitted due to microbial antibiotic resistance, presumably originating from both poultry (among other livestock) and humans (18–20). Since the exclusion of antibiotics in diets, a number of alternative supplements have been tried (**Table 1**), including prebiotics (21).

A prebiotic, as defined by Gibson and Roberfroid (35), is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health." This definition has been subsequently refined to include the requirements for resistance to the acidic gastric environment, gastric enzymes, gastrointestinal absorption, and fermentation by the gastrointestinal microbiota while stimulating growth of beneficial intestinal bacteria (22). Being indigestible by the upper gastrointestinal tract (GIT) enables it to enter the lower GIT as a substrate for health-promoting bacteria, such as bifidobacteria and lactobacilli, thereby modulating the microbiota (35). Many feed additives currently used do not fit wholly into the strict prebiotic classification; they may lack one or more of the criteria set by Roberfroid (22). Although these substances have differing modes of action compared to prebiotics, they have a similar end result of a healthy and mature GIT microbiome. They may inhibit pathogenic invasion, reduce pathogens in the environment, modulate the host immune response, or enhance the host GIT morphology to enable the host to better limit pathogens in the GIT lumen. These substances will be referred to as prebiotic-like substances for the remainder of this review.

The objective of this review is to provide an overview of the effects of prebiotic-like substances, particularly those that are yeast-derived, while assessing the influence on microbial diversity of the poultry gut microbiota when using single or complex mixtures. In order to achieve this, both the gut microbiota as well as prebiotics is reviewed. Additionally, the characteristics of complex mixtures of prebiotic-like substances are assessed, including their effects on the gut development and physiology, the interactions that occur between host and microorganisms, and the potential use of prebiotic-like substances in creating a more healthy gut microbiota. This review includes findings from not only poultry but also human and animal models, which may provide insight into potential effects in poultry.

Gut Microbiome: Terminology and Definitions

The microbiota is defined as the diverse population of microorganisms in a given environment, while the microbiome can be defined by either its genetic or ecological capacities (36). Genetic diversity is the entire collection of genes of the microorganisms in an environment, while the ecological diversity is all the microorganisms that make up an ecosystem (36). The term "microflora," once commonly used, is now often replaced by "microbiota" to avoid the plant connotation from the suffix "flora." (36). Regardless of the term used, it is essential to use a modifying adjective when referring to a specific anatomical region. For example, "gut microbiome" is indicating only the microorganisms in the GIT. There are numerous microbiome sites in addition to the gut microbiome, as they can be any shared anatomical sites between a community of microorganisms (commensal, pathogenic, or symbiotic) (37–39). An oral microbiome, for example, is the community of microorganisms that interact with and live within the oral cavity. It has several distinct microbial habitats within the oral cavity (gingival, tongue, and teeth) and extensions of the oral cavity (esophagus, middle ear, and nasal passages). Each different habitat within the oral cavity has its own distinct bacterial

TABLE 1 | Commonly researched feed additives for host health, including growth promotion and pathogen prevention, used in animal feed, their modes of action, and reviews for references.

Compound	What they do	How they work	Reviews for reference
Prebiotic	Food ingredient to act as substrate for beneficial bacteria in the host GIT microbiota	Host consumes prebiotic and it endures through the GIT relatively intact to the lower intestines where it selectively acts as substrate for beneficial bacteria	(22–24)
Probiotic	Live microbial feed supplements that beneficially impact intestinal microbial balance	Competes with pathogenic bacteria to colonize the intestines; ferments substrates to produce short-chain fatty acids; stimulates the immune response of the host	(23, 25, 26)
Mannan-oligosaccharide	Specific oligosaccharide that inhibits pathogenic bacteria from binding the mucosal epithelial lining	Pathogens have receptors specific for mannan residues, the pathogenic bacteria binds the mannan and does not bind to the host epithelial cells	(27–29)
Organic acid	Reduce the number of pathogens	Undissociated form traverses the bacterial cell membrane; once inside the bacterial cell, the organic acid dissociates to produce H^+ ions, which lowers the pH. The bacterial cell then has to expend its energy to restore it natural balance rather than promote its own growth	(30–34)

population in the form of complex biofilms (40). Research has shown that even the distinctive sites of the tongue – the dorsal and the lateral regions – possess differing bacterial profiles (41). Other frequently studied sites of microbiomes are the skin and the respiratory tract (42–44). The various regions and diversity among bacterial communities of the microbiota are indicative of the inherent complexity of microbiome research.

The gut microbiome is a widely studied topic because of its impact on health as well as its characteristic intricacy. The gut microbiome is home to one of the densest bacterial populations on earth, with numbers ranging from 10^8 to 10^{14}/g of digesta (45, 46). The microbiome encompasses biochemical and metabolic pathways not found in the host genome; this attests to the extent to which the microbiome has evolved (47). Microorganisms that comprise the gut microbiota have been found to directly impact the health of the host, providing protection against epithelial damage, aiding in digestion, and promoting development of a healthy immune system (48, 49). Commensal bacteria, in the GIT of animals, aid in absorption of nutrients as well as enhance nutrient utilization (50). Additionally, research conducted thus far has shown that earlier development of a mature and diversified microbiota leads to better growth and fewer health issues, such as obesity, allergies, and asthma (51, 52). This is in part due to healthy competition among microorganisms.

Avian Gut Anatomy, Structure, and Functionality

For a thorough understanding of the microbial communities that inhabit the GIT of poultry and the effects they may have, a brief description of the poultry GI system is warranted. The GIT of poultry, chickens specifically, begins at the esophagus and continues down past the crop, proventriculus, and gizzard, through the intestines (duodenum, jejunum, ileum, and ceca), and ends at the colon and cloaca (53, 54). The gut microbiota generally refers to the intestinal regions and the studies included in this review focus on the duodenum, jejunum, ileum, ceca, and fecal contents as well as the structural characteristics to illustrate the gut microbiome of poultry. The ceca and their contents are most often studied based on their slow passage rate [comparatively, gut transit time from mouth to the lower ileum is approximately 3 h, while contents may be retained in the ceca as long as 35 h (55–57)] as it exhibits the most diversification in the bacterial communities it harbors, in turn, indicating its impact on host health (54).

The intestines are multi-layered tubes, containing epithelial, muscular, and mucosal layers (58). Each section of the intestine, from the most proximal duodenum passing through the jejunum and out to the most distal ileum, contains numerous folds and is lined with villi and crypts. The villi are finger-like projections on the surface of the mucosal lining responsible for increasing surface area to maximize nutrient absorption and containing a meshwork of capillaries to allow nutrients entry into the bloodstream (59). When moving in the distal direction from the duodenum down toward the ileum, the mucosal lining reduces in thickness. The villi length and crypt depth also decrease in a continual gradation, which supports the notion of the majority of nutrient absorption occurring in the small intestine (58). Reduced intestinal weight is associated with improved nutrient absorption (60). Microscopic analysis has revealed that the reduction of intestinal weight is due to thinning of the epithelial lining rather than to the reduction in intestinal length, which is suggested to allow for improved nutrient absorption (61, 62).

The pancreas functions in hydrolysis of macromolecules, releasing digestive enzymes into the duodenum responsible for the hydrolysis of proteins, carbohydrates, and lipids supplied by the diet. In addition to enzyme production, the pancreas also produces hormones and bicarbonate that aid in metabolism regulation and buffer the intestinal pH, respectively (59, 63). The addition of enzymes to the duodenum allows for the small intestine to be the primary site of nutrient digestion and absorption. Having a general understanding of the digestive system of poultry allows for a more thorough insight into how microorganisms may impact GIT physiology. Turk (58) provides a more encompassing review of the entire avian GIT.

Avian Gut Microbiome Characterization

Characterization of microbial communities native to the poultry GIT began in 1901 and has since revealed these communities to be both diverse and dynamic (64). As biased culture-based methods advanced to molecular and sequencing techniques, a broader, more comprehensive representation of the microbiome has been recognized (64, 65). Researchers have attempted to determine a bacteriological profile of the poultry GIT via 16S rRNA gene-based studies; the findings have demonstrated that the majority of the 16S rRNA sequences in the cecal contents are not-yet-identified bacterial species (64, 66, 67). These discoveries uncovered the shortcomings of previously employed culture-based methods. For example, comparison of results obtained from Zhu et al. (64) and Rada et al. (68) found differing levels of *Bifidobacteria*-species present in untreated chicken cecal contents. Zhu et al. (64) used temporal temperature gradient gel electrophoresis followed by sequencing of the 16S rRNA fragments, while Rada et al. (68) used selective media; the experimental designs of both were comparable. The works of Zhu et al. (64) and Rada et al. (68) are two such examples for the characterization of the GIT microbiome; various techniques have been attempted to ascertain the microbial populations present in the different regions of the intestinal tract (**Table 2**).

Each area of the intestinal tract harbors distinct microbial communities. For example, the cecal contents exhibit greater levels of *Clostridiaceae*-related sequences as opposed to the ileum where more abundance of *Lactobacillus*-related sequences occurs (75). Apajalhti et al. (70) used G + C content to demonstrate similar results: the measurement of bacterial communities present in the ceca and ileum exhibited considerable variation when comparing the two G + C profiles. Variation in microbial communities is not only limited to differing organs, there is also a temporal factor in the nature of the microbiome (76). The cecal contents of younger birds appeared to possess more transient communities that matured into communities with much greater complexity, while the ileum indicated an overall constant microbiome except at days 3 and 49 (the youngest and oldest sampling points) (75). The response to newly introduced microorganisms also appears

TABLE 2 | Research conducted on commensal bacteria in poultry GIT based on location.

Host	Site(s)	Age(s)	Commensal or pathogenic	Method of investigation	Reference
Chicken	Ileum, cecum	7, 13 days	Commensal	PCR-based DGGE; 16S rRNA gene library analysis; qPCR	(69)
Chicken	Ileum	4, 8, 14, 21, 35 days	Commensal	DGGE; RFLP	(6)
Chicken	Ileum, cecum	4 weeks	Commensal	Percent G + C profiling	(70)
Chicken	Cecum, intestines	4, 14, 25 days	Pathogenic	Primers (species-specific) of 16S rDNA	(71)
Chicken	Cecum	1 day, 1, 2, 4, 6 weeks	Commensal	TTGE; 16S rRNA gene sequencing	(64)
Chicken	Crop, ileum, cecum, rectum	40, 41 days[a]	Commensal	16S rDNA sequencing	(72)
Chicken	Ileum, cecum	28 days	Commensal	FISH with 16S rRNA oligonucleotides	(73)
Chicken	Crop, duodenum, colon	2 months	Commensal	FCM-FISH	(74)

[a]*Indicates differing rearing methods: conventionally raised and organically raised, respectively.*

to be dependent on sex of the host when analyzed in a mouse model; male and female GIT microbiota influence the metabolic activities and immune system differently (77). The concept of host factors affecting microbial diversity offers the opportunity to use established and healthy microbiomes to generate a working GIT microbial profile. However, this may prove to be quite challenging as it has been found that chickens interacting together in the same conditions, receiving the same feed, and of the same age and sex still display uniquely dominant bacterial communities (78). Although the exact quantities and qualities of a healthy microbiota have yet to be determined, a relationship appears to exist between the establishment of a mature intestinal microbiome and positive impacts on the host, resulting in improved growth and health (79).

Avian Gut Microbiome-Metabolic Activities

The poultry GIT is essentially coated in a dense layer of commensal bacteria in a diverse array of niches. Generally, the most complex microbial communities are found in the crop and the ceca. There is less colonization in the intestines based on the unfavorable environment. For example, the duodenum contains numerous enzymes, high concentrations of antimicrobial compounds, such as bile salts, and also has a rapidly changing environment due to reflux from the jejunum up to the gizzard (80). Traveling further down the GIT, the ileum and ceca become more favorable environments with fewer enzymes and antimicrobial compounds; this is reflected in the increased concentrations of commensal bacteria, around 10^9 and 10^{11} bacteria/g, respectively (46). The unique anatomical structure of the cecum allows for the occupancy of fermentable substrates not widely available in different areas of the GIT; this enables differing microorganisms to reside and produce large amounts of energy metabolites to aid in achieving the bird's energy requirements (81).

Research profiling whole body energy consumption patterns has attributed 22.8% to being utilized by the GIT and liver (82), but not all of that energy is actually being used by and for the host. It was reported that the presence of GIT microbiota significantly increased the dietary metabolizable energy in the broiler chicken host, indicating that the microbiota are responsible for utilizing the additional dietary energy (83). The commensal bacterial communities utilize nutrients from the host's diet as energy sources, making those nutrients unavailable to the host. However, they are able to produce short-chain fatty acids (SCFAs) from the fermentation of those nutrients (84). Research suggests the GIT microbiota aid in digestion and energy release from starch and fibrous contents, especially in the ceca. It is proposed that the amounts and types of SCFAs that are generated in the ceca are in proportion to differing starches that enter the ceca (85). Although SCFAs serve as additional energy sources for the host, it is suggested that only a proportion (up to 25%) of the overall SCFA energy is recovered by the bird (85, 86). In high-fiber and low-energy diets, bacterial digestion of the fiber also releases energy in the form of SCFA (84, 87). Along with generating accessible energy, the gut microbiome is associated with conservation of energy when nutrient sources (proteins, fats, and sugars) are low (88, 89). The production and absorption of SCFAs in the intestine are occurring continuously, with more or less being produced due to alterations in the diet or cecal microbiome (85).

Conversely, the resident microbiome has also been associated with unfavorable effects to the host's utilization of dietary energy. Although the presence of the GIT microbiota has indicated a significant increase in levels of metabolizable energy in conventionally raised broiler chickens when compared to germfree (89), the metabolizable energy is attributed to the products generated by the GIT microbiota. The variation can be associated with the digestibility of those energy sources (dietary fiber and starches) being broken down into monosaccharides and SCFA. The SCFA are portrayed as possessing a high metabolic energy value, yet they are inefficiently utilized by the host. Therefore the levels of SCFA present are not reflective of the net deposition of energy to the host (86, 89). Another potential explanation may be that the presence of the gut microbiota increases the cost of energy by altering the rate of energy-consuming reactions (89, 90). For example, pathogens attach to the epithelial lining, alter its integrity and function, and in turn stimulate the renewal of epithelial lining, which increases the amount of dietary energy spent on gut maintenance (27, 91). It has also been observed that conventionally raised birds have higher energy requirements for maintenance when compared to germfree birds (92). This may be due to the addition of the host's microbiota usage of metabolizable energy, or the host's microbiota making dietary energy unavailable to the host (92).

Avian Microbiome and Foodborne Pathogens

The complex lining of the lower intestines with bacteria serves as a barrier against colonization of pathogenic bacteria, which if allowed to occur, could lead to infection. The bacteria that settle first in the lining of the intestines necessitate that any other microorganisms in search of new residence must compete for space and nutrients in order to survive and colonize (80, 93, 94). Establishing the early foundation of a mature GIT microbiota has been associated with prevention of infection with pathogens, namely *Salmonella*, by beneficial bacteria outcompeting the pathogenic bacteria for space and nutrients (95–98). In nature, chicks are hatched in the presence of maternal fecal contents, allowing rapid colonization of members from the maternal gut microbiome (25). In an attempt to colonize newly hatched chicks with a mature and healthy microbiome that will discourage pathogenic bacteria from colonizing, chicks have been experimentally inoculated with competitive exclusion culture mixtures (97, 99–102). Introduction of the competitive exclusion cultures has proven to be effective in protecting young chicks from enteric pathogens and several reviews have been written on various aspects of this research (103–106).

As previously mentioned, commensal bacteria produce SCFA, which are recognized as having growth-inhibiting effects on enterobacteriaceae (107–109). The presence of the SCFA causes a drop in cytoplasmic pH, which is recognized as a contributing factor to the inhibition of pathogen growth (110). Although the mechanisms of SCFAs are not well understood, they are known to exhibit bactericidal and bacteriostatic properties (30–32, 111). Russell (30) suggested that it is not only the result of a drop in pH caused by the SCFA but also the uncoupling reactions produced by the translocation of protons by SCFA that contribute to the growth inhibition effects seen. In accordance with this notion, Davidson et al. (112) suggested that because the fatty acids produced are weak acids, they are effective as antimicrobials in their undissociated forms as they are able to easily diffuse through the cytoplasmic membrane of the microorganism. The fatty acids dissociate into anions and protons once in the cytoplasm of the microorganism (maintained relatively neutral or slightly alkaline), in turn decreasing the pH and causing conformational changes of cytoplasmic proteins, enzymes, and nucleic acids. In an attempt to reestablish a neutral/slightly alkaline pH, microorganisms utilize ATP-dependent pump systems to transport the anions and protons outside of the cell. This is in accordance with findings of Cherrington et al. (113), where incubation of *Escherichia coli* with propionic and formic acids resulted in reduced rates of macromolecular synthesis initially, yet it partially regained synthesis rates after continued incubation.

Anion accumulation is suspected to be another factor in uncoupling reactions that attributes to growth inhibition of bacteria in the presence of SCFA. It is suggested that the accumulation of acid anions causes an uncoupling effect of the electron transport chain from oxidative respiration (via the passage of molecules in their dissociated and undissociated forms, transferring protons into the cell to dissipate the proton motive force) as well as a chaotropic effect (disrupting hydrogen bonding in water causing macromolecules in solution to lose stability) that are accountable for the increased hydrogen ion leakage into the cell. The cell is unable to excrete hydrogen ions rapidly enough, making it difficult for the cell to regain its neutral/slightly alkaline intracellular pH (30, 110, 114, 115). The intracellular increase in hydrogen is unable to counteract the accumulation of acid anions (116). Another inhibitor of bacterial growth by SCFA is the disruption of the membrane of a microorganism by means of permeabilization or intercalation, allowing for the release of macromolecules and the destabilization of the membrane (117, 118). However, there are instances of pathogenic bacteria acquiring resistance to SCFAs (32). For example, pre-incubation of *Salmonella* with high concentrations of SCFA at neutral pH resulted in an acid tolerance response and has also been demonstrated to be responsible for modulation of virulence gene expression and attachment/invasion of *in vitro* tissue culture cells (119–122).

While the production of fatty acids is inhibitory to invading bacteria, studies suggested that the fatty acids are inactive against the species that produced them (123). Smulders et al. (124) found that acid-producing bacteria are tolerant to acids and in turn, the acidic environments that they generate. Therefore, the influences of the SCFAs produced by autochthonous bacteria may provide protection against pathogenic bacteria – *Salmonella*, coliforms, and *Campylobacter* – intent on colonizing in the intestine while leaving commensal bacteria unscathed (125). However, little else has been reported on the effects of the fatty acids on the producing species.

Key Players in the Gut Microbiota

In the past, the microorganisms colonizing the GIT were thought to be commensal, neither beneficial nor harmful to the host, as opposed to being mutualistic (37). However, numerous germfree experiments in various animal models have indicated the value of these indigenous microorganisms (126–128). There has been overwhelming data collected revealing the beneficial impacts on both host physiology as well as immunology (75, 129). Several studies have indicated that introducing a balance of beneficial microorganisms to the poultry microbiota improves body weight gain and feed conversion ratio as well as warding off common diseases in poultry, such as Newcastle disease and infectious bursal disease (130–132). However, in order to better promote strategies for increasing the presence of beneficial bacteria, those bacteria and their interacting counterparts must be identified.

Although being incredibly diverse, the most abundant microorganisms in the gut microbiota of poultry are primarily anaerobic (54). This is somewhat expected since there is little to no oxygen available as an electron acceptor in the lumen, although the concentration of oxygen is greater toward the epithelium, thus forcing bacteria to use fermentation to produce pockets of organic acids within the lumen (133). Moreover, Sun and O'Riordan (133) suggest that as a result of this environment, it is necessary to investigate SCFAs more in depth because bulk analysis does not reveal the true nature and spatial arrangement of these acids (which would further indicate the location and family of anaerobic bacteria). There is no consistent data available indicating the overall Gram status of poultry GIT microbiota. Investigation into

the commensal bacteria present in an untreated chicken ceca has resulted in an array of bacterial communities (Gram-positive Y-branched, Gram-positive non-sporulating, Gram-negative) and may be attributed to the rearing conditions, chicken breed, diet, or even the cultivation and enumeration methods applied for bacterial characterization (125). Nevertheless, there are trends observed in available data investigating the microbial populations in broiler chickens grown in a conventional poultry flock and those raised under laboratory conditions (76, 134).

Lactobacilli and bifidobacteria are two of the more well-known beneficial bacteria, however, there are numerous others: *Bacillus*, *Enterococcus*, *E. coli*, *Lactococcus*, *Streptococcus* as well as undefined mixed cultures (**Table 3**) (23). These bacteria are indigenous to the GIT, occupy space, and consume nutrients along the intestinal tract, limiting the colonization of pathogenic bacteria. In addition to competing for space and nutrients, these bacteria have been recognized for exporting bacteriocins, which can target and kill invading pathogens (133). All of these microorganisms fit under the umbrella term probiotics. Like prebiotics, probiotics also have specified criteria and characteristics: (1) non-pathogenic and of host origin, (2) resistant to gastric pH and processing/storage, allowing them to persist in the intestinal tract, (3) able to adhere to epithelial and mucosal membranes, (4) modulate immune responses, and (5) produce inhibitory compounds (23). It is the complexity and broad diversity of the beneficial microorganisms that make up the microbiome and allow for a mature and healthy host (51, 52).

Bacteria may be beneficial to the host by aiding in degradation of polysaccharides otherwise indigestible to the host. The monosaccharides produced can be subsequently broken down further into SCFAs and lactic acid (37). As previously mentioned, both lactobacilli and bifidobacteria are beneficial and indigenous to the human and chicken GIT (145). Lactobacilli are members of a group collectively referred to as lactic acid bacteria, which metabolize carbohydrates to produce lactic acid as the primary end product (146). Oligosaccharides are their main nutritional source, which is reflected in their residence in ecological niches rich in carbohydrate-containing substrates, most commonly plant material, spoiled or fermented foodstuffs, and mucosal membranes of humans and animals (147). Along with their broad range of habitats, lactobacilli are able to adapt to various conditions by altering their strictly fermentative metabolism accordingly; they may be obligately homofermentative, facultatively homofermentative, or obligately heterofermentative (148). Their fermentative status is based on the levels and proportions of end products they generate from fermentation of differing substrates (although other factors, such as chemical and physical environment, play a role in determining fermentative status). Obligately homofermentative indicates that their primary fermentation product is lactic acid (>85%) generated by fermenting hexoses (149). Facultatively homofermentative indicates that they are capable of fermenting hexoses and pentoses using different pathways to generate lactic acid (although under low substrate concentration and strictly anaerobic conditions, they are capable of producing acetic acid, ethanol, and formic acid). Obligately heterofermentative lactobacilli ferment hexoses generating equimolar amounts of lactic acid, CO_2, and acetic acid (148–150). Although the end products produced are a fair indication of fermentative status, they are not the sole factor. These microorganisms are aerotolerant and acidophilic, allowing for the GIT to be an optimal residence (146, 151).

Bifidobacteria are another well-documented example of beneficial bacteria. They are often associated with lactic acid bacteria for their production of lactic acid, however, they are phylogenetically distinct. Bifidobacteria are Gram-positive, heterofermentative, and non-motile (152). Like lactobacilli, bifidobacteria digest oligosaccharides to use as carbon and energy sources, to produce lactic acid, acetic acid, ethanol, and formic acid (153). They are not exclusive to the utilization of dietary compounds, they can also digest carbohydrates produced by other members of the GIT (154). Additionally, they are capable of internalizing simple sugars remaining in the environment, thus preventing pathogenic bacteria from utilizing them as a nutrient source (155).

Both lactobacilli and bifidobacteria are known to be members of the intestinal microbiota in animals and humans; their presence is important for the maintenance of the GIT microbiota (156–158). Being that lactobacilli and bifidobacteria are autochthonous and dominant in the GIT, they can be utilized as a control method of pathogenic bacteria by competition, for example *Clostridium perfringens* (156). Lactobacilli and bifidobacteria possess characteristics that allow them to out-compete pathogenic bacteria. Various strains of lactobacilli adhere to intestinal epithelial-like cells and exhibit antimicrobial activity against bacteria typically found in the (human) GIT (157). A link between the lactobacillus strain's pH tolerance and antimicrobial properties has been reported, both *in vitro* and *in vivo* (157).

TABLE 3 | Suggested microorganisms for potential probiotic use based on various characteristics.

Microorganism	Host	Site isolated	Rationale	Reference
Enterococcus faecium	Chicken	Intestines	Bacteriocin-producing ability	(135)
Pediococcus pentosaceus	Chicken	Intestines	Bacteriocin-producing ability	(135)
Mixed culture[a]	Chicken	Cecum	Inhibition ability of *Salmonella*	(99, 136–139)
Lactobacillus reuteri	Chicken	GIT	β-glucanase gene enhances growth and nutrient digestion	(140)
Lactobacillus fermentum	Chicken	GIT	Intestinal adherence, pathogen inhibition, tolerance to gastric enzymes	(141)
Bifidobacterium longum	Chicken	GIT	Anti-Campylobacter activity	(142)
Streptococcus faecium	Chicken	GIT	Impacts of body weight, feed conversion, carcass yield, *Salmonella* colonization	(143)
Streptococcus bovis	Cattle	Rumen	Inhibition ability of *Salmonella*	(144)

[a]*Mixed culture composed of 29 cecal bacterial strains that have shown to inhibit Salmonella colonization.*

Different species of lactobacilli and bifidobacteria produce various antimicrobial agents, which allow them to be inhibitory toward pathogenic bacteria. Many species of lactobacilli and bifidobacteria produce SCFA; the production of these acids causes a drop in intestinal pH. The lowered pH level extends the lag phase for sensitive microorganisms (124). The undissociated forms of these acids are able to penetrate the microbial cell and hinder metabolic functions (further information on the mechanisms of these acids was discussed in a previous section of the current review). Another end product generated from lactobacilli and bifidobacteria is CO_2, which has demonstrated inhibition of microbial growth (149). The inhibitory mechanism of CO_2 is unclear, although Eklund (159) was able to rule out the proposed mechanism of CO_2 accumulation in the membrane of the microorganisms, physically interrupting the bacterial membrane. Growth of *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Bacillus cereus* has been shown to be inhibited in the presence of CO_2 at various concentrations (159). Succinic acid is produced by both lactobacilli and bifidobacteria, although at minimal levels (160, 161), and is associated with antibacterial activities in a multitude of environments (162, 163). Diacetyl is an end product of lactobacilli that exhibits antimicrobial effects. It is suggested that diacetyl is more effective in a lower pH ($\leq$7) causing it to be lethal to Gram-negative bacteria and inhibitory of yeasts (164). Bacteriocins, produced by lactobacilli, may have a narrow or broad range of activity. Lindegren and Dobrogosz (149) have reviewed the various antimicrobial agents produced by lactic acid bacteria in more detail.

Overgrowth of any single type of bacteria can have unfavorable effects on the host. Lactobacilli are considered beneficial bacteria, however, antibiotic growth promoters that stimulate improved growth of broilers were also associated with heightened sensitivity of lactobacilli to those antibiotics (165). Although the host may benefit from the commensal bacteria competing with pathogenic bacteria, an overgrowth of commensal bacteria can be detrimental to the host by excessive uptake of nutrients making them unavailable to the host (166). Additionally, overgrowth of lactobacilli can impair host fat absorption by not allowing proper biotransformation – deconjugation and dehydroxylation – of bile acids (14). Overgrowth of bacteria can also lead to overproduction of fermentation end products to the detriment of the host. For example, overgrowth of *Streptococcus bovis*, a commensal lactic acid-producing bacteria can generate considerable acid production and a concomitant lowering of the surrounding environment pH. This sequence can be advantageous for competing against pathogens. Consequently, under *in vitro* incubation conditions in co-culture with *Salmonella typhirmurium* growth of *S. bovis* can behave as a probiotic and directly limit *Salmonella* growth as a function of carbon source and time of inoculation (144). However, when easily fermented carbohydrates are fed to ruminants, excessive *S. bovis* growth can occur in the rumen resulting in rapid lactic acid overproduction, subsequent lowering of the ruminal pH, and the eventual development of a harmful ruminal lactic acidosis condition in the animal (167). Therefore, even though *S. bovis* might be considered a gut commensal organism, and in some cases a probiotic candidate, it can also be associated with host clinical disease states, such as bacterial endocarditis and colon cancer in humans (144).

Introduction and History of Prebiotics

The most widely accepted definition of prebiotics are non-digestible feed ingredients that are selectively fermented by beneficial bacteria in the lower GIT (capable of withstanding harsh conditions in the upper GIT) so as to provide energy to promote bacterial growth and metabolism in the colon which contributes to specific changes that lead to improved host health (22, 35, 168). Colonic food is a non-digestible ingredient that makes it past the upper GIT and into the colon, serving as a substrate for non-specific bacterial inhabitants, both beneficial and harmful (169, 170). Not all colonic foods are necessarily prebiotics; the rationale for designating a compound as a prebiotic or not depends upon whether beneficial bacteria alone are able to digest it. Some miscellaneous compounds that serve as colonic food, but do not fall into the category of prebiotics because of the non-specific targeting of microbiome bacteria include resistant starch, non-starch polysaccharides, non-digestible oligosaccharides, and yeast fermentation products (171). There have been numerous studies conducted and reviews written covering common prebiotics and their beneficial impacts; therefore they will not be discussed in detail here (**Table 4**) (35, 172–174).

Some lesser-studied prebiotic-like compounds are *Saccharomyces cerevisiae* fermentation products (SCFPs) or yeast culture (YC) components; these compounds do not fall

TABLE 4 | Published reviews on the considerations of common prebiotics in various hosts.

Prebiotic	Considerations	Host	Reference
Inulin-type	Structure overview	Not applicable	(172)
Short-chain carbohydrates	Gut function and health	Human	(175)
Inulin-type	Bifidogenic, resistant to digestion	Non-specific	(176)
Resistant starch	Production of SCFA, microbiome modulation, gut-associated immunomodulation	Human	(177)
Mannan-oligosaccharides	Modulation of gut microbiome	Poultry	(27)
Fructo-oligosaccharide, galacto-oligosaccharide, lactulose	Criteria for prebiotic classification	Human	(168)
Inulin-type, oligofructose	Quantification of inulin and oligofructose in Western diet	Human	(173)
Fructo-oligosaccharide	Bifidogenic, lack carcinogenic and toxic effects	Poultry, swine	(174)
Fructo-oligosaccharide, inulin-type	Selective to beneficial bacteria, prevent pathogen colonization	Poultry	(178)

into the precise definition of prebiotics as set by Roberfroid (22), among other classical definitions. However, they have prebiotic-like effects in that they have been shown to enhance nutrient utilization and digestibility, as well as improving the immune system and inhibiting pathogen-intestinal cell interaction by modifying the GIT microbiome (179–181). The fermentation of *S. cerevisiae* – undefined strains – produces SCFP. They include the fermentation products and metabolites, the media used in the fermentation to preserve fermentation activity, and both the yeast cell wall fragments and residual live yeast cells; thus, they share characteristics in both probiotic and prebiotic realms (179). There are commercial YC products available that are being more thoroughly investigated to identify their exact effects and maximize the directed influence(s) they may have.

Because yeasts are most often associated with the wine making, brewing, baking, and other fermenting industries, it is critical to consider why these unique organisms were initially promoted for use in improvements of animal and human health. In order to do this, a brief review of the history of yeast that led to its usage as a feed additive is discussed in the following section.

Introduction to Yeast: History and Background

To understand the current use of yeast and yeast products in food and agricultural settings, it is important to at least briefly describe the history of yeast in scientific applications and the evidence for the close relationship among yeast strains originally uncovered and those used in today's laboratory-based research. Humans began using yeast over 7,000 years ago, with its earliest usage dating back to the Neolithic times for wine making (182, 183). In the past century, yeasts have been investigated on a genetic level after the Carlsberg Laboratory introduced scientific concepts to the brewing industry, as discussed by Greig and Leu (184). In the 1930s, the genetic analysis of yeast became accepted based on its potential as an experimental organism; it was pioneered by Øjvind Winge and Carl Lindegren (185). Winge used a strain isolated from the Carlsberg Laboratory, while Lindegren used a strain, EM93, isolated from rotting Californian figs (182). Yeast continued gaining popularity in the scientific field for its ease in gene manipulation (182). In the 1950s, Robert Mortimer constructed the strain S288C, which has been purported to share more than 85% of its genome with EM93, Lindegren's original strain (most laboratories involved in the analysis of yeast use a derivative of EM93 – a strain of *S. cerevisiae*). This strain was subsequently sequenced in 1996, making it the first fully sequenced yeast genome (186, 187). For further purposes of the current review, *S. cerevisiae* is the main species of yeast discussed unless otherwise indicated.

Yeast in the Laboratory

A renowned model organism, yeast is a single-celled fungal eukaryote that most often divides by budding. Yeasts are used in various industries because of their ability to ferment sugars in the absence of oxygen to produce CO_2 and alcohol. In a laboratory setting, yeasts are most often used for analysis as a model template to study higher eukaryotic organisms. Yeasts are ideal for studying processes known to occur in more complex eukaryotic organisms because even though yeasts are unicellular, they encode similar proteins and are thus representative of more complex organisms at the cellular level (188). When comparing all yeast protein sequences to mammalian sequences, of the potential protein encoding regions in yeast, "statistically robust" homology among the two was observed (189). Because of the lack of mammalian protein families and proteins sequenced, there may be much greater similarities between the two.

Part of the attraction of yeast as an experimental model is the ability to easily manipulate and mutate genes, either on plasmids or in the yeast chromosome itself, to view the resulting phenotypic effects (182). An insight into its fairly simple manipulation is evident in research performed by both Caspeta et al. (190) and Liu et al. (191). Caspeta et al. (190) manipulated *S. cerevisiae* into expressing thermotolerance to temperatures ≥34°C (typical response to these temperatures is serious impairment of function) by exposing the isolate for short stretches of time to increased heat followed by serial batch transfers. This resulted in non-inheritable heat tolerant strains that exhibited increased growth rates as well as increased glucose consumption rates at higher temperatures when compared to thermolabile strains (190). Thermotolerance has also been bestowed upon *S. cerevisiae* by the introduction of genes from organisms that are naturally thermotolerant. This transfer of genes allows for inheritable alteration in future generations of *S. cerevisiae*. Duina et al. (182) illustrated the extent to which yeast has proven its efficacy as a model organism, discussing research advancements and accolades (Nobel Prize and Lasker Award) in an array of fields achieved by utilizing yeast.

Although great progress has resulted from the study of yeast, it has also stimulated further inquiry. Yeast researchers began with the goal of determining functions of single genes and proteins, but now seek a "systems level" approach. The benefit of understanding how proteins interact to maintain cellular functions (metabolism, reproduction, growth, regulation, signaling, and homeostasis) is now at the forefront for yeast biology (192). Yeast's position as a model organism for various scientific fields is reviewed more thoroughly in several articles and therefore will not be further discussed here (192–194). A review by Siddiqui et al. (195) encompasses the potential of engineering yeasts to contain secondary metabolite pathways for pharmacological purposes. Additionally, Sherman (196) has generated a comprehensive review (both extended and truncated versions available) on the biological basics of yeast, which includes a section on a variety of outside literature references for yeast.

Yeast Metabolism

Yeasts are capable of cellular respiration in the presence and absence of oxygen; for this review, we will discuss respiration only in the absence of oxygen, as it is most applicable to the topic of the current review. Anaerobic respiration, or fermentation, is the process of breaking down sugars to generate energy for carrying out cellular processes. In anaerobic cellular respiration, sugars are broken down into pyruvate and subsequently decarboxylated and reduced to form CO_2 and ethanol. For fermentation to begin, any complex sugars must be broken down into simple sugars

(e.g., sucrose to glucose and fructose) via enzymes from yeasts, adding an additional step to the fermentation process (197). In the process of understanding this, it is recognized that complex carbohydrates (starches and fiber) are more challenging for yeasts to ferment than simple sugars. Investigation into the types of sugars and environments yeasts are capable of fermenting is necessary to optimize the production and utilization of yeast fermentation products. By understanding the conditions in which yeast fermentation is optimized, they can be engineered to generate additional metabolites that may prove to be beneficial for use in animal feed.

Yeast as an Animal Feed Additive

The usage of live yeast and yeast products in animal feed is not a new concept, although pinpointing the exact point of its conception has proven to be challenging. It is suggested that the introduction of YC in animal feed was not until the 1980s (198). It appears that the majority of research has been dedicated toward ruminants, while equine, porcine, poultry, and companion animals received attention to a lesser extent. Initially, yeast was used in an array of modes because of the large quantities of yeast biomass waste generated by distilleries (and other yeast utilizing industries) (199). It was used as a feed additive because it was a rich source of protein, fiber, and minerals. It has been hypothesized that both viable and non-viable yeast cells provide essential B vitamins and organic acids (200). In the past, both viable and non-viable yeast cells have been added to animal feed – including poultry feed – and resulted in increased host growth and improved health (199).

It is essential to have a precise definition for YC, so it is not confused with using live yeast (probiotic/direct fed microbial form) or yeast extract (only soluble portion of yeast autolysis) products (201). As described in a previous section of the current review, YC contains the cellular constituents as well as residual viable cells. It is effective when used because it contains lysed yeast cells; this allows for the nutrients within the yeast cells to be available for digestion and absorption (202). These yeast cells are lysed by autolysis; they are subjected to temperature or osmotic shock, thereby killing the yeast cell while leaving the endogenous enzymes undamaged. The yeast cell's own enzymes begin to degrade the yeast cell, releasing its contents and further degrading its proteins into amino acids (203). Some yeast cells that are capable of tolerating the temperature or osmotic shock, do not autolyze, and remain metabolically active.

The mode of action of YC is seen to enhance digestive and fermentative functions of the GIT, while modifying activities of the GIT microbiota, although the mechanisms are less clear (198). Based on *in vitro* and *in vivo* studies, supplemented YCs appear to have several impacts on the rumen microbiota including increased numbers of beneficial bacteria and fiber digesting bacteria as well as shifting away from hydrogen consuming methanogens and toward bacteria capable of converting hydrogen and CO_2 to acetic acid, all of which could, in turn, potentially benefit the ruminant host animal either directly or indirectly (204, 205). Enhanced growth performance resulting from the supplementation of YC with probiotics (*Lactobacillus acidophilus* and *Streptococcus faecium*) has indicated its potential effect of increasing digestion and absorption of the GIT microbiota occurring in broiler chickens (206). de Oliva Neto et al. (207) conducted studies on the antibacterial properties of YC supernatant, which indicated a reduction of pathogenic bacterial growth when tested against a common distillery bacterial species. Interestingly, the supernatants were tested as both fresh and post freeze/thaw, and reported similar results indicating the antimicrobial activity could withstand freezing. Conversely, when heat (90°C for 20 min) was applied, the antibacterial activity was destroyed. Accordingly, YC and yeast extract have yielded varying results, which suggests the necessity for metabolically active yeast cells. When supplementing heat-treated inactive yeast cells to steer diets, there was no effect on the concentrations of cellulolytic bacteria, while supplementing live, metabolically active yeast cells increased the concentration of cellulolytic bacteria (208).

In addition to their ability to interfere with bacteria due to their relative large size, supplementation with live yeast products has led to a few suggested modes of action (209). One mechanism suggested by Jouany et al. (204) involves metabolic competition with bacteria that may be adhering to and digesting fiber or starch molecules. In this scenario, the yeasts ferment the carbohydrates produced, prohibiting their usage by other bacteria. Another mechanism of action of live yeast cells is their ability to produce protective products with antitoxin effects (210). Yeast intake has resulted in a stimulation of activity of host intestinal brush border enzymes, which has counteractive effects to those of pathogens, along with supplying the host with additional enzymes (211). Elimination of oxygen has been deemed the most influential mode of action in ruminants (212). Although there is little oxygen present in the GIT, live yeast cells scavenge for excess oxygen introduced by food and water intake; this allows for a more optimal environment for anaerobic bacteria (204, 212). Most all implications regarding the mechanism of oxygen elimination have been derived from studies conducted on ruminants.

As noted previously, the majority of the studies on the effects and mechanisms of YC have been performed on ruminants. Although such studies may be a good indicator of the potential use of YC in other animals, it can also be expected that there will be differences seen among ruminants and non-ruminants. For example, considerable research has been conducted on the effects of milk production in cattle, while this is beneficial for other lactating animals, the information gleaned from these studies holds little merit for poultry researchers. Instead, conducting *in vitro* and *in vivo* studies on specific animal subjects of interest would be more useful in identifying the mechanisms of YC in those animals rather than projecting ruminant/rumen microbiota results onto non-ruminant species.

Impact of YC on Host–Microorganism Interactions

The effects of YC on the intestinal morphology in swine have indicated increased jejunal villi width, which allows for greater digestive and absorptive intestinal capacity leading to better body weight gain when compared to controls (180). In contrast, poultry data obtained has thus far indicated significant differences in intestinal morphology (213–215). Supplementation of YC has resulted in more shallow crypt depths, indicating less necessity for cell renewal and turn-over, allowing for decreased

host energy utilization for intestinal epithelial maintenance (216). Feed efficiency and body weight gain have both resulted in significant increases when YC, yeast derivatives, and live yeast cells are added to the poultry diet (215, 217, 218).

Inclusion of YC in animal feed has led to suggestions that they may aid in the clearing of pathogens from infected animals. A study involving the inoculation of pigs with *Salmonella* suggested that the inclusion of YC in the diet allowed for rapid shedding of the pathogen from the GIT (180). Supplementation of broiler feed with YC has also been seen to enhance adaptive immune system T lymphocytes, allowing for better clearing of the pathogens (181). El-Husseiny et al. (219) observed that commercial YC were able to significantly increase antibody production against SRBC, much in agreement with the findings of Al-Homidan and Fahmy (220), who reported significantly higher antibody titer concentrations in response to Newcastle disease in broilers fed YC.

Further examination into the components of yeasts' cell walls indicates the beneficial structural polysaccharides present and released into culture when yeast cells autolyze. Mannan-oligosaccharide (MOS) is included in the YC as it is derived from the outer cell wall of *S. cerevisiae*. MOSs bind to pathogenic bacteria in the GIT, preventing their attachment to the mannan residues on intestinal epithelia (221). This not only protects the host from pathogens but also allows for host energy reserves to be utilized for their own growth rather than to the repair and regeneration of the epithelial lining (222). β-glucans are also released when the yeast cell wall is degraded; presence of these molecules can lead to pathogen inhibition along with immunomodulating effects. Similar to MOS, β-glucans act by preventing pathogens from binding to the villi of the gut mucosa (214, 216). Additionally, β-glucans are known to activate phagocytes, natural killer cells and B and T lymphocytes as well as increase cytokine production and phagocytic activity of macrophages (223).

Mannan-oligosaccharide supplementation has been reported to increase broiler growth performance when supplemented in their diet (224, 225). *In vitro* experimentation has indicated that addition of MOS inhibits the attachment of enteropathogenic *E. coli* to the gut mucosa as well as removing attached *E. coli* from the mucosa (226). Inclusion of yeast fermentation products, like MOS, appears to reduce pathogenic bacterial populations. The mechanism is unclear, although the agglutination of the pathogens with sugars from the yeast cell wall occurs rather than attachment to the host intestinal lining is one hypothesized mechanism (227). Yang et al. (228) indicated MOS altered the gut microbiota of broilers and reduced the number of mucosal-associated coliforms.

Although some studies suggest a positive association between yeast and growth promotion (229, 230), other studies have indicated no positive effects on inclusion of YC in broiler diets (231). Paryad and Mahmoudi (229) indicated that inclusion of 2% yeast (*Saccharomyces cerevisae*) in broiler chicken diets resulted in significant differences in body weight gain, feed intake, and feed conversion rate when compared to controls. Similarly, investigation into YC on growth promotion in lambs suggested its efficacy, resulting in increased feed intake and growth by 8 and 26%, respectively. Conversely, similar research conducted on lambs evaluating the efficacy of three yeast strains and a mixed culture resulted in little consistency and lacked an overall effect when compared among yeast strains (232). Adebiyi et al. (231) also showed no significant differences in body weight gain in broiler chickens when fed varying percentages of YC.

Yeast Metabolites and Metabolism as Prebiotic-Like Substances

In addition to the structural polysaccharides derived from the yeast cell wall, yeasts generate a number of metabolites that may offer benefits to the host animal when supplemented to animal feed. Metabolites include carotenoids, vitamins, enzymes, amino acids, and some miscellaneous products (200). Several yeast species are naturally capable of producing carotenoids (including β-carotenes), which are subsequently metabolized into vitamin A (200). Vitamin A aids in cellular differentiation and proliferation, making it critical for intestinal maintenance and health (233). The enzyme responsible for the synthesis of vitamin A from β-carotene is β,β-carotene 15,15′-monooxygenase, which has been isolated and characterized from the intestines of poultry, among other animals (234, 235). Although *S. cerevisiae* is not capable of naturally producing carotenoids, it is capable of and has been engineered to express a biosynthetic pathway for the production of β-carotene (236).

Other vitamins (vitamin precursors) produced by yeasts include ergosterol, L-ascorbic acid, and D-erythroascorbic acid. Ergosterol is particularly abundant in *S. cerevisiae*, accounting for up to 90% of the total sterols (237). It is located in the membrane of yeasts and is responsible for its fluidity, structure, permeability, and activity of membrane-bound enzymes (238). Ergosterol is a precursor to both vitamin D_2 and cortisone (239). Vitamin D_2 is responsible for the proper absorption and transport of calcium, among other minerals (240). D-Erythroascorbic acid is also synthesized by *S. cerevisiae* and depending on the substrates available, that pathway can be manipulated into producing L-ascorbic acid (vitamin C) (241). The ingestion of vitamin C has been suggested to alleviate some of the repercussions of heat stress: poor immune function and growth performance (242). However, instances of supplementation of L-ascorbic acid in poultry diets have had varying results; some resulted in increased levels of superoxide dismutase in 45-week-old broilers, while others revealed no effect on the activities of antioxidative enzymes, superoxide dismutase included in 7-week-old broilers (243, 244).

Yeasts are recognized for their production of enzymes expressing various activities (245). Jones (246) wrote a comprehensive review documenting the activities of the proteolytic systems in *S. cerevisiae*, along with mentioning other enzymes elucidated in *S. cerevisiae* (carboxypeptidases, aminopeptidases, and dipeptidyl aminopeptidases). An enzyme in *Saccharomyces boulardii*, a subtype of *S. cerevisiae*, was found to degrade the ileal receptors in rats for toxin A generated from *Clostridium difficile* (a food-associated pathogen causing gastroenteritis; one study isolated *C. difficile* from 2.3% of broiler chickens tested) (247, 248). The degradation of the receptors prohibits the toxin from binding and prevents infection from occurring (249, 250). There have been multiple other proposed mechanisms of action for yeast on the immunoprotective effect in the GIT, specifically the prevention of *C. difficile* infection: (1) *S. boulardii* releases proteases that hydrolyze toxins and prevent its binding to the intestinal receptor (250),

(2) *S. boulardii* is capable of stimulating the activity of disaccharidases in the intestinal brush border with no additional alterations of the intestinal mucosa (211), and (3) *S. boulardii* increased the production and secretion of glycoproteins, namely the secretory component of immunoglobulin A (251). Potentially, by narrowing the focus on the exact mechanism of action, *S. cerevisiae* could be engineered to confer said mechanism and supplemented into animal (poultry) feed to prevent colonization of *C. difficile*.

Invertase is another enzyme produced by *S. cerevisiae*; it hydrolyzes sucrose into glucose and fructose (252). Invertase efficiency and sucrose availability allows for glucose to be a carbon source for *S. cerevisiae* (252). Ideally, provided the diet contained appropriate levels of sucrose, one could engineer *S. cerevisiae* to overproduce invertase and subsequently add it to poultry feed. This would allow increased production of glucose, available not only for its own needs but also for other microorganisms in the surrounding environment. This mode of action would not be selective toward beneficial bacteria in the microbiome.

Yeasts have multiple amino acid transport systems; amino acids are incorporated into proteins or they are broken down and utilized as nitrogen and carbon sources to promote growth (253). Yeasts and yeast derivatives are capable of producing amino acids; therefore supplementation to animal feed would provide both the host and the microbiome with amino acids. Almquist (254) reviewed the essential amino acid requirements in young chicks, laying hens, and turkeys; Almquist included a table outlining the percentages of each amino acid to reach a specific protein level. Amino acids are necessary for poultry to have proper growth and promote efficient weight gain and feed conversion ratios (255). Lysine appears to be one such amino acid that plays a significant role in the body composition of poultry (256). Mutants of *S. cerevisiae* have been revealed to produce up to 17 times as much lysine as wildtype; thus this rich source of lysine may prove to be valuable to the growth and development of poultry (257).

Miscellaneous metabolites are also produced in *S. cerevisiae*, including toxins responsible for the "killer phenomenon." Originally, this phenomenon was considered to be lethal only toward members of the same species; however, further investigation has led to the recognition of these toxic species to have destructive consequences reaching both prokaryotic and eukaryotic organisms (258–261). Polonelli and Morace (261) acknowledge that the inhibition of outside species may not be a direct impact on the toxins secreted, but more of a concerted effort from multiple metabolites. Nevertheless, these toxic species of *S. cerevisiae* are displaying lethality toward unrelated species. This can be utilized to the advantage of commercial poultry production, provided further research is conducted on characterizing whether this toxicity also occurs toward beneficial bacteria.

Conclusions: Impact on Poultry Industry and Future Directions

In the search for a replacement to antibiotic growth promoters, the poultry broiler industry has two main objectives, a substance that (1) increases the growth of broiler chickens (body weight gain and feed conversion ratio) and (2) prevents the colonization of invading pathogens. Ideally, a single feed additive would prevent pathogen colonization while developing beneficial microbiota to aid in bird growth and feed conversion (262). Multiple feed additives have been attempted: antimicrobial agents, probiotics, prebiotics, and prebiotic-like substances. Probiotics need to be clearly identified and carefully analyzed to understand the influence they may have on the poultry GIT microbiota. As discussed previously, lactobacilli and bifidobacteria are two known groups that provide the host health and well-being based on their end products. These bacteria both ward off pathogens by creating an unfavorable environment against pathogen retention in the gut and also generally aid host GIT health, in turn resulting in enhanced bird growth (133).

To increase the efficacy of supplying probiotics to the host, the concept of synbiotics has been suggested. Synbiotics entail equipping the beneficial bacteria with substrates specific to their metabolic needs (23). Potentially, this allows for the greatest impact as it reduces the substrates taken by the probiotics from the host. Prebiotic-like substances are often times non-selective, therefore, combining a probiotic and a prebiotic-like substance does not fit into the synbiotic definition (263). Understanding the effects and specificity of probiotics, prebiotics, and prebiotic-like substances will allow for the best match of known commensal bacterial communities and substrates for a given host.

Yeast cells and YC products developed thus far have been extensively examined for their effects as supplements in animal feed. Numerous studies report the positive association with growth performance, immunostimulation, and microbiome modulation in animals and humans (209). In addition to being explored for their positive impacts as supplements in animal feed, yeasts and their derivatives have been investigated for their low risk and assurances of safety in their usage. Yeasts are cost efficient in both production and formulation (200). They do not have the ability to transfer genes they may acquire to pathogenic or commensal bacteria, or to the host. Yeasts are able to resist acquisition of antimicrobial resistance as well as not allowing for the transfer of such resistance (209). This also allows yeast to be safely used in parallel with antibacterial agents. Yeasts also have multiple mechanisms of action, allowing them to be productive in a range of environments (200).

A more thorough understanding of the microbiome can elucidate the mechanisms of prebiotics and prebiotic-like substances. The GIT microbiome is distinct and unique in its functionality relying on the presence of a definable, and potentially identifiable, microbial consortia. Understanding the influences of the members of the microbiome and also the microbiome as a single entity will allow for a more directed approach in the search of therapeutics and growth promoters. The GIT microbiome may be more appropriately considered as an additional organ; it has impact on host growth and development, and host health.

The limitations in previous research conducted have made future research necessary to resolve unanswered questions. It is imperative to define universal and standardized detection methodology to identify the bacterial communities present in the healthy, mature poultry microbiome. This would alleviate the issue of having varying results based on detection methods utilized. In addition, evaluating the currently suggested probiotic candidate organisms (**Table 3**) indicates the potential advantages of involving multiple potential probiotic bacterial and yeast strains to exhibit

a concerted effort in maintaining GIT health. This would allow for the identification of potentially more uniform mixed probiotic cultures consisting of functionally well-defined individual bacterial members that when used to inoculate newly hatched chicks ensures more rapid development of a mature beneficial microbiome.

Further work with yeast, YC, and yeast extracts needs to be conducted on poultry. Much of the discussion in the current review was based on the results from yeast products applied to animals and humans but not poultry. To gain an accurate sense of the effects in poultry, such experimentation needs to be conducted in poultry (*in vitro* and *in vivo*). Additionally, many of the metabolites mentioned previously were investigated independent of yeast, YC, or yeast extract. It would be beneficial to assess the impact of metabolites and components from yeast individually as well as when combined. This would allow for the identification of beneficial metabolites and their respective individual and combined functional impacts on the corresponding host.

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REVIEW
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Regulation of the Intestinal Barrier Function by Host Defense Peptides

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Intestinal barrier function is achieved primarily through regulating the synthesis of mucins and tight junction (TJ) proteins, which are critical for maintaining optimal gut health and animal performance. An aberrant expression of TJ proteins results in increased paracellular permeability, leading to intestinal and systemic disorders. As an essential component of innate immunity, host defense peptides (HDPs) play a critical role in mucosal defense. Besides broad-spectrum antimicrobial activities, HDPs promotes inflammation resolution, endotoxin neutralization, wound healing, and the development of adaptive immune response. Accumulating evidence has also indicated an emerging role of HDPs in barrier function and intestinal homeostasis. HDP deficiency in the intestinal tract is associated with barrier dysfunction and dysbiosis. Several HDPs were recently shown to enhance mucosal barrier function by directly inducing the expression of multiple mucins and TJ proteins. Consistently, dietary supplementation of HDPs often leads to an improvement in intestinal morphology, production performance, and feed efficiency in livestock animals. This review summarizes current advances on the regulation of epithelial integrity and homeostasis by HDPs. Major signaling pathways mediating HDP-induced mucin and TJ protein synthesis are also discussed. As an alternative strategy to antibiotics, supplementation of exogenous HDPs or modulation of endogenous HDP synthesis may have potential to improve intestinal barrier function and animal health and productivity.

Keywords: host defense peptides, barrier function, tight junction, gut health, innate immunity

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INTRODUCTION

The gastrointestinal (GI) tract is lined by a single layer of epithelial cells that serve to facilitate digestion and absorption of nutrients and also act as a barrier to invading microorganisms, toxins, and dietary antigens. Intestinal barrier function is achieved through coating of the epithelial cells with a

Abbreviations: aPKC, atypical protein kinase C; CCR2, CC chemokine receptor 2; CD, Crohn's disease; DSS, dextran sulfate sodium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; ETEC, enterotoxigenic *E. coli*; GI, gastrointestinal; GSK-3, glycogen synthase kinase 3; GUK, guanylate kinase; HBD, human β-defensin; HDPs, host defense peptides; JAM, junction adhesion molecule; LPS, lipopolysaccharides; MAMPs, microbe-associated molecular patterns; MAPK, mitogen-activated protein kinase; MBD, mouse β-defensin; MLCK, myosin light chain kinase; MUC, mucin; P2X7, P2X purinergic receptor 7; PAMPs, pathogen-associated molecular patterns; PBD, porcine β-defensin; PDZ, post-synaptic density 95, disk-large, and zonula occludens; PI3K, phosphatidylinositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PRRs, pattern recognition receptors; SEA, sea urchin sperm protein, enterokinase and agrin; TACE, TNF-α-converting enzyme; TEER, transepithelial electrical resistance; TGF, transforming growth factor; TJ, tight junction; TR, tandem repeat; UC, ulcerative colitis; VWF, von Willebrand factor domain; ZO, zonula occludens.

mucus layer and the formation of a selectively permeable barrier across and between epithelial cells (1). The mucus layer consists primarily of mucin glycoproteins that are secreted by goblet cells, functioning as a physical barrier between the luminal contents and the host and also to facilitate nutrient digestion and absorption (2). However, the primary barrier function of the GI tract resides with epithelial cells, which transport water, ions, and macromolecules through either of two routes, i.e., the transcellular and paracellular pathways (1, 3, 4). The transcellular pathway refers to the movement of small molecules through epithelial cells either by active or passive transport, whereas the paracellular pathway refers to the diffusion of water, macromolecules, and immune cells between epithelial cells. In the presence of intact epithelial cells, the paracellular pathway dictates the intestinal permeability and is regulated by inter-epithelial connections known as tight junctions (TJs) (1, 3, 4).

Maintenance of mucin and TJ assembly ensures proper absorption and transport of nutrients, water, and electrolytes, while shielding the host from pathogens, toxins, intestinal microbiota, and dietary antigens. Disruption of the mucus layer and TJ complex, on the other hand, results in an increase in intestinal permeability, followed by heightened bacterial translocation, inflammation, and possibly the onset of various enteric and systemic disorders (1, 3, 4). In livestock production, impaired intestinal barrier function leads to reduced animal health and growth performance (5, 6). Therefore, it is critically important to understand how the intestinal barrier function is maintained and regulated in order to achieve optimal animal health and productivity.

Host defense peptides (HDPs), also known as antimicrobial peptides, are an important component of the animal innate immune system, and a majority of HDPs are expressed on mucosal surfaces, including the GI tract (7, 8). With potent antimicrobial and immunomodulatory activities, HDPs exert a pleiotropic effect on innate adaptive immune responses (9–11). Recent research has further shed light on the direct involvement of epithelial HDPs in regulating intestinal mucin and TJ protein expression and microbiota composition. The focus of this review is to summarize the latest advances regarding the emerging role of HDPs in maintaining intestinal barrier and homeostasis with a goal of exploring HDP-based therapies to improve gut health and performance of food-producing animals.

HOST DEFENSE PEPTIDES: A CRITICAL COMPONENT OF INNATE IMMUNITY

A variety of HDPs with direct antimicrobial activities are produced by host cells in response to infections. Among them are several major families found in vertebrate species such as defensins, cathelicidins, the S100 family, the RNase A superfamily, regenerating islet-derived III (REGIII) C-type lectins, and peptidoglycan-recognition proteins (12, 13). Defensins are primarily identified by three conserved disulfide bridges that form several antiparallel β-sheets due to the presence of multiple cysteine residues (14). Based on the spacing pattern of six cysteines, vertebrate defensins are further categorized into three subfamilies, including α-, β-, and θ-defensins. While β-defensins are present in all vertebrate animals, α-defensins are found in most but not all mammals, and θ-defensins only exist in primates (14). Cathelicidins are structurally recognized by the highly conserved cathelin domain found in the precursor that is cleaved off to release the biologically active peptides adopting a variety of structures such as α-helix (15).

The S100 family proteins are 9–14 kDa in mass containing two highly conserved Ca^{2+}-binding EF-hand domains that are separated by four α-helical domains with a variable C-terminal region (16, 17). The RNase A superfamily are characterized by the presence of 6–8 conserved cysteines forming distinct disulfide bridges, together with two invariantly spaced histidines and a lysine (18, 19). The REGIII family proteins are a group of soluble C-type lectins with a conserved carbohydrate-recognition domain that binds to sugars in a Ca^{2+}-dependent manner (20). Peptidoglycan-recognition proteins constitute a family of phylogenetically conserved host defense molecules with a PGRP domain that binds to bacterial peptidoglycans through specific interactions with the muramyl-tripeptide fragments (21).

Expression of HDPs

Six α-defensins (22) and a minimum of 39 β-defensins (23) have been reported in humans. The genomes of cattle and pigs encode at least 57 and 29 β-defensin genes, respectively (24, 25), while the chicken genome harbors a total of 14 β-defensin genes (26, 27), with no α-defensins being found in cattle, pigs, or chickens. All β-defensin genes are located in tandem in a single genomic region in the chicken (26) and are expanded to 4–5 different clusters in humans, cattle, and pigs (23–25). Interestingly, all human α-defensin genes form a single cluster within a β-defensin gene cluster (22), suggesting that α-defensins likely diverged from β-defensins. While four human α-defensins (HNP1–4) are abundantly present in neutrophil granules, the other two α-defensins (HD5 and HD6) are specific to Paneth cells in the crypts of the human small intestinal tract (14). On the other hand, a majority of β-defensins are expressed in a wide range of cell types, particularly the epithelial cells lining the skin, GI, respiratory, and urogenital tracts of all livestock species as well as humans (14).

A single cathelicidin known as LL-37, CAMP, or hCAP-18 is present in humans (28) and four cathelicidins are reported in chickens (29, 30). In cattle and pigs, 10 and 11 cathelicidins have been identified, respectively (31, 32). All cathelicidin genes are located in a syntenic chromosomal region in vertebrate species. Expressions of cathelicidins are widespread with abundant presence in neutrophil granules as well as various epithelial mucosal surfaces of cattle, pigs, and humans. Four chicken cathelicidins are similarly expressed in a broad range of tissues as well as in heterophils (29, 30, 33, 34), which are equivalent to neutrophils in mammals. Additionally, chicken cathelicidin-B1 is highly expressed in M cells of the bursa of Fabricius (30), a type of specialized epithelial cells involved primarily in antigen transportation from the intestinal lumen to submucosal immune cells (35).

The S100 family members have been found in all vertebrates (17). A total of 21 S100 proteins are present in humans, with 17 members clustered in the same 2-Mb region on chromosome

1q21 (17). The tissue expression pattern of S100 proteins is unique and isoform specific (16). For example, S100A7 (also known as psoriasin) isolated initially from the skin of psoriatic patients is mainly expressed in the skin and breast tissues, whereas the heterodimer S100A8/S100A9, or calgranulin A/B, is expressed in keratinocytes, neutrophils, monocytes, and macrophages.

Biological Functions of HDPs

Host defense peptides are an integral part of the innate immune system. Historically, HDPs are known for their ability to function as natural antibiotics with broad-spectrum antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, viruses, protozoa, and even cancerous cells (7, 36). Human LL-37 and α- and β-defensins are all capable of killing a broad spectrum of pathogens (7, 36). All four chicken cathelicidins have been demonstrated to be active at low micromolar concentrations against both Gram-positive and Gram-negative bacteria, including antibiotic-resistant strains (29, 30, 37–39). Several chicken β-defensins are also potent against a range of human and zoonotic pathogens (40–42). Similarly, β-defensins and cathelicidins in the cattle and pigs are broadly active against multiple pathogens as well (32).

Because of the cationic and amphipathic properties associated with a majority of HDPs, they kill bacteria primarily through disruption of cell membranes and/or interaction with intracellular macromolecules (43). A net positive charge allows HDPs to bind to negatively charged phospholipid groups on the bacterial membrane through electrostatic interactions. The amphipathic nature of HDPs facilitates their insertion into target cellular membranes allowing them to disrupt its integrity. Multiple models of membrane disruption, such as "barrel-stave", "carpet," or "toroidal-pore" models, have been proposed (43). Intracellularly, certain HDPs are also capable of inhibiting protein, DNA and RNA synthesis, or binding to specific targets (43). Because of their primary membrane-lytic activities, HDPs are generally equally active among drug-resistant and -susceptible pathogens. It is conceivably more difficult for pathogens to develop resistance to HDPs, although certain bacteria have developed mechanisms to resist their action in order to infect and colonize the hosts (44). It appears that commensal bacteria are generally resistant to the action of the constitutively expressed HDPs, but sensitive to certain inducibly expressed HDPs in the human intestinal tract (45). However, the mechanism by which commensal and probiotic bacteria show a reduced sensitivity to HDPs remains elusive.

Besides their antimicrobial activity, HDPs are involved in the modulation of innate and adaptive immune responses (10, 11) (**Figure 1**). Many human HDPs have been shown to promote the recruitment of neutrophils or monocytes and suppress proinflammatory response. Human HDPs also induce the differentiation and activation of macrophages and dendritic cells. Additionally, human cathelicidin LL-37 facilitates the resolution of inflammation by promoting re-epithelialization and wound healing as well as autophagy and apoptosis (10, 11). Three chicken cathelicidins known as fowlicidin 1–3 bind to bacterial lipopolysaccharides (LPS) directly with a strong capacity to neutralize LPS-induced production of inflammatory cytokines in macrophage cells (37–39). Furthermore, chicken fowlicidin-1 is chemotactic to neutrophils, but not monocytes or lymphocytes (46). Fowlicidin-1 also activates macrophages by inducing modest synthesis of inflammatory cytokines and chemokines and further potentiates the antibody response if co-administered with a model antigen (ovalbumin) in mice (46). Importantly, a single application of fowlicidin-1 is not only able to protect animals from an established infection (47) but also to prevent the disease beyond a 2- to 4-day window (46) in a murine model of methicillin-resistant *Staphylococcus aureus* (MRSA) infection (46).

Among 14 bovine β-defensins examined, three (BNBD3, BNBD9, and EBD) are chemotactic to immature monocyte-derived dendritic cells (48). Porcine cathelicidin PR-39 is also capable of inhibiting phagocyte NADPH oxidase activity and attenuating myocardial ischemia–reperfusion injury (49) by blocking the assembly of the enzyme complex through binding to p47phox, a cytosolic component of the NADPH oxidase (50). PR-39 accelerates wound repair by inducing syndecans (51). Furthermore, PR-39 facilitates angiogenesis and formation of functional blood vessels by inhibiting the ubiquitin–proteasome-dependent degradation of hypoxia-inducible factor (HIF)-1α (52). Several porcine cathelicidins also help with the update of bacterial DNA and subsequent activation of dendritic cells (53). HDPs with potent antimicrobial activity and the ability to modulate innate and adaptive immunity are, therefore, being actively exploited as novel antibiotics.

Additionally, recent emerging evidence has highlighted the beneficial effect of HDPs on mucosal barrier permeability by directly regulating mucin and TJ protein expression and shaping microbiota composition. This emerging role of HDPs in intestinal barrier function and homeostasis will be the focus of this review.

MUCUS LAYER: A LAYER OF INTIMATE PROTECTION FOR MUCOSAL SURFACE

An intact mucus layer that is composed primarily of secreted mucins plays a critical role in maintaining the intestinal barrier function (54, 55). Mucins are large, highly glycosylated proteins ranging from 0.5 to 20 MDa. Synthesized and released by goblet cells, mucins function to coat the mucosal surface to facilitate the passage of substances, maintain proper cell hydration, act as a permeable barrier for the exchange of gas and nutrients, and also protect the epithelial cells from invading pathogens and toxins (54, 55). Structurally, a hallmark of all mucin protein backbones is the presence of 1–5 tandem repeat (TR) domains, which consist of an excessive number of identical or nearly identical TR sequences rich in serine, threonine, and proline residues (56) (**Figure 2**). The TR domain is heavily glycosylated because of attachment of oligosaccharides to serine and threonine through *O*-linked glycosylation, giving rise to 50–80% glycans in mass. Saturated sugar coating is beneficial to increase the water-holding capacity and the resistance of mucins to proteolytic cleavage.

In humans, the mucin family consists of up to 20 members, including both secreted and membrane-bound forms. Secreted mucins form homo-oligomeric, gel-like structures constituting the mucosal layer, while the membrane-bound mucins are part

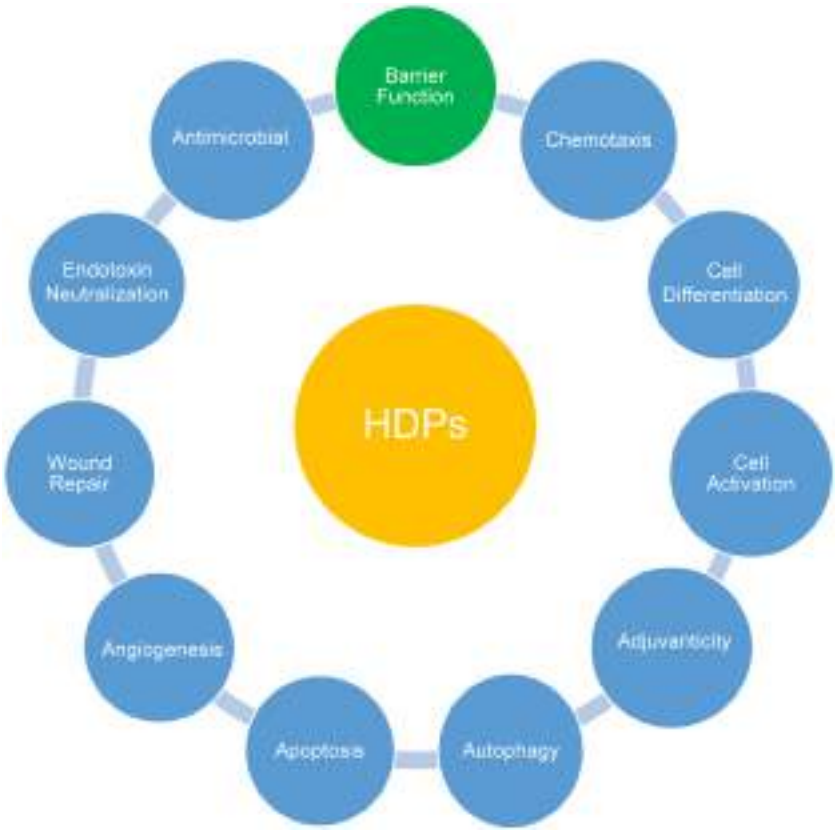

FIGURE 1 | Multifunctional roles of host defense peptides (HDPs). Besides direct antimicrobial activities, HDPs actively participate in systemic and mucosal epithelial defense by modulating a range of host innate and adaptive immune responses as indicated. Recent accumulating evidence has highlighted a direct involvement of HDPs in improving intestinal and epidermal barrier function.

of the epithelial glycocalyx that are involved in cell signaling and interactions with the environment without forming gel or oligomerization (57). Secreted human mucins include MUC2, -5AC, -5B, -6, -7, and -19, and the transmembrane mucins consist of MUC1, -3, -4, -12, -13, -15, -16, -17, and -20 (54, 55). Structurally, most secreted mucins are unique in the presence of multiple von Willebrand factor (VWF) domains and a C-terminal cysteine-knot domain, while a majority of membrane-bound mucins specifically consist of a transmembrane domain, a cytoplasmic tail, 1–2 epidermal growth factor (EGF)-like domains, and a sea urchin sperm protein, enterokinase, and agrin (SEA) domain (56, 58) (**Figure 2**). The VWF domains and cysteine-knot domain of secreted mucins are responsible for formation of higher-order structures through oligomerization, while the EGF-like and SEA domains of membrane-bound mucins mediate signaling transduction and cleavage of the extracellular portion of mucins, respectively (58). Among all secreted mucins, MUC2 is the most abundant in the human small intestine and colon, and MUC5AC is predominant in the stomach. Structurally, similar mucins have been found in most other vertebrate species, including cattle, pigs, and chickens (59–62).

The mucus layer formed by secreted mucins varies in composition along the GI tract. The stomach and large intestine consist of two distinct mucus layers: a "loose" outer layer and a "thick" inner layer (2). The inner layer closest to epithelial cells is densely packed and holds firmly to the cells. The inner mucus layer is largely free of bacteria, providing a sterile protective environment for the epithelium. The outer mucus layer is much more soluble due to proteolytic cleavages that allow the mucus layer to expand without disrupting mucin polymers. This outer layer provides a habitat for commensal bacteria to bind via specific adhesins and to thrive via breaking down the mucin glycans as a food source. Specificity of bacteria for different glycans is speculated to be important for developing species-specific microbiota (2).

Altered expression or glycosylation of mucins is often associated with intestinal barrier dysfunction (57). For example, Muc2 deficiency in mice causes increased permeability, gross bleeding, spontaneous development of inflammation in the GI tract, as well as severe growth retardation (63, 64). Muc1- or Muc2-deficient mice become more prone to infections with *Campylobacter jejuni*, *Helicobacter pylori*, *Salmonella enterica* serovar Typhimurium, and *Citrobacter rodentium* (64–67). Moreover, mice lacking the enzyme, β1,3-*N*-acetylglucosaminyltransferase that synthesizes *O*-glycans on mucins, exhibit a thinner mucus layer showing an enhanced susceptibility to enteric bacterial infections (67) and dextran sodium sulfate (DSS)-induced colitis (68). Additionally,

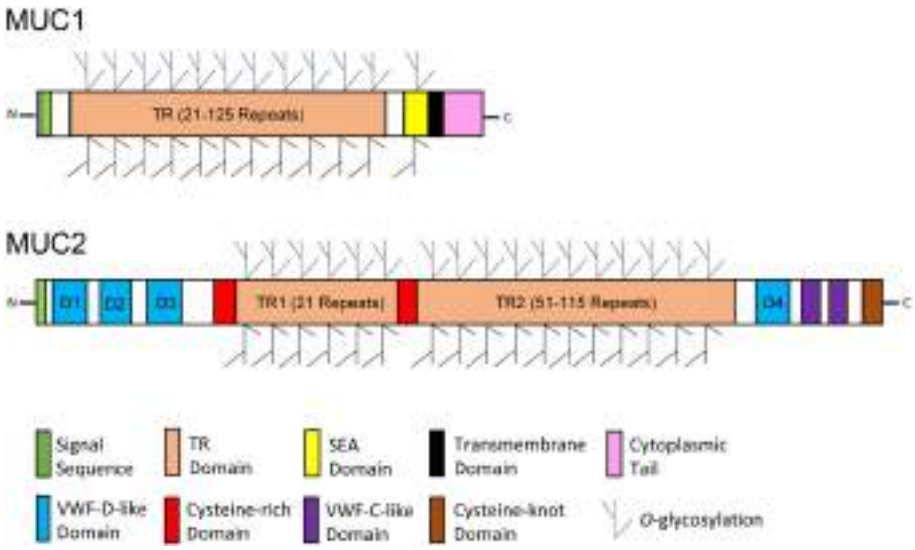

FIGURE 2 | Schematic diagrams of glycosylated mucin monomers. Representative membrane-bound and secreted mucins are exemplified by mucin 1 (MUC1) and MUC2, respectively. The protein backbone of mucins is characterized by the presence of an excessive number of tandem repeat (TR) sequences. Mucins are heavily *O*-glycosylated through enriched threonine and serine residues in the TR domain. A transmembrane domain, a cytoplasmic tail, and a sea urchin sperm protein, enterokinase, and agrin (SEA) domain are unique to most membrane-bound mucins, whereas the presence of several von Willebrand factor (VWF) domains and a C-terminal cysteine-knot domain is specific to a majority of secreted mucins. The diagrams were modified primarily from reference (56).

significantly reduced expressions of multiple mucins such as MUC1, MUC3, MUC4, and MUC5B are observed in ileal mucosa of Crohn's disease (CD) patients (69), although the expression changes of mucins are less clear in ulcerative colitis (UC) patients (70).

TIGHT JUNCTIONS: GATE GUARDS FOR BORDER PROTECTION

The intestinal epithelium is made up of several different cell types organized into crypts and villi. These include intestinal epithelial stem cells, enterocytes, and secretory cells, such as Paneth, goblet, and enteroendocrine cells (71). Intestinal stem cells give rise to all other epithelial cell types, while enterocytes primarily function in nutrient absorption with the ability to synthesize and release HDPs and mucins. Paneth and goblet cells are major producers of HDPs and mucins, respectively, while enteroendocrine cells have a primary role of secreting numerous hormones that act as regulators of digestive function (71). All intestinal epithelial cells are linked at lateral membranes through formation of three major types of junctional complexes, i.e., TJs, adherens junctions, and desmosomes (1, 3, 72). Collectively, they form a virtually impermeable seal to the paracellular space. Besides the barrier function, these junctional complexes maintain cell polarity by separating the apical from basolateral membranes. TJs are multi-protein complexes located at the most apical end of the lateral membrane. The TJ assembly is composed of both transmembrane and cytoplasmic plaque proteins that interact directly with the cytoskeleton (**Figure 3**). Among all three major junctional complexes, only TJs have the ability to control the selective paracellular permeability for ions, water, and other small molecules (1, 3, 72). Therefore, TJs are the major determinant of mucosal epithelial permeability.

Tight Junction Structures

Among the proteins involved in TJ assembly, claudins, occludin, junctional adhesion molecules (JAM), and tricellulin are the major transmembrane proteins that constitute a selective paracellular barrier, whereas zonula occludens (ZO) and cingulin are the main cytoplasmic plaque proteins located at the peripheral membrane (1, 3, 72). All TJ proteins are highly conserved in vertebrate species. Claudins are a large family of small proteins of 21–34 kDa that make up the backbone of the TJ structure, with at least 26 members reported in humans (73). Remarkably, each claudin shows a unique tissue expression pattern with varied levels of expression in different segments of the GI tract. Occludin (65 kDa) was the first transmembrane TJ protein identified (74), with no homologs being found (75). On the other hand, the JAM family is comprised of three classical members (JAM-1, -2, and -3) and four related molecules (JAM-4, JAM-L, CAR, and ESAM) at ~40 kDa each (76). Tricellulin is a 64-kDa protein located preferentially at tricellular junctions, although it is also present in bicellular junctions (77). Tricellulin shares 32% identity in the amino acid sequence with the C-terminal tail of occludin. ZO proteins belong to the family of membrane-associated guanylate kinase (GUK) homologs that include three members, i.e., ZO-1 (~220 kDa), ZO-2 (~160 kDa), and ZO-3 (~130 kDa) (78), whereas cingulin is an ~140-kDa protein that links ZO proteins to the actin cytoskeleton (79).

Claudins, occludin, and tricellulin are all membrane proteins with four transmembrane domains, one intracellular and two

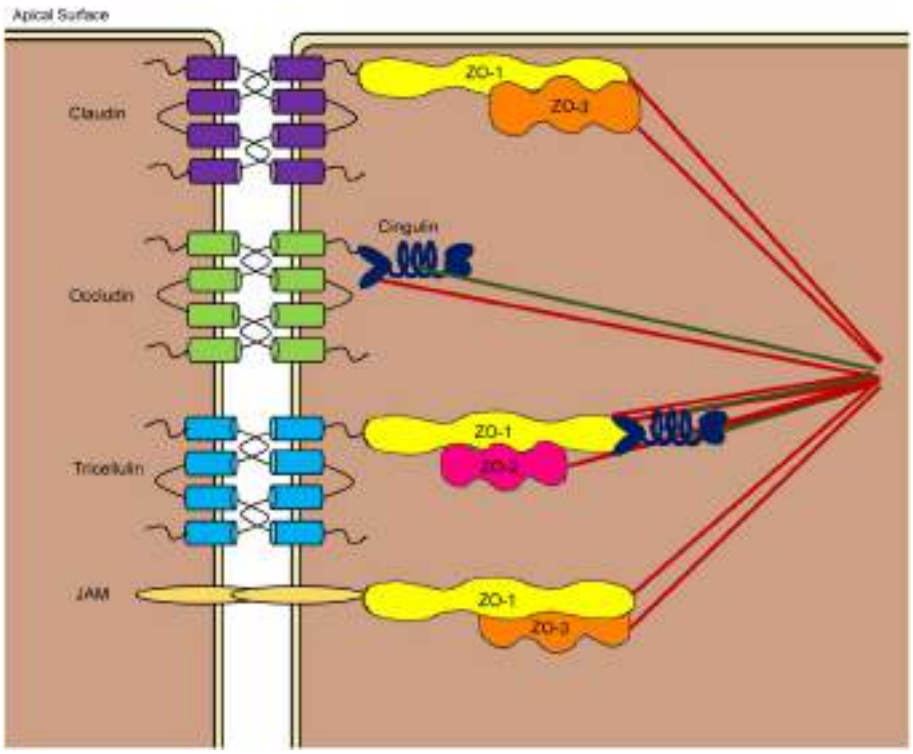

FIGURE 3 | Schematic drawing of tight junction structures at the apicolateral membranes of the paracellular space. Tight junctions are comprised of transmembrane proteins such as claudins, occludin, junctional adhesion molecules (JAM), and tricellulin as well as cytoplasmic plaque proteins such as three zonula occludens (ZO) proteins and cingulin. Claudins, occludin, and tricellulin consist of four transmembrane domains, while JAMs have a single transmembrane domain. Through largely homophilic interactions, neighboring cells are sealed at the apicolateral region. Cytoplasmic proteins link the tight junction assembly to the cytoskeleton to control the opening of paracellular pores.

extracellular loops, and two cytoplasmic tails, whereas the JAM proteins are single-pass transmembrane proteins consisting of two extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain, and a short intercellular C-terminal tail (72) (**Figure 4**). While the N-terminal tail is generally short, the longer C-terminal tail of claudins consists of a post-synaptic density 95, disk-large, and zonula occludens (PDZ)-binding motif that interacts with the first PDZ domain of ZO-1 (73). The crystal structure of claudins as exemplified by mouse claudin-15 indicates that the four transmembrane segments form a tight four-helix bundle with parts of the two extracellular loops forming a "palm-shaped" structure (80). A model for the architecture of claudin-formed TJ strands in the membrane has been proposed based on the results of crosslinking experiments and electron microscopy (81). In this model, claudins show an antiparallel, double-layer arrangement (**Figures 5A,B**). The association of claudin double layers in neighboring lateral membranes results in the formation of multiple extracellular β-barrel-like pores parallel with the membrane plane to allow the passage of ions through the paracellular space (**Figure 5C**).

As for occludin, its two extracellular loops mediate homophilic interactions and permeability to macromolecules. The occludin–ELL domain in the C-terminal tail of occludin is responsible for interacting with the GUK domain of ZO proteins, while the N-terminal tail lacks a defined function (75) (**Figure 4**). In comparison with claudins and occludin, tricellulin consists of two long tails, with the C-terminal tail containing an occludin–ELL domain that is likely to interact with the GUK domain of ZO proteins (82). JAM proteins also contain a cytoplasmic tail with a PDZ-binding motif that interacts with the third PDZ domain of ZO-1 (83) (**Figure 4**). The extracellular Ig-like domains of JAM proteins are responsible for homophilic and heterophilic interactions.

The cytoplasmic ZO proteins contain three PDZ domains, a Src homology-3 (SH3) domain, and a GUK domain (78). As stated above, ZO-1 directly interacts with claudins, ZO-2, and JAM-1 through the first, second, and third PDZ domain, respectively. The GUK domain of ZO proteins is known to associate with occludin, while the C-terminal actin-binding region is responsible for bridging with actin, raising the possibility of forming a large protein complex through simultaneous interactions of many TJ proteins with ZO-1 (78). Cingulin is another intracellular plaque protein that is predicted to form a homodimer with globular head and tail at both ends connected by a coiled-coil "rod" domain in the central region. The head of cingulin is known to bind to ZO proteins and the coiled-coil domain interacts with myosin (79). The interactions between the TJ protein complex and cytoskeleton are critical in maintaining and regulating the TJ

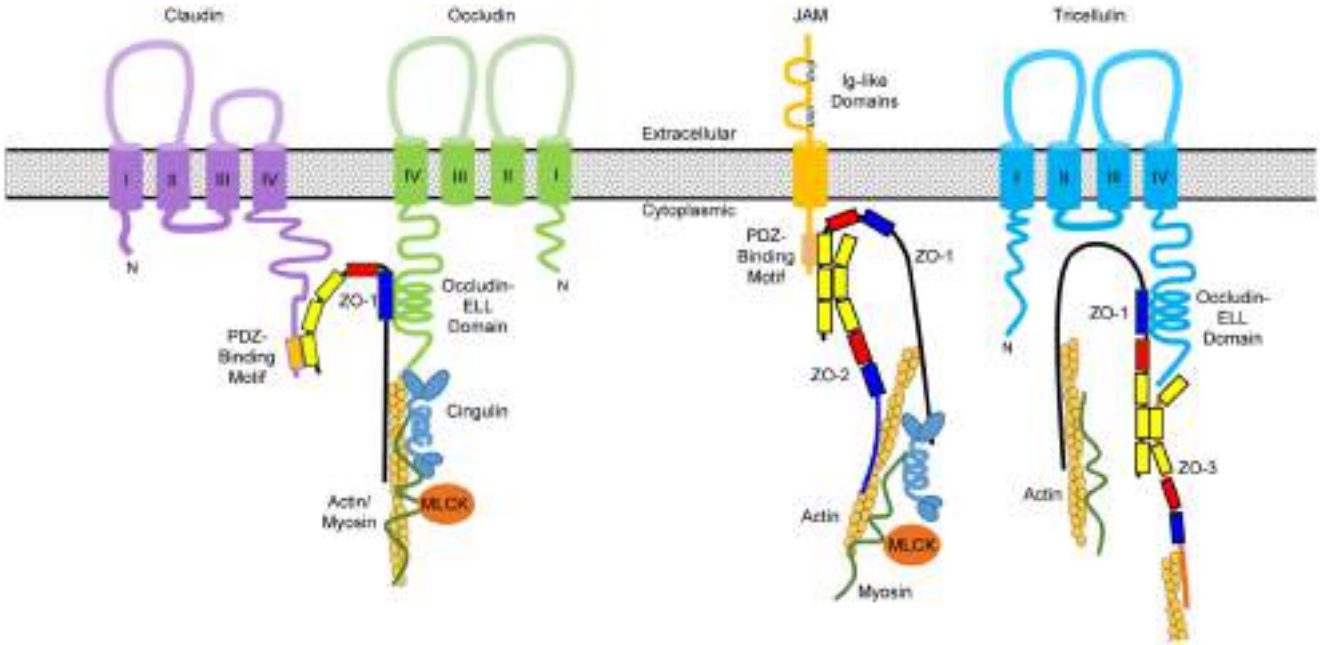

FIGURE 4 | Domain structures of primary tight junction proteins and their interactions. Claudins consist of four transmembrane domains with a PDZ-binding motif at the C-terminal tail, which interacts with the first PDZ domain (in yellow) of zonula occludens (ZO)-1. The GUK domain (in blue) of ZO-1 binds to the occludin–ELL domain at the C-terminal tail of occludin and possibly tricellulin as well. Junctional adhesion molecules (JAM) contain a single transmembrane domain, two immunoglobulin (Ig)-like domains in the extracellular region, and a PDZ-binding motif at the C-terminal tail that interacts with the third PDZ domain of ZO-1. ZO molecules form heterodimers through interactions at their second PDZ domain. The C-terminal segment of ZO proteins binds directly to actin filaments as well as the globular head of cingulin that interacts with myosin through its rod domain. The globular head of cingulin also binds to the C-terminal occludin and actin filaments. Phosphorylation of myosin light chain by myosin light chain kinase (MLCK) will cause contraction of the perijunctional actomyosin ring and opening of the tight junction channels.

structure and function, as the mucosal permeability is regulated heavily by the phosphorylation status of myosin light chain, which can be modified by the kinases such as myosin light chain kinase (MLCK) (72, 73) (**Figure 4**).

Tight Junctions' Function in Selective Permeability

Tight junctions are distributed at the apical surface of epithelial and endothelial cells throughout the body in vertebrate animals, including the skin, GI, respiratory, and urogenital tracts as well as the blood vessels (72). TJs are the major determinant of mucosal barrier permeability. Ions, water, and macromolecules pass TJs through either of the two major types of pores. The non-restrictive pathway, also known as the "leak" pathway, allows the transport of macromolecules through large pores with no charge selectivity, while the restrictive or "pore" pathway is only permeable to small ions through pores of ~4 Å in radius with charge selectivity (1, 3, 4). Claudins are mainly responsible for the "pore" pathway, and the two extracellular loops work as an "electrostatic selective filter" to select the size and charge for small pores (**Figure 5**). The charge selectivity of individual claudins is determined by the net charge of the amino acid residues in the first extracellular loop. For example, claudin-1 is selective for anions, while claudin-2 prefers cations. In the intestinal tract, claudin-1, -3, -4, -5, -8, -9, -11, and -14 decrease paracellular permeability and are regarded as barrier-forming claudins, while claudin-2, -7, -12, and -15 are to increase permeability and generally referred as pore-forming claudins (72, 73).

The non-restrictive "leak" pathway is primarily dependent on occludin as evidenced by an increase in paracellular flux of macromolecules without a noticeable effect on transepithelial electrical resistance (TEER) both *in vitro* and *in vivo* after occludin knock-down (84). JAM proteins also enhance the TJ function by decreasing permeability and facilitating the assembly of occludin to the TJ complex (83). In addition, JAMs can regulate the paracellular barrier for the transmigration of leukocytes from the blood vessel to inflamed sites in response to inflammation (76). It is noteworthy that paracellular water permeability is mainly dictated by claudin-2.

Modulation of Paracellular Permeability

A number of agents such as cytokines, growth factors, pathogens, probiotics, nutrients, and phytochemicals have been found to impact TJ permeability and mucosal barrier functions through transcriptional regulation and posttranslational modification of TJ proteins (72, 73). Increased expressions of barrier-forming claudins, occludin, and JAM proteins are commonly associated with reduced paracellular permeability and improved barrier function, whereas an elevation in the expression of pore-forming claudins often leads to barrier dysfunction. For example,

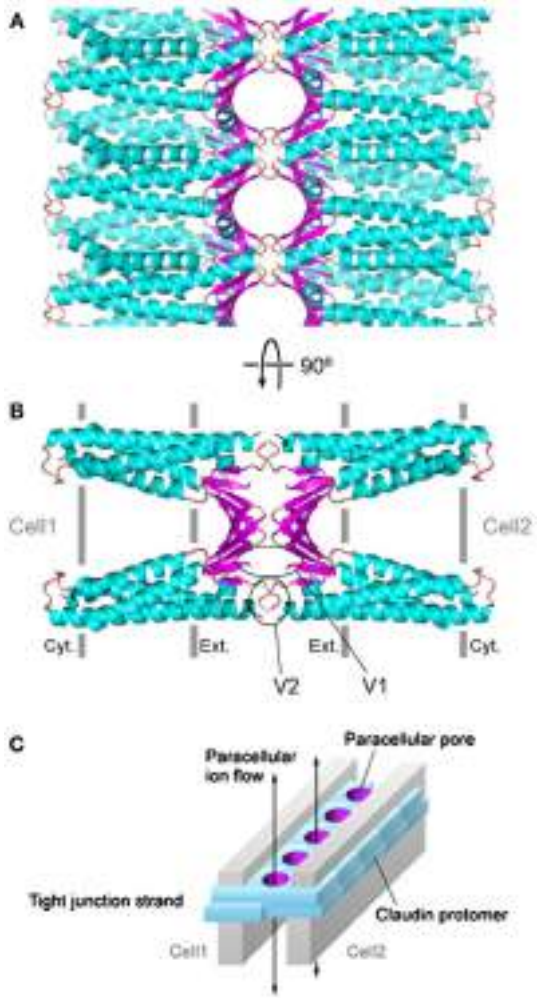

FIGURE 5 | A model of paracellular claudin-based tight junction channels. Putative β-barrel-like channels are formed by the extracellular domains (in magenta) of double-layered claudins, whose transmembrane helices are depicted in cyan. The tight junction complex is shown in parallel **(A)** and perpendicular **(B)** views, respectively, from the apical surface of the cells. **(C)** A schematic drawing of claudin-based tight junction channels. The paracellular pores are colored in magenta and double-layered claudins are represented by cyan blocks. Two gray plates indicate two neighboring cell membranes, and the arrows indicate the directions of ions passing through the tight junction channels. The graphs are adopted from the open-access reference (81).

transforming growth factor (TGF)-β enhances barrier integrity of intestinal epithelial cells by augmenting claudin-1 and -4 expression, while IL-1β, IL-6, and TNF-α increase intestinal cell permeability by increasing claudin-2 expression and/or reducing occludin and ZO-1 expression (72).

Tight junction barrier integrity is also affected by post-translational modifications of transmembrane and cytoplasmic proteins and associated regulatory proteins. Phosphorylation, glycosylation, and/or ubiquitination of the TJ proteins have a profound impact on barrier permeability. For example, claudin-1 is phosphorylated by atypical protein kinase C (aPKC), protein kinase A (PKA), and mitogen-activated protein kinases (MAPK) and dephosphorylated by protein phosphatase 2A (72, 73). Phosphorylation of claudins generally promotes their assembly into the TJ, whereas dephosphorylation often results in the dissociation of claudins from the TJ (72, 73). Similarly, phosphorylation of occludin enhances the barrier function, while dephosphorylation delays the TJ assembly resulting in barrier dysfunction (72, 75).

Besides those proteins involved directly in the TJ assembly, paracellular permeability is also heavily influenced by actin–myosin filaments that are linked to the TJ proteins. It is well known that up-regulation of MLCK is linked to an increase in the TJ permeability by catalyzing the phosphorylation of myosin light chain, which in turn induces the contraction of actin–myosin filaments and opening of the TJ barrier (85). Both IL-1β and TNF-α are strong inducers of the MLCK gene transcription and activation, resulting increased myosin light chain phosphorylation and TJ barrier permeability (86, 87).

The expression and posttranslational modifications of TJ proteins are influenced by a complicated network of signaling pathways that intertwine with each other. Activation of nuclear factor (NF)-κB signaling by pathogens, pathogen-associated molecular patterns (PAMPs), and proinflammatory cytokines often causes an increase in intestinal epithelial permeability through induction of pore-forming claudin-2 and suppression of barrier-forming claudins such as claudin-1, -3, -4, -5, -7, and -8. On the other hand, activation of TGF-β/SMAD and PPAR-α/γ signaling are generally barrier protective by enhancing claudin-1 and -4 expressions while downregulating claudin-2 (73).

Implication of Tight Junction Dysfunction in Disease Pathogenesis

The intestinal barrier helps to maintain homeostasis between gut microbiota and the immune system. TJ dysfunction is associated with many enteric disorders such as CD, UC, and celiac disease (3, 72). In CD patients, the expressions of barrier-forming claudin-3, -5, -8, occludin, and JAM-1 are decreased, while pore-forming claudin-2 is significantly increased; and in UC patients, a down-regulation of claudin-1, -4, and JAM-1, and up-regulation of claudin-2, is observed (3, 72). Moreover, increased MLCK expression and activity are evident in both CD and UC patients (85). These factors collectively exacerbate the intestinal paracellular permeability leading to a "leaky gut syndrome." However, many of these clinical conditions are also accompanied with increase synthesis of proinflammatory cytokines, which are known to cause barrier dysfunction. Thus, it is difficult to determine whether barrier dysfunction is a cause or effect of many of these diseases.

Several enteric pathogens such as *Vibrio cholera*, enteropathic *Escherichia coli*, *Clostridium perfringens* are known to cause diarrhea mainly through disruption of the intestinal barrier function by secretion of exotoxins (72). For example, claudin-3 and -4 are receptors for *C. perfringens* enterotoxin, and the binding of enterotoxin to the extracellular loops of claudins causes internalization of claudins and disintegration of the TJ assembly (88). Early weaning (<3 weeks of age) is known to impair the development of intestinal barrier functions of pigs leading to

more pronounced diarrhea (89), and was recently found to lead to reduced expressions of occludin, claudin-1, and ZO-1 in the jejunum (90).

REGULATION OF TIGHT JUNCTION AND MUCIN PRODUCTION BY HOST DEFENSE PEPTIDES

Association of HDP Expression with Barrier Dysfunction

Along with decreased TJ protein expression, aberrant HDP expression is common in CD and UC patients (91). The expression of Paneth cell α-defensins (HD5 and HD6) is significantly reduced in ileal CD patients, but unaffected in colonic CD patients Z (92). Instead, a reduced expression of HBD-1 and HBD-2 is observed in colonic CD (93). Moreover, induction of cathelicidin LL-37 and HBD-2, -3, and -4 is also reduced in colonic CD relative to healthy subjects (94–97). This lack of HDP induction in CD patients is thought to play a key role in CD pathogenesis as it indicates a lack of intestinal immune response. A deficiency in HD5 and HD6 synthesis is even more pronounced in patients carrying a mutation in the intracellular NOD2 receptor (98), which is expressed by Paneth cells. Consistently, NOD2-knockout mice show a diminished expression of Paneth cell α-defensins known as cryptdins in mice (99). In contrast to CD patients, UC patients display unchanged HD5 and HD6 expressions (92), while LL-37, HBD-2, -3, and -4 are upregulated (94–97). A thin or even absent mucus layer is evident in UC intestinal segments, which causes intestinal inflammation due to direct adhesion and invasion of bacteria to mucosal epithelial cells. Although UC patients produce HDPs, these peptides are not retained in the intestinal tract. Perhaps the most convincing evidence linking the positive role of HDPs in barrier function comes from the studies with cathelicidin (CRAMP)-deficient mice. These mice show delayed recovery of barrier permeability in response to acute disruption of epidermal barrier, albeit with subtle barrier abnormalities in the epidermis (100). Collectively, these lines of evidence suggest a direct impact of HDPs on intestinal barrier function and homeostasis.

Transcriptional Regulation of Mucins and TJ Proteins by HDPs

Accumulating pieces of evidence suggest a direct involvement of HDPs in regulating the synthesis of mucins and TJ proteins in the intestinal tract. HBD-2 upregulates MUC2, MUC3, but not MUC1 or MUC5AC in human HT-29 colonic epithelial cells (101). MUC2 expression is also enhanced in human Caco-2 colonic epithelial cells in response to HBD-2 (101), and upregulated MUC2 in turn promotes HBD-2 expression (102), suggestive of a positive feedback mechanism between MUC2 and HBD-2. LL-37 also enhances MUC1, MUC2, and MUC3 expressions in HT-29 cells (103, 104) and MUC3 expression only in Caco-2 cells (104). Buforin II, a 21-amino acid HDP isolated from the stomach of an Asian toad (*Bufo bufo garagriozans*), improves intestinal barrier function in weaned piglets challenged with three enterotoxigenic *E. coli* (ETEC) strains (105). Oral administration (twice daily) of buforin II leads to an increase in claudin-1, occludin, and ZO-1 expression in the jejunal segments of *E. coli*-challenged piglets (105). Importantly, buforin II also improves intestinal morphology and growth performance and reduced bacterial shedding in fecal swabs (105). Additionally, administration of a banded krait HDP known as cathelicidin-BF induces ZO-1 expression in the jejunum of healthy mice and also restores LPS-mediated impairment of ZO-1 and intestinal barrier function (106). Furthermore, porcine β-defensin-2 (PBD-2) is capable of restoring the expression of MUC1, MUC2, claudin-1, ZO-1, and ZO-2 as well as the barrier integrity of the colon of DSS-treated mice (107).

Besides direct regulation of the intestinal paracellular permeability, several HDPs also positively influence the barrier effect of the respiratory tract and the skin. LL-37 induces MUC5AC in human NCI-H292 airway epithelial cells (108). LL-37, HBD-3, and S100A7/psoriasin are all able to augment the expression of TJ proteins in human skin keratinocytes (109–111). HBD-3 induces the expressions of all 14 claudins examined, but not occludin or ZO 1–3 in human keratinocytes (110). Similarly, LL-37 dose-dependently enhances the expressions of 11 claudins and occludin in skin keratinocytes, but not JAM 1–3 or ZO 1–3 (109). S100A7 also promotes the expressions of multiple claudins and occludin, but not JAM 1–3 or ZO 1–3 in human keratinocytes (111). Multiple signaling pathways are involved in HDP-induced barrier protein synthesis as detailed below.

MOLECULAR MECHANISMS OF HDP REGULATION OF BARRIER FUNCTIONS

A number of extracellular and intracellular receptors have been reported to be responsible for a range of physiological functions of cathelicidins and defensins in humans and mice. LL-37 and the mouse ortholog (CRAMP) are ligands for P2X purinergic receptor 7 (P2X7), formyl peptide receptor-like (FPRL) 1/2, glyceraldehyde 3-phosphate dehydrogenase, and sequestosome-1/p62, whereas several human and mouse β-defensins bind to CC chemokine receptor 2 (CCR2), CCR6, CXC chemokine receptor 2 (CXCR2), and toll-like receptor 1/2/4 (112, 113). The receptors and signaling pathways by which HDPs induce the expression of mucins and TJ proteins have been studied, but remain elusive in most cases. It is worth noting that, although most published mechanistic studies were based on skin keratinocytes, the following overall conclusions are believed to be applicable to intestinal epithelia as well: (1) HDPs vary greatly in their ability to modulate barrier function, albeit with structural similarities, (2) mucins and TJ proteins are differentially regulated by HDPs, (3) multiple signaling pathways are employed by the same HDP, and (4) receptors appear to be differentially engaged in mediating the induction of mucins and TJ proteins by different HDPs. The current findings are summarized below.

Signaling Mechanisms of HDP-Mediated Mucin Induction

LL-37-induced MUC5AC expression in lung epithelial cells appears to be mediated mainly through transactivation of the EGF receptor (EGFR) (108), although EGFR is not a direct receptor for LL-37 (**Figure 6A**). Initially, LL-37

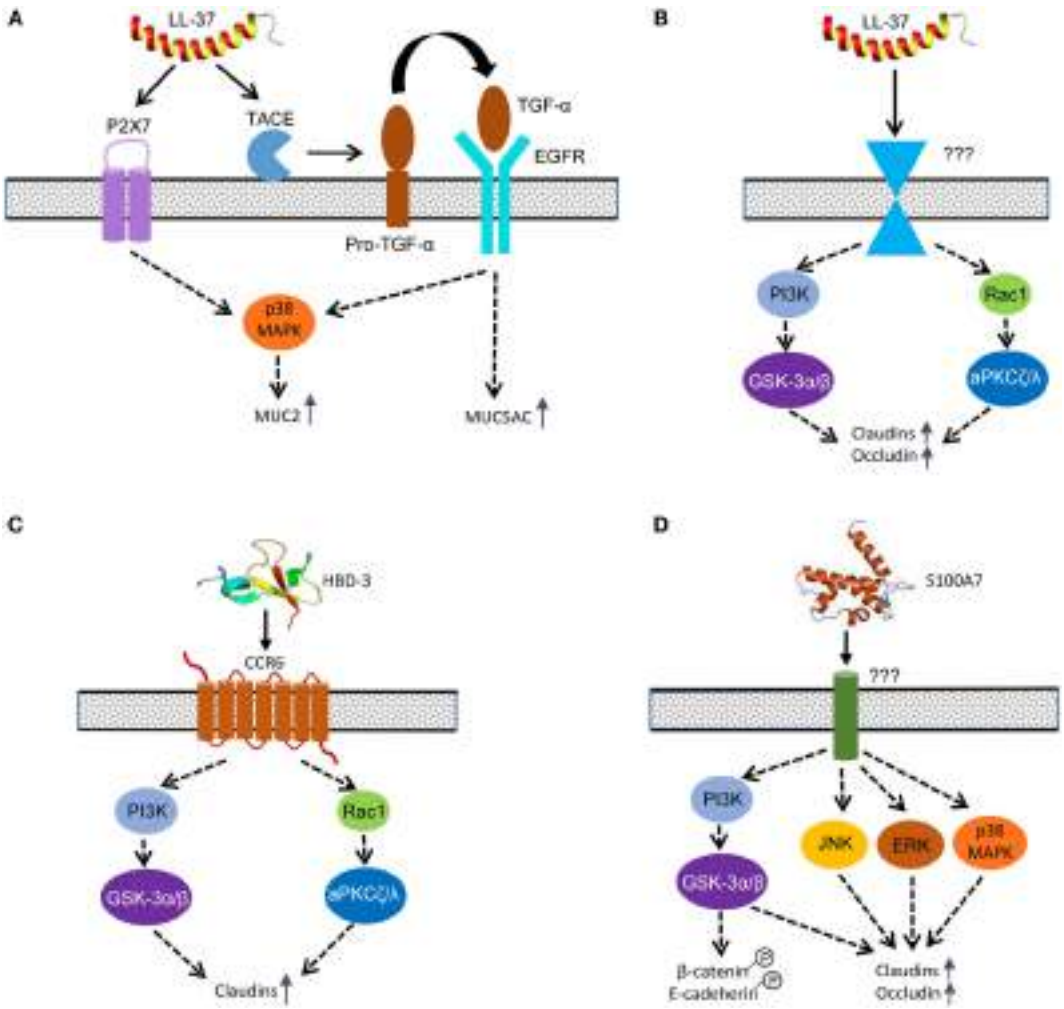

FIGURE 6 | Host defense peptide-mediated signaling pathways to induce mucins and tight junction proteins in epidermal and intestinal epithelial cells. **(A)** LL-37 primarily utilizes purinergic receptor P2X7 and transactivates EGFR to mediate MUC2 and MUC5AC induction. **(B)** LL-37 induces the synthesis of multiple claudins and occludin in skin keratinocytes mainly through PI3K-GSK-3α/β and Rac1-aPKCζ/λ pathways; however, the receptor that mediates the effect is currently unknown. **(C)** HBD-3 mainly engages CCR6 to induce claudin synthesis in skin keratinocytes through the PI3K-GSK-3α/β and Rac1-aPKCζ/λ pathways. **(D)** S100A7 enhances the synthesis of claudins and occludin through PI3K-GSK-3α/β and three canonical MAPK pathways. S100A7 also triggers phosphorylation of β-catenin and E-cadherin to enhance the adherens junction. A solid arrow indicates a direct effect, whereas a dashed arrow refers to an indirect action. It is noted that cross-talks among different signaling pathways likely exist, but they have not been reported.

triggers the activation of TNF-α-converting enzyme, which in turn cleaves the membrane-bound form of TGF-α, but not heparin binding-EGF. Released TGF-α subsequently interacts with and phosphorylates its receptor, EGFR, which induces MUC5AC gene expression through activation of multiple signaling pathways (108). For LL-37 to induce MUC2 and MUC3 expression in human intestinal epithelial cells, both EGFR and P2X7, but not G-protein-coupled receptors, are involved (104). HBD-2-induced mucin expression in human intestinal epithelial cells is shown to be partially mediated through CCR6 (101). The p38 MAPK, but not extracellular signal-regulated kinase (ERK) or PI3K, is involved in mediating P2X7- and EGFR-activation of MUC2 production in human Caco-2 cells (104).

LL-37-Mediated TJ Protein Induction

Rac1, aPKCζ/λ, glycogen synthase kinase (GSK)-3α/β, and PI3K are all phosphorylated and activated in human skin keratinocytes in response to LL-37, and blockage of any enzyme with a specific inhibitor results in a substantial reduction in the TEER and a significant increase in the permeability to FITC-dextran (109) (**Figure 6B**). Rac1 is a small GTPase that functions upstream of aPKCζ/λ as part of the Par3/Par6/aPKCζ/λ polarity complex, which in turn phosphorylates the C-terminal domain of occludin (114) or JAM-1 (115), promoting its assembly into the TJ complex and enhancing the barrier function (116). GSK-3α/β is involved in a number of signaling pathways (117) and is required for induction of occludin and claudin-1 in intestinal and kidney epithelial cells (118). Consistently, LL-37 triggers phosphorylation

and activation of GSK-3α/β at Tyr 216 and Tyr 279 in human keratinocytes at 1–2 h after exposure, leading to the improvement of epidermal barrier function (109).

PI3K functions upstream of GSK-3, and the PI3K signaling cascade has been implicated in both the degradation and stimulation of TJ barrier function depending on the stimulating agent (119). PI3K is quickly phosphorylated within 30 min in human keratinocytes upon stimulation with LL-37 (109). In intestinal cells, PI3K plays a key role in directing proper occludin localization and subsequent tightening of epithelial barrier function in response to prostaglandins (120). Inhibition of PI3K in porcine ischemia-injured ileal mucosa attenuates the ability of prostaglandin to recover proper barrier function. In rat Con8 mammary epithelial cells, glucocorticoid recruits Ras and the p85 subunit of PI3K to the TJ complex and increases barrier function (121). However, the specific cellular receptor(s) mediating LL-37-induced TJ protein expression remain unknown and warrant further investigation.

HBD-3-Mediated TJ Protein Induction

Although HBD-2 is capable of inducing mucin expression (101), only HBD-3 triggers the synthesis of multiple TJ proteins (110). CCR6 has been shown to be primarily responsible for HBD-3-induced enhancement of barrier integrity in epidermis (110). Similar to LL-37, HBD-3 is also capable of phosphorylating and activating Rac1, aPKCζ/λ, PI3K, and GSK-3α/β in similar kinetics in human skin keratinocytes (110) (**Figure 6C**). Of note, toll-like receptors, PKA, and MAPK pathways are not involved in mediating HBD-3-induced barrier function improvement (110). Although HBD-1, HBD-2, and HBD-4 fail to alter the epidermal permeability, they also have weak activities in activating Rac1, aPKC, GSK-3, and PI3K (110), suggesting those pathways may not be solely devoted to the TJ functions. It is important to note that, although similar in the tertiary structure, only HBD-3, but not HBD-1, -2 or -4, has the ability to trigger the induction of TJ proteins (115).

S100A7-Mediated TJ Protein Induction

The role of GSK-3α/β and MAPK in human epidermal barrier function mediated by S100A7 has been studied (111) (**Figure 6D**). GSK-3α/β is phosphorylated and activated at Tyr 216 and Tyr 279 within 30 min following exposure of human keratinocytes to S100A7/psoriasin. Specific inhibition of GSK-3 activation abolishes induction of claudins and epidermal TEER by S100A7 (111). Because β-catenin is regulated directly by GSK-3 (117), S100A7 is revealed to phosphorylate and activate β-catenin, which is vital to the assembly of adherens junctions. E-cadherin, another essential component of the adherens junction complex, is also phosphorylated by S100A7 (111), suggesting that, besides the TJs, S100A7 also improves the assembly of adherens junctions.

The MAPK pathway includes three canonical signaling cascades that consist of ERK, c-Jun N-terminal kinase (JNK), and p38 (122). Collectively, they are critical to many important physiological processes ranging from cell division and differentiation to stress and immune responses. Unlike HBD-3, S100A7 is capable of activating all three canonical MAPK cascades (111). ERK is quickly phosphorylated in 2 min in human skin keratinocytes following exposure to S100A7, while JNK and p38 MAPK are also phosphorylated in 30 min. Inhibition of individual MAPK signaling cascades leads to a substantial reduction in claudin induction and epidermal TEER (111), implying that all three major MAPK pathways are required. However, the involvement of any specific receptors or other signaling pathways remains to be studied.

ROLE OF HDPs IN INTESTINAL MUCOSAL HOMEOSTASIS, IMMUNE DEFENSE, AND GROWTH PERFORMANCE

One of the major functions of the intestinal epithelium is to act as a barrier against the invasion of microorganisms. This task is especially difficult considering that the intestinal mucosa is colonized by over 1013 microorganisms (123), with the majority being commensal bacteria that are beneficial to the host through their ability to improve digestion, absorption, and vitamin synthesis while also limiting pathogen growth (124). The two most dominant bacterial phyla present in the intestinal tract of humans and mice are Gram-negative Bacteriodetes and Gram-positive Firmicutes, which together comprise about 70–80% of the total bacteria present (125). Commensal bacteria are vital to the development of normal intestinal morphology and immune system (126, 127). While commensal bacteria are beneficial to the host under homeostatic conditions, a state of dysbiosis, or imbalance of the microbial community, leads to inflammation and disturbed epithelial homeostasis. This is particularly seen in the CD patients in which the host immune system displays increased activation against commensal microbiota.

The intestinal epithelium continuously monitors resident microbes through interactions between pattern recognition receptors (PRRs) and microbe-associated molecular patterns (MAMPs). Activation of PRRs stimulates the synthesis and release of HDPs and mucins from intestinal cells (126, 127). A large amount of HDPs secreted from Paneth cells and enterocytes are retained in the mucus layer to create a strong barrier against bacterial invasion (128). Studies with HDP-knockout and -transgenic mice have illuminated the role of HDPs in intestinal homeostasis and immune defense. Knockout of the mouse cathelicidin CRAMP gene causes exaggerated colitis in the colon of mice, and the disease symptoms are further exacerbated following DSS treatment (129). Adoptive transfer of bone marrow cells from the wild-type mice to CRAMP-knockout mice alleviates DSS-induced colitis (129). Mice carrying the transgene for HD5 show an augmented ability to fight off orally challenged *S. enterica* serovar Typhimurium (130). Conversely, matrix metalloproteinase 7 (MMP7)-knockout mice with a deficiency in producing biologically active enteric defensins display reduced capacity to clear enteric pathogens (131). Furthermore, a comparison between those two complementary mouse models has revealed dramatic defensin-dependent reciprocal shifts in the intestinal bacterial composition. In comparison with wild-type mice, Firmicutes are reduced and Bacteriodes are increased in small intestine of HD5-transgenic mice, while the opposite is true with MMP7-deficient mice (125, 132). Moreover, overexpression

of HD5 in mice causes a significant loss of segmented filamentous bacteria in the distal small intestine and a reduced presence of Th17 cells in the lamina propria (132), suggesting clearly that enteric HDPs represent a critical factor in shaping the microbiota composition and inflammatory status of the GI tract.

Multiple studies have highlighted the beneficial effects of direct feeding of HDPs on growth, intestinal morphology, and immune status in pigs (133, 134). Dietary supplementation of an *E. coli*-producing bacteriocin, colicin E1, significantly improved weight gain and feed efficiency of ETEC-challenged weanling pigs in a 4-day trial, relative to the control pigs (135). Colicin E1 inclusion also reduced the *E. coli* titers in both the fecal and ileal samples as well as the incidence and severity of diarrhea (135). Moreover, the expression levels of proinflammatory cytokines (IL-1β and TNF-α) were reduced in the ileum of pigs in response to colicin E1 feeding (135). Similarly, feeding a recombinant silkworm HDP, cecropin A/D, improved growth and feed efficiency and reduced diarrhea incidence in ETEC-challenged weanling pigs, without an obvious impact on intestinal morphology or nitrogen/energy utilization over a period of 6 days (136). Dietary inclusion of a recombinant fusion HDP derived from bovine lactoferrin also enhanced growth performance and decreased the incidence of diarrhea in ETEC-challenged piglets over a 21-day period (137). Across five different commercial farms, feeding a mixture of four recombinant HDPs, including lactoferrin, cecropin, defensin, and plectasin resulted in an enhancement of growth and feed efficiency and a reduction in diarrhea incidence in normally reared weanling pigs (138). Parallel to the studies above, supplementation of a synthetic HDP (AMP-A3 or P5) improved nutrient digestibility, intestinal morphology, and growth performance of normally reared weanling pigs in a 4-week trial, without affecting serum concentrations of IgA, IgG, or IgM (139, 140). Additionally, AMP-A3 and P5 appeared to reduce the titers of potentially harmful *Clostridium* spp. and coliforms in the ileum, cecum, and feces (140). Feeding a combination of two HDPs and a probiotic yeast led to an improvement in intestinal morphology and feed efficiency of piglets challenged with deoxynivalenol, a mycotoxin commonly found in grains (141, 142). In most trials above, HDPs performed indistinguishably with in-feed antibiotics in promoting growth, feed efficiency, and intestinal morphology (133).

The beneficial effects of direct feeding of HDPs are not limited to pigs. Supplementation of AMP-A3 to broiler chickens resulted in an increase in weight gain and feed efficiency over control birds, which was comparable to the birds fed avilamycin, an in-feed antibiotic (143). Intestinal morphology was also improved in broilers as measured by increased villus heights and villus height:crypt depth ratios in the small intestine (143). Similar to the results in pigs, broilers also displayed an improvement in nutrient utilization and a reduction in *Clostridium* spp. and coliforms in the intestinal tract (143). Supplementation of a yeast broth containing recombinant cecropin A/D improved intestinal morphology and nutrient utilization, with a tendency to enhance growth performance of broiler chickens in a 4-week trial (144). Cecropin A/D inclusion also reduced the total aerobic bacterial counts in both the jejunal and cecal contents of 42-day-old chickens (144). Collectively, these animal results have suggested the beneficial effects of HDP feeding, justifying dietary supplementation of HDPs as an antibiotic-alternative strategy in growth promotion and disease control.

However, because of HDP's proneness to enzymatic degradation and high production costs with either the synthetic or recombinant form, it may not be biologically efficient and economically effective for direct supplementation of HDPs in animal diets. Recently, several classes of small-molecule compounds, such as butyrate, have been found to induce HDP synthesis and enhance bacterial clearance in humans, chickens, pigs, and cattle without triggering inflammatory response (145–151). Dietary supplementation of these simple HDP-inducing compounds or their combinations may prove to be an alternative, cost-effective approach to antibiotics for livestock applications (152). However, the efficacy of these HDP-inducing compounds in promoting growth, intestinal health, and microbiota balance is yet to be demonstrated in animal trials.

CONCLUDING REMARKS

A comprehensive understanding of intestinal barrier function and its regulation is paramount to ensuring the sustainability of the food animal industry because disruption of the barrier results in disease states and decreased production efficiency (6). With potent antimicrobial and immunomodulatory properties, HDPs are further revealed to hold a new capacity to directly regulate barrier function. Aberrant synthesis of epithelial HDPs often leads to barrier dysfunction, and the diseases with impaired barrier integrity are commonly associated with reduced HDP synthesis, raising the possibility of treating barrier dysfunction with HDPs. However, a number of questions remain before HDP-based therapies can be devised for augmentation of intestinal barrier function, animal health, and production efficiency.

On the one hand, several structurally diverse HDPs (e.g., cathelicidins, defensins, and S100A7) have a similar ability to induce the expression of mucins and TJ proteins in both epidermal and intestinal epithelial cells. On the other hand, certain structurally similar HDPs (e.g., HBD-3 vs. HBD-1, -2, and -4) behave quite differently in their capacity to induce TJ proteins. Only HBD-3, but not other HBDs, has the capacity to enhance the barrier effect (110). Structure–activity relationship studies of HDPs may reveal whether there is an optimal physicochemical or structural feature for maximal induction of mucins and TJ proteins.

A number of different extracellular and intracellular receptors have been identified for human HDPs to mediate different physiological functions. However, the identities of the receptors utilized by different classes of HDPs to regulate paracellular permeability remain largely unclear in most cases. There are a number of questions on the signaling mechanisms of HDP-mediated barrier function that need to be answered. For example, what are the major receptors involved in HDP-induced synthesis of mucins and TJ proteins in humans? Do the same set of receptors that are utilized by human HDPs work similarly in the livestock species? Are there any new, unidentified receptors specific for regulation of barrier function? Besides the Rac1-aPKC, PI3K-GSK-3, and MAPK pathways, what are other major signaling pathways that

mediate the barrier effect? How do these pathways cross-talk with each other? What are those major transcription factors that are required for induction of different mucin and TJ proteins? How and whether are mucins and TJ proteins differentially regulated? Do epithelial cells on epidermis, GI, respiratory, and genitourinary tracts engage in different receptors and signaling pathways?

Besides the abundance of TJ proteins, both posttranslational modifications of TJ proteins and the status of associated actomyosin ring have a strong impact on barrier permeability. Many agents are shown to alter the barrier function through phosphorylation of certain TJ proteins or through activation of MLCK, which in turn phosphorylates myosin light chain and causes contraction of the perijunctional actomyosin ring and opening of the paracellular pores. It will be important to examine whether and how HDPs influence posttranslational modifications of TJ proteins as well as the transcription and activity of MLCK.

Nevertheless, it is exciting to reveal a direct involvement of HDPs in barrier function and the potential of HDPs in enhancing gut health and animal performance. Additional studies along this line may someday turn the HDP-based therapies into reality. Although administration of synthetic peptides may be feasible in human medicine, it is cost-prohibitive in the livestock industry. Supplementation of exogenous recombinant HDPs or dietary compounds with the capacity to induce the synthesis of HDPs in the GI tract has emerged as a cost-effective strategy in antimicrobial therapy and may have potential to replace antibiotics in food animal production.

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Immunometabolism and the kinome peptide array: a new perspective and tool for the study of gut health

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Immunometabolism is a relatively new research perspective, focusing on both metabolism and immunology and the cross-talk between these biological processes. Immunometabolism can be considered from two perspectives; 1) the role that immune cells play in organ metabolism and metabolic disease, and 2) the metabolic processes that occur within immune cells and how they affect overall immunity. The gut may be the prototypical organ of immunometabolism. The gut is the site of nutrient absorption and is a major, if not the major, immune organ. We also describe the integration of kinomics and the species-specific peptide array to the study of the gut. This unique immunometabolic tool combined with the unique immunometabolic nature of the gut provides significant research potential to many animal health applications.

Keywords: immunometabolism, gut health, kinome, immunity, metabolism, animal agriculture

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IMMUNOMETABOLISM

The interface of the immune system and metabolism is an emerging field of study. Relatively recently, immunity and metabolism were treated as distinct processes carried out by an organism. Immunity was focused on the recognition and resistance to a pathogen and involved its own set of cells and tissue activities. Metabolism was solely the chemical processes that provided the energy to carry out the various functions of the organism; this included immune functions, but metabolism was simply the source of energy for the immune system.

The perspective linking immunity and metabolism is styled as immunometabolism. Immunometabolism can be considered from two sides: 1) the role of immune cells in metabolism in organs and the effects on whole organism metabolism or 2) the role of metabolic pathways in immune cells and the effect on broader immunity (1). Early studies in immunometabolism from the first perspective involved human health concerns related to obesity, diabetes, and metabolic disorder (2). Excessive fat deposition can lead to an innate immune inflammatory response. Chronic low-grade inflammation has been linked to metabolic diseases, such as type 2 diabetes, fatty liver disease, and atherosclerosis. Studies typifying the second perspective involved the role of some of the classic metabolic energy sensors and energy switches, such as the signaling proteins AKT1–3, AMPK, mTOR, and LKB1; these were shown to be linked to CD8+ T cell (3) and other immune cell functions. From there, links between metabolism, immunity, and host response to infectious disease grew.

Within animal agriculture, a consideration of immunometabolism in animal production has been ongoing, though not coined as such. It has been clear to poultry producers that a focus solely on maximizing animal growth can be detrimental to immune potential, while an innate immune response has negative consequences on growth (4). Integrating metabolism and immunity provides

a research avenue for the ultimate goal of maximizing growth and animal production without having a negative impact on animal health and immunity. Our own research has shown the nearly innumerable links between cellular signaling proteins classically characterized as members of either the immune or metabolic functional groups (5). Due to these links, we feel that an integrated immunometabolic approach is worth considering for anyone researching animal production from either a nutrition/metabolism or immunity/disease perspective. Below, we describe some research categories that fall under the immunometabolism umbrella and their relevance to animal agriculture.

Growth/Immunity Balance

A significant avenue of research combining immunity and metabolism in animal production was how mounting an immune response affected energy levels and the transfer of energy from growth to immunity (4). Research into the energy consequences of immunity is relatively advanced in animal science. It has been well understood for many decades that an animal that initiates an innate immune/inflammatory response will likely grow slower and have worse feed conversion (6, 7). It is thought that one mode of action of growth-promoting antibiotics given to food animals is a general reduction in inflammation. Indeed, it has been argued that the anti-inflammatory effects of growth-promoting antibiotics are even more important than their reduction/elimination of disease-causing pathogens (8). In human medicine, disease early in life has been linked to ultimate growth potential. Less incidence of disease as infants results in greater growth and ultimate height in adulthood (9).

Obesity, Inflammation, Immunity, and Metabolism

With growing research into obesity and associated ailments, including metabolic syndrome, diabetes, and heart disease, a new perspective on the interaction of immunity and metabolism emerged (10). It was found that chronic low-level inflammation was a symptom of obesity (11); this inflammation could lead to diseases like diabetes, among other ailments.

In the animal science field, feed-induced inflammation has been a concern. Certain feed ingredients can lead to an inflammatory gut response; examples include non-digestible components of wheat and rye in chickens (12) and soybean meal in fish (13, 14). Even an excess of feed can lead to changes in immune response (15). One current animal feed strategy involves adding enzymes to break down certain indigestible and/or inflammatory feed components in the gut, with the aim to reduce immune response and redirect this energy to growth (16). A current feed trend involves trying to find natural additives that enhance the animal's resistance to disease, either by influencing the host immune response or the gut microbiota. Caution must be exercised when evaluating these feed additives; robust scientific methodology must be used to determine efficacy and understand the mechanism of action.

In dairy cattle, the transition period immediately before and after calving is an important immunometabolic period. During this time, a dairy cow's immune functions are impaired, as the mobilization of lipids causes susceptibility to both metabolic and infectious disease (17). This increase in fatty acids (FAs) in the blood can lead to uncontrolled inflammation and oxidative stress. The dysfunction in the inflammatory response, due to the free FAs increase, is the link between metabolism and immunity during the calving period.

In poultry, there has been a significant amount of research into nutrition's effects on immunity (18) and the use of pre- and probiotic feed ingredients to improve growth and disease resistance (19–21). However, the literature is limited on the immunometabolism link between stress or disease and production issues. The links between disease and production issues are certainly there, and poultry production problems ranging from lameness (22) to muscle fat deposition (23) have been explored.

As discussed in the following section, the study of immunometabolism now incorporates the metabolic pathway changes in immune effector cells, such as macrophages and T-cells that lead to changes in their activation or immune activity. A promising new avenue of research is the targeting of metabolic machinery and metabolic pathways of immune cells as an alternative means of modulating the immune response.

Intracellular Immunometabolism Interactions

The recent expansion of the immunometabolism field involves characterizing the direct intracellular pathway links between metabolism and immunity (1, 24). Research is focusing on signaling molecules that integrate both metabolic energy sensing and immune response signals; some examples include mTOR, AMPK, and sirtuins. The protein synthesis pathway is regulated by mTOR and is also involved in T-cell fate (25), determining whether the cell becomes an effector T-cell or a regulatory T-cell (3). AMPK is an energy sensor that monitors the ratio of AMP:ATP, altering anabolic and catabolic processes; it is also involved in innate immune response and has a direct link to mTOR (26). Evidence also points to metabolic-induced epigenetic reprograming of immune pathways via the sirtuins (27). The past perspective of separating immunity and metabolism meant a focus on targeting immune pathways in infectious disease and metabolism pathways for growth/metabolic disorders. With an integrated approach, we can broaden our potential targets for disease intervention and our understanding of how metabolic processes can influence health.

Oxidative Phosphorylation, Glycolysis, Warburg Effect, and Immune Response

Studies of the metabolism of immune cells have shown that metabolic processes determine immune function. In dendritic cells and macrophages, the switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, which can be triggered by immune ligands like LPS, leads to profound immune activity changes (24). These changes include a release of proinflammatory cytokines, an increase in cell migration, and the utilization of OXPHOS machinery (28, 29) for the production of immune effectors such as nitric oxide (NO) and reactive oxygen species (ROS) (30, 31). In fact, without the change to aerobic glycolysis initiated by immune ligands, the electron transport chain proteins in the mitochondria would not be available to generate these potent antibacterial molecules. The change in metabolism when

immune cells are activated has been described as akin to the Warburg effect in cancerous cells, leading to aerobic glycolysis-fueled proliferation and activity (32).

T-Cells and Metabolism

In T-cells, the metabolic processes that are activated can help determine the ultimate function of the cell. Effector T-cells undergo active glycolysis, utilizing this energy to carry out immune activities and proliferate. Quiescent T-cells rely on glycolysis, the citric acid cycle, and OXPHOS (1). Memory T cell metabolism is biased toward free FA metabolism, and the proliferative machinery is turned off. This gives memory T-cells a lower metabolic rate and a longer life span, allowing them to survive and circulate longer than effector T-cells (33).

IMMUNOMETABOLISM AND THE GUT

The gut is the prototypical organ for considering immunity and metabolism as an integrated whole. The gastrointestinal system is the site of significant food breakdown and nutrient absorption, and it is the largest lymphoid organ in the body secreting the most antibodies in humans (34) and animals (35).

In the production of food animals, the gut is a major focus. Effective and efficient nutrient absorption is the first and major step in cost-effective animal production. With the gut as the site of nutrient absorption, any defect in the ability to extract nutrients from feed can have a profound impact on growth and disease susceptibility. In addition, feed efficiency is of critical importance to animal producers, as higher feed efficiency increases the amount of commodity produced and reduces costs. Any increases in feed efficiency from an animal perspective must take place in the gut.

The gut is a major, if not the major, immune organ. It is the main mucosal immune site, and a majority of a body's immune tissue and immunoglobulin producing cells are found in the gut. Infections of the gastrointestinal tract can have huge implications on animal health, gut function, meat contamination, and the spread of disease. A large proportion of disease-causing microbes in food animals enter the host via the gut (36). Manipulation of the gut is a multi-billion dollar target for animal industry products, including prebiotics, probiotics, antibiotics, anti-parasitics, feed enzymes, and feed additives, among others. Many of the pathogens that are considered a food safety concern originate or reside in the animal gut. A proper understanding of the gut can lead to more efficient animal production, less disease, and safer food.

The microbiome, the central component of gut physiology, should be considered in any discussion of the gut and immunometabolism. The microbiome is a key nutritional/metabolic component of the gut, as gut microbes break down otherwise indigestible components of food, providing absorbable and further digestible metabolites (37). The microbiome is an immune component of the gut, as the resident microbes are competitors for pathogens that enter the gut (37, 38). Adding microbes or altering the ratio of microbe species in the animal gut to competitively exclude pathogens is undergoing a significant amount of research and development. The commensal microbes are also critical to proper stimulation and development of the neonatal immune system and help the gut immune system to maintain a balance between tolerance and active immune response (38, 39).

IMMUNOMETABOLISM AND THE KINOME PEPTIDE ARRAY

Species-Specific Peptide Arrays for Kinome Analysis

Peptide arrays have become a productive, high-throughput method of studying the active kinome, the kinase complement of a cell or tissue (40). The principle involves immobilized kinase-target peptide sequences printed on a glass array. Exposing the array to lysate containing active kinases, from gut tissue, for example, results in peptide phosphorylation and can generate a visual signal of substrate–enzyme phosphorylation. By comparing the relative signal of experimental cell or tissue samples, one can identify changes in signal transduction pathways and phosphorylation-regulated events.

It is often the case that new, high-throughput methodologies are designed for the standard laboratory species, mice, and rats, or for work with human samples. This was also the case with the kinome peptide arrays. Through extensive research and development, a methodology for designing and using species-specific kinome arrays was developed (41–43). The use of this technology in agricultural species has been reviewed extensively elsewhere (44) and has been used to design peptide arrays to study important biological questions in a number of agriculturally important species, including bovine (45) and poultry (46).

Immunometabolism Array

Not only are the peptide arrays designed to be species-specific but also they can be process-specific. The initial species-specific arrays were designed to study the innate immune response and contained numerous signaling pathways intermediates involved in this response, such as toll-like receptor (TLR) signaling members, inflammatory intermediates, and others (45). Subsequently, in order to study the metabolic consequences of stress responses, a metabolic peptide array was designed. This array incorporated protein, carbohydrate, and FA metabolic signaling intermediates as well as key energy regulating proteins (23, 47). We have conducted numerous studies on the physiology and host–pathogen interactions in agricultural species using the species-specific peptide arrays. Our analysis of these data showed that the metabolism and immune processes may be distinctions without differences, in that they are two integrated parts of a single cellular process (5). It became clear how much protein–protein interaction there was in the pathways represented on each array. This level of interaction was the impetus for our design of an immunometabolic, species-specific peptide array for both poultry (chicken and turkey) and cattle. This latest generation of species-specific peptide arrays provides an integrated immunometabolic approach to studying kinome response (5). These large arrays, representing approximately 1,000 individual peptides, have been designed to cover the entire network of immunometabolism, including innate and adaptive immunity, protein, carbohydrate, and FA metabolism, as well as hormone and stress response. This integrated peptide array will allow for the study of both the immune and metabolic

consequences of an environmental condition, disease, treatment, additive or intervention, and the interactions between them.

In the realm of animal agriculture, nutrition/metabolism and immune performance have been two fields that have been converging for many years. Producers, veterinarians, and animal scientists have come to understand that a sole focus on growth can often come at the expense of health and disease susceptibility, and a strong response to disease can have significant effects on growth. With a tool that can study both metabolism and immunity simultaneously, these two areas of animal science no longer have to be at odds. We can study nutrition and observe effects on immune responses or, conversely, study disease and see how this may effect growth. Our group has already published data that show that *Salmonella* infection of chicken can have effects on the fat deposition and carbohydrate metabolism in peripheral muscle (23). The results indicated that the dysbiosis caused by the *Salmonella* in the gut effected metabolic processes in the skeletal muscle. We are currently working on projects to in which we hope to show an immunometabolic response to infectious diseases and feed-induced inflammation of the gut. This represents only a small fraction of the research potential of this approach.

CONCLUSION

The search for antibiotic alternatives in animal production has renewed the research focus on gut health. It seems likely that any effective alternative will center in the gut. Immunometabolism has expanded from the study of chronic, low-level inflammation, and obesity to a full research perspective, encompassing a variety of fields. The adaption of kinomics to animal agriculture is a relatively recent development and has provided valuable insight into animal biology. Integrating gut health, immunometabolism, and kinomics have significant potential in animal production/health, feed additive development, drug discovery, reproduction, and disease research. Here, we have described this new perspective in gut health and animal production research and a useful tool to carry it out.

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A role for the non-canonical Wnt-β-Catenin and TGF-β signaling pathways in the induction of tolerance during the establishment of a *Salmonella enterica* serovar Enteritidis persistent cecal infection in chickens

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Non-typhoidal *Salmonella enterica* induce an early pro-inflammatory response in chickens. However, the response is short-lived, asymptomatic of disease, resulting in a persistent colonization of the ceca, and fecal shedding of bacteria. The underlying mechanisms that control this persistent infection of chickens by *Salmonella* are unknown. Recently, we found an expansion of the Treg population and subsequent increased *in vitro* immunosuppressive functions of the $CD4^+CD25^+$ cells isolated from the ceca of the *Salmonella*-infected chickens by day 4 post-infection that increased steadily throughout the course of the 14 days of infection, whereas the number of $CD4^+CD25^+$ cells in the non-infected controls remained steady throughout the study. $CD4^+CD25^+$ cells from cecal tonsils of *S. enteritidis*-infected birds had greater expression of IL-10 mRNA content than the $CD4^+CD25^+$ cells from the non-infected controls at all the time points studied. These results suggest the development of a tolerogenic immune response in the cecum of *Salmonella*-infected chickens may contribute to the persistance of *Salmonella* cecal colonization. Using a chicken-specific kinome peptide immune array, we have analyzed the signaling pathways altered during the establishment of this tolerogenic state. This analysis has revealed a role for the non-canonical Wnt signaling pathway in the cecum at 4 days post-infection. Infection induced the significant ($p < 0.01$) phosphorylation of the G-protein-coupled transmembrane protein, Frizzled 1 (FZD1), resulting in an influx of intracellular Ca^{2+} and the phosphorylation of the Ca^{2+}-dependent effector molecules calcium/calmodulin-dependent kinase II (CamKII), β-catenin, protein kinase C, and the activation of the transcription factor, NFAT. Nuclear translocation of NFAT resulted in a significant increase in the expression of the anti-inflammatory cytokines IL-10 and TGF-β. Increased expression of TGF-β4 mRNA activates the TGF-β signaling pathway that phosphorylates the receptor-activated Smads, Smad2 and Smad3. Combined with the results from our Treg studies,

these studies describe kinome-based phenotypic changes in the cecum of chickens during *Salmonella* Enteritidis infection starting 4 days post-infection that leads to an anti-inflammatory, tolerogenic local environment, and results in the establishment of persistent intestinal colonization.

Keywords: *Salmonella*, chickens, kinome array, Wnt signaling pathway, tolerance

Introduction

Salmonellosis is a zoonotic disease produced by the Gram-negative enteric bacterium *Salmonella*. *Salmonella* are not restricted to particular host species, with more than 2500 serotypes having been described mostly belonging to the species *Salmonella enterica* (1), with most having asymptomatic colonization of the gastrointestinal tract of animals. The most prevalent serovars, *S. enterica* serovar Typhimurium (*S.* Typhimurium) and serovar Enteritidis (*S.* Enteritidis) are major causes of intestinal infections in a wide range of host species worldwide (2, 3). Both serovars have a broad host range able to infect poultry, livestock, and humans (4). *S.* Typhimurium and *S.* Enteritidis infections of humans, cattle, and pigs cause self-limiting gastroenteritis manifested by abdominal pain, vomiting, and inflammatory diarrhea (5); whereas, infection of birds more than a few days old with either serovar produces asymptomatic cecal colonization with persistent shedding of bacteria that may persist for months, causing carcass contamination at slaughter with potential human food safety issues (6–10).

The host responds to infection with pathogens by activating the innate and adaptive immune mechanisms. However, some pathogens, such as *Salmonella*, have evolved the ability to survive the initial host immune response and persist. The interactions between the host and pathogen during this persistent phase are multifaceted and reflect the co-evolution of bacterial virulence mechanisms and host immune responses. Very little is known about the regulatory interactions between the host immune response and virulence mechanisms that lead to *S. enterica* persistence in the avian intestine. Chronic colonization of the intestinal tract is an important aspect of persistent *Salmonella* infection in poultry because it results in propagation of bacteria in the birds due to the impossibility to isolate contaminated animals (11).

A better comprehension of the host factors that are exploited by the bacteria in order to establish a persistent infection would be invaluable for the identification and development of therapeutic targets. Recently, Chausse and colleagues (12) found that genes involved in the inflammatory response were down-regulated during the carrier state, suggesting a bias toward a Th2 response in susceptible chickens. Furthermore, in a murine model of long-term S. *typhimurium* infection, the bacteria preferentially associated with anti-inflammatory/M2 macrophages during the later stages of infection (13). Lastly, the immune-suppression role of regulatory T cells has been shown to play a role in *Salmonella* persistence in a murine model (14). All told, we speculate that the bacterium is involved in redirecting the host response toward immune tolerance. The present study was designed to address the question on the induction of immune tolerance during a persistent paratyphoid *Salmonella* infection in chickens.

When considering the effects of an infection on a host, such as asymptomatic salmonellosis in poultry, studying the protein level as opposed to the gene or transcript level reduces complicating variables. The proteome contains the final effectors resulting in the organism's phenotype. Such studies can provide a dramatically different perspective on the avian host's biochemical and physiological properties to this asymptomatic enteric bacterial pathogen. Our recent report of the development of chicken species-specific peptide arrays for kinome analysis of host signaling responses to *Salmonella* provided us with the prospect to characterize a more detailed understanding of the host–pathogen interactions in the chicken (15). Using a metabolism kinome array, we have documented altered metabolic signaling pathways in the skeletal muscle of *S.* Typhimurium-infected chickens that affected fatty acid and glucose metabolism through the AMPK and the mTOR signaling pathways over the first 3 weeks post-infection (16). Additionally, using a chicken-specific immune array, we have detailed the toll-like receptor (TLR) signaling pathways stimulated in monocytes by TLR ligands, CpG (TLR 21) and poly I:C (TLR3), but also identified a unique signaling pathway stimulated by the combination of CpG/poly I:C treatment that was not observed by treatment with the individual ligands (17).

Therefore, in the present study we hypothesized that *S. enterica* serovar Enteritidis (*S.* Enteritidis) induces an immune tolerance in chickens that results in the bacteria's ability to persistently colonize the cecum of poultry. To test this hypothesis, we analyzed temporal chicken-specific kinomic immune peptide arrays anti-inflammatory cytokine gene transcription of avian cecal tissue during a persistent infection by *S.* Enteritidis. Using these approaches, we were able to begin to characterize the specific immune post-translational signaling events during a persistent *Salmonella* colonization in chickens. Furthermore, we characterized the cellular and cytokine profiles that provide confirmation for the transition of an early pro-inflammatory mucosal response to the development of an immune tolerogenic mucosal response.

Materials and Methods

Experimental Animals

Experiments were conducted according to the regulations established by the U.S. Department of Agriculture Animal Care and Use Committee. Straight-run broiler chickens used in this study were obtained from a commercial breeder and were all of the same genetic background and were not vaccinated at any time. Chicks were placed in floor pens containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal-based chick starter diet *ad libitum* that met or exceeded the levels of critical nutrients recommended

by the National Research Council (18). *Salmonella* was not detected in the feed or from the paper tray liners using standard procedures (19).

S. Enteritidis Challenge

A poultry isolate of *S. enterica* serovar Enteritidis [*S.* Enteritidis; (ID 9711771, part 24)] was obtained from the National Veterinary Services Laboratory (Ames, IA, USA), and was selected for resistance to nalidixic acid and novobiocin and maintained in tryptic soy broth (Difco Laboratories, Sparks, MD, USA) containing antibiotics (20 μg/ml nalidixic acid and 25 μg/ml novobiocin; Sigma Chemical Co., St. Louis, MO, USA). A stock culture was prepared in sterile PBS and adjusted to a concentration of 1×10^9 colony forming units (cfu)/ml. The viable cell concentration of the challenge dose for each experiment was determined by colony counts on XLT4 agar base plates with XLT4 supplement (Difco) and nalidixic acid and novobiocin (XLT-NN).

Experimental Design

One-day-old broiler chickens were randomly distributed into either non-infected control or infected groups each with 50 birds per group. The birds were fed a balanced, unmedicated corn and soybean meal-based diet. At 4 days post-hatch, all chickens were orally challenged with 1 ml of either 5×10^6 CFU/ml *S.* Enteritidis or mock challenged with 1 ml sterile PBS. Four, 7, 10, and 14 days after challenge, 10 chickens from each group were humanely euthanized, cecal contents were analyzed for *S.* Enteritidis colonization, cecal tonsils were collected for quantitative real-time PCR (qRTPCR), and cecal tissue from 3 of the 10 chickens per treatment was flash frozen in liquid nitrogen and stored for use in the peptide arrays (see below).

All experiments were replicated three times. Therefore, for the mRNA expression, the ceca from a total of 40 chickens for each of the two groups (10 chickens at each of four time points) were used to prepare the mRNA for the qRT-PCR assays. RNA from each bird ($n = 10$) was isolated and assayed separately and not pooled. Each RNA sample was replicated three times per immune gene per experiment).

Sample Collection for Peptide and Antibody Arrays

At 4, 7, 10, and 14 days post-infection, a 25 cm^2 section from one cecum (from the middle of the cecal pouch) was removed from each of the three randomly selected birds from each group (non-infected and infected) and immediately flash frozen in liquid nitrogen to preserve kinase enzymatic activity, and then transferred to a −80°C freezer until used in the peptide array. Following microbiological analysis of the cecal contents (see below), the cecal tissues from three confirmed non-infected and three confirmed infected chickens (out of the 10 birds per group peer time point) were used for the peptide and antibody arrays.

Peptide Arrays

At each of the time points and under each condition (infected and uninfected), three cecal samples from three different animals were taken from storage for analysis (24 samples total). Cecal tissue samples were weighed to obtain a consistent 40 mg sample for the array protocol. Samples were homogenized in lysis buffer and the homogenates were used in the peptide array protocol as described previously (19, 20).

Antibody Array

The Wnt pathway antibody array assay kit was obtained from Full Moon BioSystems (Sunnyvale, CA, USA) and the protocol was carried out as per the manufacturer's instructions. The antibody array was used as an alternative to performing several western blot assays.

Data Analysis for Peptide and Antibody Arrays

Data normalization and PCA analysis were performed for both the peptide and antibody microarrays as described previously (21). Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis were performed by uploading the statistically significant peptide lists to the search tool for the retrieval of interacting genes (STRING) (22).

Sample Collection for Bacterial Contents

The ceca from each chicken were removed aseptically and the contents (0.25 g) from one cecal pouch was serially diluted in sterile saline to 1:100, 1:1000, or 1:10,000 and spread onto XLT-NN plates. The plates were incubated at 37°C for 24 h, and the number of NN-resistant *S.* Enteritidis cells per gram of cecal contents was determined. The data from each experimental group were pooled from three separate trials for statistical analysis.

Sample Collection for mRNA

Chickens from each experimental group were euthanized at 4, 7, 10, and 14 days post-infection. A 25-mg piece of tissue was removed from the cecal tonsils and was washed in PBS, placed in a 2-ml microcentrifuge tube with 1 ml of RNAlater (Qiagen, Inc., Valencia, CA, USA), and stored at −20°C until processed. In addition, for comparison purposes, a 25 mg piece of ceca from the 10 extra birds from each group (see above 50 birds total per group, 10 used per time point = 40) was collected at 2 days post-infection.

RNA Isolation

Tissues (25 mg) were removed from RNAlater and transferred to pre-filled 2 ml tubes containing Triple-Pure™ 1.5 mm zirconium beads. RLT lysis buffer (600 μl) from the RNeasy mini kit (Qiagen) was added and the tissue was homogenized for 1–2 min at 4,000 rpm in a Bead Bug microtube homogenizer (Benchmark Scientific, Inc., Edison, NJ, USA). Total RNA was extracted from the homogenized lysates according to the manufacturer's instructions, eluted with 50 μl RNase-free water, and stored at −80°C until qRT-PCR analyses were performed. RNA was quantified and the quality evaluated using a spectrophotometer (NanoDrop Products, Wilmington, DE, USA). Total RNA (300 ng) from each sample was prepared.

Quantitative Real-Time PCR

Primer and probe sets for the cytokines and 28S rRNA were designed using the Primer Express Software program (Applied Biosystems, Foster City, CA, USA) have been described (23, 24) and are provided in **Table 1**. The qRT-PCR was performed using the TaqMan fast universal PCR master mix and one-step RT-PCR

TABLE 1 | Real-time quantitative RT-PCR probes and primers for IL-6 and TGF-β4.

RNA target		Probe/primer sequence	Accession number[a]
28S	Probe	5′-(FAM[d])-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	F[b]	5′-GGCGAAGCCAGAGGAAACT-3′	
	R[c]	5′-GACGACCGATTGCACGTC-3′	
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′	
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′	
TGF-β4	Probe	5′-(FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)-3′	M31160
	F	5′-AGGATCTGCAGTGGAAGTGGAT-3′	
	R	5′-CCCCGGGGTTGTGTGTTGGT-3′	

[a]*Genomic DNA sequence.*
[b]*Forward.*
[c]*Reverse.*
[d]*5-carboxyfluorescein.*

master mix reagents (Applied Biosystems). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast real-time PCR system with the following cycle profile as described previously (23, 24). Quantification was based on the increased fluorescence detected by the 7500 Fast sequence detection system due to hydrolysis of the target-specific probes by the 5-nuclease activity of the r*Tth* DNA polymerase during PCR amplification. Normalization was carried out using 28S rRNA as a housekeeping gene. To correct for differences in RNA levels between samples within the experiment, the correction factor for each sample was calculated by dividing the mean threshold cycle (*CT*) value for 28S rRNA-specific product for each sample by the overall mean *CT* value for the 28S rRNA-specific product from all samples. The corrected cytokine mean was calculated as follows: (average of each replicate × cytokine slope)/(28S slope × 28S correction factor). Fold changes in mRNA levels were calculated from mean 40 *CT* values by the formula $2^{(40\ CT \text{ of infected group} - 40\ CT \text{ in non-infected group})}$.

Calcium Detection

Intracellular Ca^{2+} in cecal lysates from non-infected and infected chickens was measured with a colorimetric Ca^{2+} Detection Kit (Abcam, Inc., Cambridge, MA, USA). Preparation of cell extracts was done according to the manufacturer's instructions. Total amount of Ca^{2+} was determined using a standard curve.

Statistical Analysis

The data from these replicated experiments were pooled for presentation and statistical analysis. The mean and SEM were calculated and differences between groups were determined by analysis of variance. Significant differences were further separated using Duncan's multiple-range test (23). A p value of <0.05 was considered statistically significant.

Results

S. Enteritidis Infection

Infection status of the chickens was confirmed by *S.* Enteritidis culturing of cecal contents and feces from each bird with and without enrichment. Cultures confirmed that greater than 70% of the chickens in the infected group displayed *S.* Enteritidis throughout the experiment while *Salmonella* was not isolated from the birds in the control group (**Tables 2** and **3**).

TABLE 2 | Number of chickens positive for *Salmonella* Enteritidis ceca colonization for 2 weeks following challenge.

	Percent positive for *Salmonella* Enteritidis cecal colonization (total positive/total challenged)			
	Days post-challenge			
Treatment groups	4	7	10	14
Non-infected control	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)
Infected	100 (30/30)	100 (30/30)	85 (26/30)	70 (21/30)

Results shown are pooled from three separate experiments.

TABLE 3 | Cecal *Salmonella* Enteritidis for 2 weeks following challenge.

	CFU of *Salmonella* Enteritidis in cecum (log 10)			
	Days post-challenge			
Treatment groups	4	7	10	14
Non-infected control	0	0	0	0
Infected	4.11 ± 1.37	4.79 ± 1.28	5.23 ± 1.41	4.36 ± 1.78

Results are expressed as the mean ± SEM of three separate experiments.

Altered Expression of Cytokines

Characteristically, during an acute infection (within 24 h) by paratyphoid strains of *Salmonella* in chickens, there is an up-regulation of pro-inflammatory cytokines mRNA expression in the cecum (25). Few, if any, studies have measured the comparative expression of mRNA between anti-inflammatory cytokines and pro-inflammatory cytokines during persistent *Salmonella* infections. In the present studies, we profiled the pro-inflammatory (IL-6) and anti-inflammatory (TGF-β4) cytokine mRNA expression in the cecum of chickens over the first 14 days post-infection with *S.* Enteritidis and compared the results to the non-infected control birds. Initially, at 48 h post-infection, as expected, IL-6 (14.21-fold change) mRNA expression in the ceca from *S.* Enteritidis-infected chickens was up-regulated when compared to the expression in the cecum from the non-infected birds (**Figure 1**). However, by 4 days post-infection, there was a dramatic reduction in IL-6 mRNA expression (1.45-fold change)

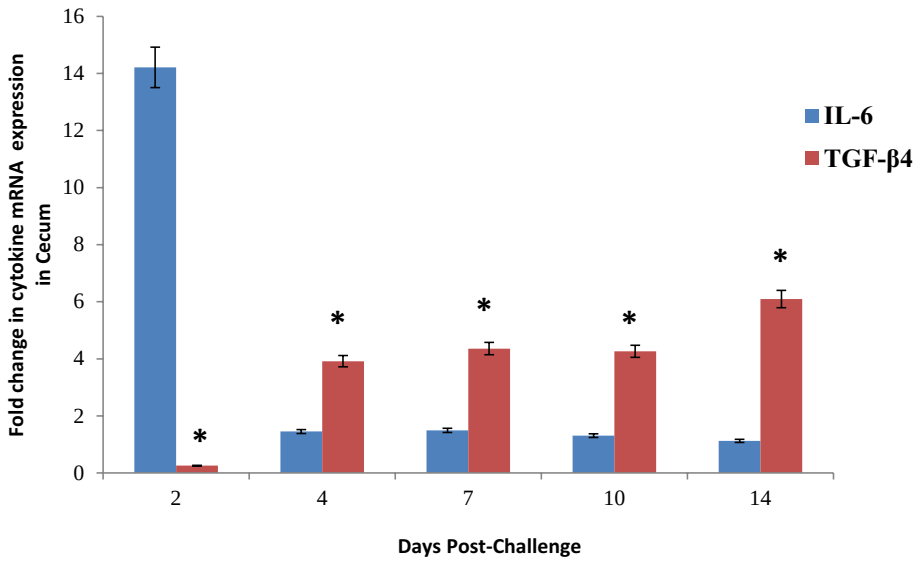

FIGURE 1 | Expression of pro-inflammatory (IL-6) or anti-inflammatory TGF-β4) cytokine mRNA in the ceca from experimental chickens with persistent colonization by *Salmonella* Enteritidis. The expression of cytokine mRNA was determined by quantitative RT-PCR. Data represent the fold change in mRNA expression in the cecum from infected chickens when compared to the mRNA expression in the cecum from non-infected chickens. Data represent the mean ± SEM from three separate experiments.

TABLE 4 | KEGG pathways generated by STRING.

		4 days		7 days		10 days		14 days	
GO ID	**Pathway**	**# peptides**	***p*-Value (FDR)**	**# peptides**	***p*-Value (FDR)**	**# peptides**	***p*-Value (FDR)**	**# peptides**	***p*-Value (FDR)**
hsa04141	Protein processing in endoplasm reticulum pathway	4	0.07	13	0.073	1	1.00	3	0.071
hsa05130	Pathogenic *Escherichia coli* infection	–	N/S	2	N/S	4	N/S	4	N/S
hsa04250	TGF-β4 signaling pathway	12	0.016	–	N/S	–	N/S	–	N/S
hsa04310	Wnt signaling pathway	16	0.0004	2	N/S	–	N/S	5	0.024
hsa04623	Cytosolic DNA-sensing pathway	4	7.01×10^{-2}	5	N/S	4	N/S	–	N/S
hsa05217	Basal cell carcinoma	–	N/S	–	N/S	2	0.338	–	N/S
hsa04672	Intestinal immune response for IgA production	1	1.00	5	N/S	–	N/S	–	N/S

Peptides that displayed a significant change in phosphorylation state were input into the STRING database for each time point. Generated pathways involved in immune activation/suppression that displayed p-value of less than 0.05 (FDR corrected) are listed. N/S indicates that the pathway is non-significant.

that remained low throughout the 14 days post-infection. In fact, there was no statistical difference in IL-6 mRNA expression between the infected and non-infected cecal tissues.

Alternatively, as the pro-inflammatory IL-6 mRNA expression was decreasing, the *S.* Enteritidis infection persisted, and the expression of the anti-inflammatory cytokine TGF-β4 mRNA were significantly up-regulated over the course of the 14-day infection period with the most profound up-regulation of both between 7 and 10 days post-infection when compared to the non-infected controls birds (**Figure 1**).

Peptide Arrays

Chicken-specific peptide arrays designed for the study of chicken immune signaling pathways were used to analyze the cecal samples from the non-infected and infected control chickens. To account for any changes in phosphorylation state that were not due to the infection, the results at each time point were corrected using their respective time matched controls.

The KEGG pathway results generated from STRING showed a large number of pathways implicated by the data at a statistically significant level [$p < 0.05$ false discovery rate (FDR) corrected]. Of particular interest were those pathways that showed a large number of statistically significant peptides that were phosphorylated at different times over the course of the study. These pathways are shown in **Table 4**. Of note are the Wnt signaling and TGF-β4 signaling pathways that were dramatically altered by the infection. Both of the pathways had multiple significantly altered peptide phosphorylation events at multiple time points post-infection; however, a total of 20 differentially phosphorylated peptides were found within these two pathways in chickens

on the fourth day post-infection with *S.* Enteritidis (**Table 4**), signifying a dramatic local post-translational modification of the infected cecum. Of the 20 peptides that were differentially phosphorylated, 13 belong to the Wnt signaling pathway and 7 to the TGF-β4 pathway. Interestingly, only nine more total peptides were found to be differentially phosphorylated within these two specific pathways over days 7–14 post-infection (**Table 4**).

Phosphorylation Events Within Specific Pathways

Wnt Signaling Pathway

Frizzled is a family of G protein-coupled receptor proteins that serves as receptors in the Wnt signaling pathway. We found frizzled 1 (FZD1) to be significantly phosphorylated in the ceca of *S.* Enteritidis-infected chickens (**Table 5**), providing us with the first indication of the involvement of the Wnt pathway. Further analysis of the peptide alterations in the Wnt signaling pathway revealed other significant changes in phosphorylation events: (1) the serine/threonine kinase, glycogen synthase kinase 3β (GSK3β), was significantly dephosphorylated in the *S.* Enteritidis-infected cecal tissue at 4 days post-infection when compared to the non-infected control cecal tissue, while β-catenin was significantly phosphorylated; (2) both protein kinase C (PKC) alpha isoforma (PRKCA) and calcium/calmodulin-dependent protein kinase type II alpha chain (CaMK2A) are significantly phosphorylated in the *S.* Enteritidis-infected cecal tissue at 4 days post-infection when compared to the non-infected control cecal tissue; (3) the serine–threonine protein calcineurin (PPSCA), an inhibitor of the canonical Wnt signaling pathway, was significantly phosphorylated in the *S.* Enteritidis-infected cecal tissue at 4 days post-infection; and (4) nuclear factor of activated T-cells (NFAT), a family of transcription factors that play a pivotal role in the transcription of cytokine genes and other genes critical for the immune response were found to be significantly phosphorylated in the ceca of *S.* Enteritidis-infected chickens (**Table 5**).

TGF-β Signaling Pathway

The TGF-β signaling pathway showed statistically significant changes in the cecal tissue from chickens 4 days post-infection with *S.* Enteritidis (**Table 6**). Smad proteins 1–3 were all significantly phosphorylated in the infected cecal tissue. Smads are intracellular proteins that transduce extracellular signals from TGF-β ligands and activate gene transcription. Smads 1–3 are receptor-regulated proteins that associate with receptor kinases and are phosphorylated. These proteins then typically bind to the common mediator Smad or co-Smad, Smad4. Smad complexes then accumulate in the cell nucleus where they regulate transcription of specific target genes.

In addition to Smad signaling, which directly impacts transcription, TGF-β induces mTORC1 (the mammalian target of rapamycin complex 1) signaling through phosphoinositide 3-kinase (PI3K) and Akt (26). Further analysis of the TGF-β signaling pathway showed a significant phosphorylation of p70S6 kinase, a serine–theonine kinase that is a target for the S6 ribosomal protein (**Table 6**). P70S6 kinase is in a signaling pathway that includes the mammalian target of rapamycin (mTOR). mTOR can be activated in distinct ways, thereby activating p70S6K.

Validation of Kinome Analysis with Antibody Array

Despite the scarcity of chicken-specific antibodies, the key proteins of interest based on the peptide array results were relatively well conserved between humans and chickens, giving us confidence that we would observe significant cross-reactivity

TABLE 5 | Peptides from the Wnt signaling pathway that displayed a statistically significant change in phosphorylation.

	Wnt signaling pathway							
	Days post-infection							
	4		7		10		14	
Peptide	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value
CAMK2A	2.789	0.008	–		–		–	
β-catenin	1.657	0.044	1.538	0.035	–		–	
EP300	1.954	0.017	–		–		1.804	0.047
Jun	3.604	0.001	–		–		–	
Jun	3.389	0.0003	–		–		–	
GSK-3β	–2.254	0.002	–		2.448	0.018	–	
NFATC1			–		–		1.957	0.009
NFATC2	1.779	0.045	–		–		–	
NFATC3	2.239	0.006	–		–		1.655	0.020
Calcineurin	2.447	0.003	–		–		–	
PRKCA Thr638	1.744	0.042	–		–		–	
PRKCA Tyr657	2.555	0.011	–		–		1.927	0.024
RAC1	1.740	0.008	–		–		–	
SMAD2 Ser345	3.434	0.008	1.756	0.013	–		–	
SMAD2 Thr255	4.532	0.006	–		–		–	
SMAD3	1.442	0.033	–		–		–	
FZD1	2.712	0.034	–		–	–	1.844	0.008

Peptides that displayed a p-value of less than 0.05 are listed.

TABLE 6 | Peptides from the TGF-β4 signaling pathway that displayed a statistically significant change in phosphorylation.

	TGF-β4 signaling pathway							
	Days post-infection							
	4		7		10		14	
Peptide	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value	Fold change	*p*-Value
EP300	1.954	0.017	–		–		–	
MAP3K7 (TAK1)	2.709	0.016	–		–		–	
MAPK8 (JNK1)	2.207	0.022	–		–		1.167	0.013
MAPK3 (ERK1)	4.269	0.001	–		–		–	
p70S6K	1.145	0.023	–		–		–	
P70S6K	3.005	0.012	–		–		–	
P70S6K	3.759	0.009	–		–		–	
SMAD1	2.698	0.021	–		–		–	
SMAD1	2.916	0.00	–		–		–	
SMAD2 Ser345	3.434	0.008	–		–		–	
SMAD2 Thr255	4.532	0.006	–		–		–	
SMAD3	1.441	0.032	–		–		–	

Peptides that displayed a p-value of less than 0.05 are listed.

TABLE 7 | Antibody array results.

	Antibody array		Peptide array			Homology
ID	Fold change	*p*-value	ID	Fold change	*p*-value	
Calmodulin (phospho-Thr286)	1.31	0.003	Calmodulin T185	2.99	0.008	100
CAMK2-beta (phospho-Ser33)	1.18	0.002	CAMK2-beta Ser33	1.66	0.044	100
MAP3K7 (TAK1) (phospho-431)	1.49	0.001	MAP3K7 (TAK1) Ser446	2.71	0.016	100
MAP3K7 (TAK1) (phospho-Thr187)	1.50	0.005	MAP3K7 (TAK1) T177	1.85	0.05	100
NFATC2 (phospho-Ser168/170)	1.05	0.009	NFATC2	1.76	0.045	100
NFATC4 (phospho-Ser203)	2.21	0.006	NFATC4 Ser203	2.24	0.01	100
PPP2CA (phospho-Ser307)	1.91	0.03	PPP2CA Ser304	2.45	0.003	93
SMAD1 (phospho-Ser206)	2.70	0.021	SMAD1 Ser206	2.09	0.02	100
SMAD1 (phospho-Ser462)	2.92	0.004	SMAD1 Ser462	3.43	0.008	100
SAMD2 (phospho-Thr220)	1.68	0.025	SMAD2 Thr255	4.53	0.006	93
SMAD2 (Ser350)	1.21	0.004	SMAD2 Ser345	3.43	0.008	100
SAMD3 (phospho-Thr199)	1.36	0.001	SAMD3 Thr180	1.44	0.006	93
PKCA (phosphor-Thr640)	1.18	0.036	PKCA Thr640	2.55	0.01	100
PKCA (phospho-Ser657)	1.34	0.0009	PKCA Ser659	1.74	0.04	100
PLCB (phospho-Tyr783)	−1.19668	0.03417	PLCG1 Y675	−2.78738	0.00067	86

Statistically significant ($p < 0.05$) phosphospecific antibody array results of Salmonella Entertidis cecal samples. Four days post-infection samples were compared to non-infected control samples to find changes in infected cecal tissue over time. Antibodies bound to phosphorylated protein having a statistically significant difference in fluorescent signal are shown. Fold Change Antibody Array is the change in fluorescent signal when comparing the infected samples to control samples. Homology indicates the % similarity between human and chicken at the 15 amino acid region flanking the phosphorylation residue. Fold Change Peptide Array is the change in fluorescent signal as indicated by the peptide array.

from the antibodies. The percent orthology between the human and chicken at the 15 amino acid phosphorylation target sites as determined by NCBI Protein Blast analysis is shown in **Table 7**. Following the data normalization, the results pointed to a similar pattern to that observed with the peptide arrays (**Table 7**).

Verification of Increased Ca^{2+} in Infected Cecal Tissues

Using a commercial ELISA kit to measure intracellular Ca^{2+} levels, we found almost five times more intracellular Ca^{2+} in the cecal tissue from chickens 4 days post-infection with *S.* Enteritidis (**Figure 2**). These results provide further proof of the activation of the non-canonical Wnt-Ca^{2+} signaling pathway during the establishment of a persistent *Salmonella* infection in the cecum.

Discussion

In chickens, *Salmonella* have evolved the capacity to survive the initial immune response and persist. Very little is known about the regulatory interactions between the host immune response and virulence mechanisms that lead to *S. enterica* persistence in the avian intestine. The carrier state, corresponding to a persistent colonization of the gut, is established, and *Salmonella* is able to stay in the ceca for months without clinical signs (10). Chronic colonization of the intestinal tract is an important aspect of persistent *Salmonella* infection because it results in a silent propagation of bacteria in poultry stocks due to the impossibility to isolate contaminated animals (11).

Collectively, the results from the current experiments demonstrate the phenotypic plasticity of the avian immune system in

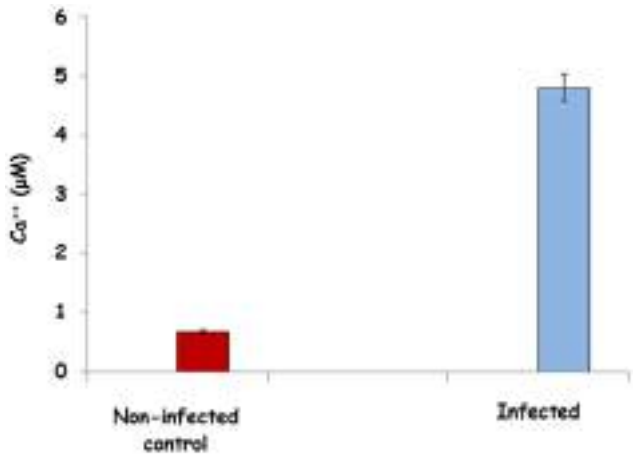

FIGURE 2 | Ca^{2+} levels in the ceca of *Salmonella*-infected and non-infected chickens 4 days post-challenge. Intracellular Ca^{2+} in cecal lysates from non-infected and infected chickens was measured with a colorimetric Ca^{2+} Detection Kit. Data represents the amount of Ca^{2+} measured in the cecal tissue from infected and non-infected chickens and are expressed as the mean micromoles of Ca^{2+} ± SEM from three separate experiments.

the gastrointestinal tract as it first orchestrates an inflammatory response against a primary *Salmonella* infection followed by a dramatic change in the immune microenvironment during the establishment of a persistent *Salmonella* infection.

The 4-day post-infection time period is the initiation of a transitional period between the acute inflammatory response to a primary *Salmonella* infection and the establishment of an "immune *status quo*" (27). As described in the present experiments, by 4 days post-infection, we see a dramatic down-regulation of pro-inflammatory cytokine expression that coincides with the up-regulation of anti-inflammatory cytokine expression (**Figure 1**). Further, by day 4 post-infection, a dramatic increase in Tregs ($CD4^+CD25^+$) in the cecum and remains elevated through the 14-day post-infection time period (27). This coordinated production of pro- versus anti-inflammatory responses is fundamental for the development of an effective initial inflammatory response and subsequent return to tissue homeostasis. Finally, we used the power of kinomics to highlight the mechanisms used by *S*. Enteritidis to alter the avian inflammatory responses and uncover host signaling events that are manipulated by the bacteria in order to establish a persistent infection. Our experiments have identified multiple effects on the host kinome during the establishment of a *Salmonella* persistent infection in the avian cecum. This comparative immune kinome analysis between the *S*. Enteritidis-infected avian cecum versus non-infected cecum provides unique information on host molecular signaling cascades that are mobilized during the establishment of *Salmonella* persistence. Additionally, the relative lack of differential phosphorylation events found in the host signaling pathways between the infected and non-infected ceca 7–14 days post-infection are suggestive that a level of homeostasis was achieved and that the *Salmonella* were no longer recognized as "foreign" and were part of the commensal population. Future experiments are planned to characterize and compare this homeostasis to that of the non-infected controls. Lastly, the identified tissue protein kinases represent potential targets for future antimicrobial compounds for decreasing *Salmonella* loads from the intestines of food animals before going to market.

TGF-β Signaling Pathway

The purpose of these studies was to begin to understand and characterize the biological and molecular mechanisms that regulate the mucosal phenotype of the chicken cecum during the establishment of a persistent infection by *Salmonella*. $CD4^+CD25^+$ regulatory T cells (Tregs) are potent regulators of immune homeostasis (28). We have recently described a dramatic increase in the number of Tregs in the chicken cecum 4 days post-infection with *Salmonella* Enteritidis, and that the number of T regs remains elevated through the 14 days post-infection (29). Understanding the signals necessary for this generation and expansion of Tregs is important for understanding persistence of *Salmonella* in poultry. The pleotropic cytokine, TGF-β, plays a major role in the regulation of inflammation, with T cells being a key target (30). TGF-β suppresses T cell proliferation and T effector cell function (30) while promoting the generation and function of Treg cells (31). Here, we show a dramatic increase in TGF-β mRNA expression between 2 and 4 days after *Salmonella* challenge that remained elevated through 14 days post-infection (**Figure 1**). Simultaneously, using our chicken-specific immune kinome array, for the first time, we have characterized dramatic changes in phosphorylation events where we observed a significant increase in phosphorylation events within the TGF-β signaling pathway (**Table 6**). The TGF-β signaling pathway is mediated by a Smad transcription factor-dependent pathway (21). Upon ligand binding, the TGF-β receptor phosphorylates and activates receptor-associated Smad2 and Smad3, which then associate with Smad4 to control TGF-β-targeted gene expression (32). Both Smad2 and Smad3 are significantly phosphorylated in the ceca from *Salmonella*-infected chickens at 4 days post-infection (**Table 6**). Mitogen-activated protein kinases can also mediate TGF-β signaling through Smad-independent pathways, including TGF-β-activated kinase (TAK1 and MAP3K7), extracellular signal-regulated kinase (ERK and MAPK3), Jun-N-terminal-3-kinase (JNK and MAPK8) (33, 34), all of which are significantly phosphorylated in the ceca from the *Salmonella*-infected chickens at 4 days post-infection. Therefore, the establishment of a persistent cecal colonization in chickens by *Salmonella* initiates the activation of both the canonical (Smad-dependent) and non-canonical (Smad-independent) TGF-β signaling pathways. Smad-dependent and -independent TGF-β signaling appear to separately control Treg and non-Treg function (35). Specifically, Smad-dependent pathways appear to be required to mediate TGF-β functions in non-Treg cells, including non-T cells, whereas Smad-independent pathways are important for Treg function (35, 36). Further, Smad 2/3 are involved in the suppression of pro-inflammatory cytokines by inhibiting the activation of different signal transducers and activators of transcription (STAT) proteins, including STAT1 and 4 to inhibit IFN-γ production (37). Therefore, it is reasonable to speculate that the change in

the cecal mucosal phenotype from pro-inflammatory to tolerance is at least partially mediated by the increased expression of TGF-β that results in the activation of both Smad-dependent and -independent TGF-β pathways and the increase differentiation and function of Tregs that provide the environment essential for *Salmonella* to establish a persistent infection.

Wnt Signaling Pathway

The Wnt signaling pathway system is evolutionarily conserved system that regulates a diverse series of essential functions (38, 39). There are three distinct pathways in Wnt signaling: the canonical Wnt/β-catenin and two non-canonical pathways, Wnt/Planar Cell Polarity and Wnt/Ca^{2+} pathways (39). Based on the results from the kinome array, a number of peptides from the Wnt signaling pathway exhibited statistically significant changes in their phosphorylation (**Table 5**). Further observation of these results indicates that two of the Wnt signaling pathways had significant changes in phosphorylation events, namely the canonical Wnt/β-catenin and the non-canonical Wnt/Ca^{2+} pathways.

Bacterial Effect on Canonical Wnt Signaling

The phosphorylation of two peptides in the canonical Wnt pathway was significantly altered at the 4 days after *Salmonella*-infection time point: glycogen synthase kinase 3β (GSK-3β) and β-catenin (**Table 5**). GSK-3β was significantly dephosphorylated, whereas β-catenin was significantly phosphorylated in these experiments. GSK-3β is a constitutively active serine/threonine kinase that regulates the phosphorylation and degradation of β-catenin (40). In response to external stimuli, GSK-3β is regulated by phosphorylation, inactivated by dephosphorylation of Tyr216 or activated by dephosphorylation of Ser9. The results found here determined that GSK-3β was dephosphorylated at Ser9. This site-specific phosphorylation of GSK-3β results in the activation of its kinase activity (41). In its activated form, GSK-3β forms a catalytically active complex that phosphorylates β-catenin inducing ubiquitylation and proteasomal degradation of β-catenin (39). In these studies, β-catenin was phosphorylated at Ser33 (**Tables 5** and **6**). This phosphorylation site promotes ubiquitylation and targeted destruction of β-catenin (39).

Bacterial Effect on Non-Canonical Wnt Signaling

Non-canonical Wnt signaling controls nuclear localization of nuclear factor of activated T cell (NFAT) transcriptional factor though Ca^{2+} and suppresses canonical Wnt signaling (42, 43). Using the chicken-specific kinome array (**Table 5**), Wnt antibody array (**Table 6**), and a Ca^{2+} ELISA assay (**Figure 2**), we have outlined the activation of the entire non-canonical Wnt/Ca^{2+} pathway in the cecum of chickens as *Salmonella* establishes a persistent infection beginning 4 days after infection (**Figure 3**). The Wnt5A (fold change = 1.82696, $p < 0.0001$)/frizzled 1 receptor complex induces the influx of intracellular Ca^{2+} that, in turn, phosphorylates calcinuerin, a Ca^{2+}, calmodulin-dependent serine/threonine protein phosphatase. Phosphorylated calcinuerin phosphorylates both calmodulin-dependent kinase II (CaMK2A)

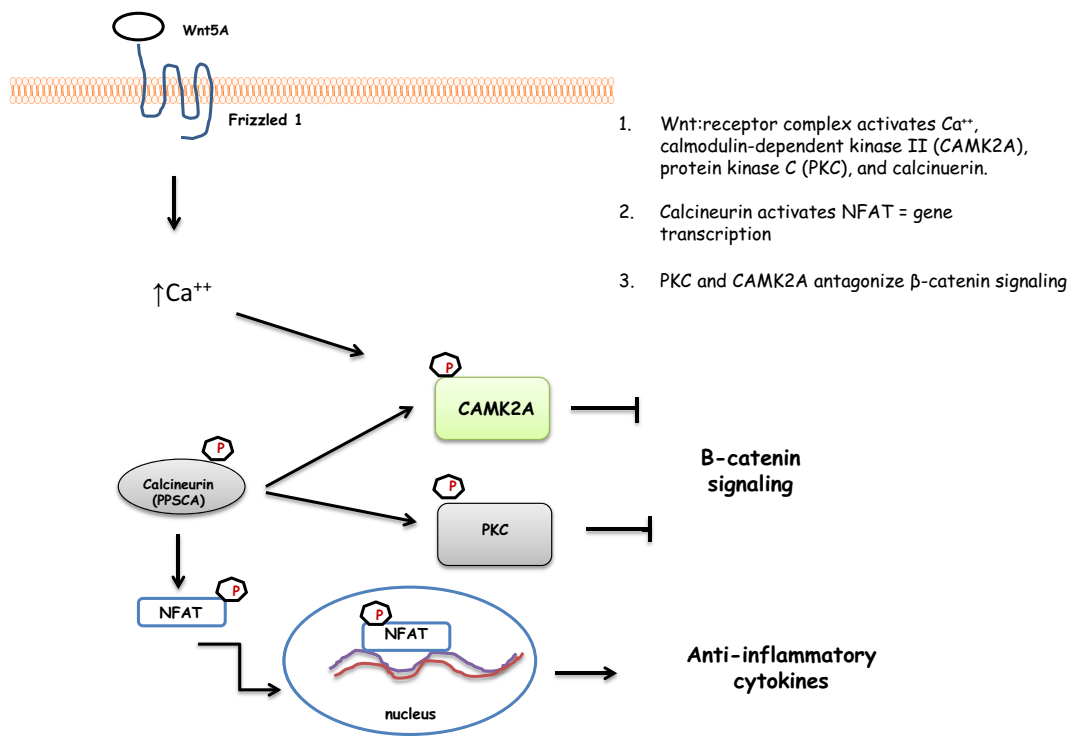

FIGURE 3 | A schematic of the proposed non-canonical Wnt signaling pathway induced in the ceca from experimental chickens with persistent colonization by *Salmonella* Enteritidis.

and PKC and activates NFAT, which can then translocate to the nuclease to induce gene transcription (44). NFAT functions to regulate the interaction of the innate immune cells with acquired immunity and to promote anti-inflammatory programs (45). Thus, the increased phosphorylation of NFAT peptides would suggest the initiation of anti-inflammatory signals. Further, Ca^{2+}/calcineurin/NFAT pathway is crucial for both development and function of regulatory T cells (46). Lastly, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcription factor that plays a key role in regulating the pro-inflammatory response whose activity is triggered in response to infectious agents and pro-inflammatory cytokines via the IκB kinase (IKK) complex (47, 48). Thus, dephosphorylation of both IKK (Thr37, fold change $= -1.63995$, $p < 0.0024$; Ser194, fold change $= -2.86736$, $p < 5 \times 10^5$) and NF-κB (NF-κB1 p105, Ser342, fold change $= -31.2387$, $p < 0.025$) would result in a down-regulation of pro-inflammatory cytokines, as we observed in the present experiments (**Figure 1**).

In summary, the results from the present studies, taken together with our previous studies with Treg, provide solid evidence of a phenotypic change in the mucosal microenvironment that allows for the establishment of a persistent infection by *S*. Enteritidis in the avian cecum. The phenotype alteration appears to be partially mediated due to the targeting of signaling cascades, such as the non-canonical Wnt/Ca^{2+} and the TGF-β signaling pathways that inhibit the transcription of pro-inflammatory responses that provide an appropriate local environment for the generation and expansion of Tregs.

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Effect of dietary exogenous enzyme supplementation on enteric mucosal morphological development and adherent mucin thickness in Turkeys

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Anti-nutritional factors (ANFs) in feed ingredients can challenge gut health and reduce nutrient utilization. Birds typically activate their innate immune system as a protective response against the adverse effects of ANF, which often involves the secretion of mucin. Although dietary supplementation of exogenous enzymes are commonly used to alleviate the adverse effects of ANF on apparent nutrient digestibility, little is known about how they affect gut health, particularly in relation to the morphological development and mucin secretion of enteric mucosa. We carried out two trials to examine the effect of dietary supplementation of different types of exogenous enzymes on gut health of by accessing the effect of jejunum morphological development and ileal enteric adherent mucin thickness layer in turkeys. Dietary β-mannanase supplementation reduced ileal adherent mucin thickness layer (804 vs 823 μg/g; $p < 0.05$), while a commercial blend of xylanase, amylase, and protease (XAP) reduced ileal adherent mucin layer thickness (589 vs 740 μg/g; $p < 0.05$); thus reducing the apparent endogenous loss of nutrients. Both enzyme supplements also affected gut morphological characteristics. In comparison to the control treatment, dietary β-mannanase supplementation improved the jejunum tip width (219 vs 161; $p < 0.05$), base width (367 vs 300; $p < 0.05$), surface area (509,870 vs 380, 157; $p < 0.05$) and villi height/crypt depth ratio (7.49 vs 5.70; $p < 0.05$), and XAP improved the crypt depth ($p < 0.05$). In conclusion, dietary supplementation of exogenous enzymes may help alleviate the adverse effects of ANF on nutrient utilization by directly or indirectly removing the mucosal irritation that stimulates enteric mucin secretion.

Keywords: supplemental enzymes, gut health, enteric mucosal morphology, mucin, turkeys

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INTRODUCTION

The gut is a very complex and diverse ecosystem, and maintenance of gut health is a high nutrient-consuming task. Croom et al. (1) reported that energy required for gut maintenance accounts for about 25% of the total basal metabolic needs of an animal. The requirements may even be higher during an event of enteric distress or microbial pathogen challenge, which can significantly impact the partitioning of energy and other nutrients away from growth, thus reducing the overall feed conversion efficiency. Gut health can be maintained by a balance between the protective function

of intestinal mucosa mucin secretion and the symbiotic community of microorganisms that competitively exclude pathogenic microbes (2).

Dietary composition plays an important role in maintaining the healthy gut ecosystem. Non-starch polysaccharides (NSPs), such as arabinoxylan and β-mannan, are one of the dietary components that influence the gut ecosystem. Their complex water-soluble structure increases digesta viscosity (3), which may entrap macronutrients, such as fat, protein, and starch, and reduces digestive enzyme–substrate interactions (4). Consequently, digestion and nutrient absorption in the foregut is impeded, which adversely effects nutrient supply for growth and reduces feed conversion efficiency. Furthermore, viscous β-mannan and arabinoxlyan may carry undigested nutrients from foregut into the hindgut where they become substrate for the fermentation of competitive microflora that alters the enteric ecosystem stability (5).

Excessive NSPs in the diet, such as arabinoxylan and β-mannan, may lead to the proliferation of undesirable pathogenic intestinal microflora, such as *E. coli* and *Clostridium spp*. (5–7). These enteric pathogens initiate a mucosal inflammatory response, leading to enteric distress, and suppressed gut morphological development. Consequently, this could have a biological effect on intestinal health by changing the mucosal morphological architecture and diverting more nutrients away from productive purposes toward intestinal maintenance. Enteric inflammation, due to enteric pathogen challenge, is associated with the activation of the innate immune system, which is positively associated with the stimulation of intestinal mucous secretion (8). Mucin, which is secreted by the goblet cells, is the protein-rich component of mucous. Although, little information is available on the influence of dietary arabinoxylan and β-mannan on enteric mucosa morphological development and health, atrophic shortening and thickening of jejunum villi, along with increased number of goblet cells per villus, has been observed in broilers fed a diet containing β-glucans which is a similar NSPs (9).

To our knowledge, there are limited published data available on the influence of enzymes on intestinal health, particularly as it affects gut morphology and intestinal adherent mucin secretion in turkey poults. This paper is a report of results from two experiments that evaluated the influence of dietary enzyme supplementation of turkeys fed high and low-energy diets, modified by dietary fat supplementation, on gut health as assessed by changes in mucosa morphometric characteristics and adherent ileal mucosa mucin layer thickness.

MATERIALS AND METHODS

Experiment 1

Experimental diet

The experiment was designed as a 2 × 2 factorial arrangement of two dietary inclusion levels of β-mannanase (0 and 0.05% CTCzyme®, CTCBio, Inc., Korea), supplying 0 and ~500 U endo-beta-D-mannanase/kg of diet, respectively, and two dietary levels of metabolizable energy (high and low) that differed by 150 kcal ME/kg by the addition of supplemental beef tallow fat. All experimental diets were corn-SBM based formulations. The high-energy basal diet was made and divided into two lots, and each was mixed with either 0.05% of vermiculite, or 0.05% of β-mannanase. A second low-energy basal diet (150 kcal/kg less than the high-energy basal diet) was also made and divided into two parts, each was also mixed with either 0.05% of vermiculite or 0.05% of β-mannanase. Vermiculite was added as a non-nutritional filler in place of the enzyme in the experimental control diets. All experimental diets were formulated to meet or exceed NRC (10) requirements for turkeys (**Table 1**). All diets were supplemented with 2.0% Celite® (Celite Corp., Lompar, CA, USA) which served as an acid insoluble ash indigestible reference marker for the determination of digestibility coefficients. The calculated and analyzed composition of the experimental diets, including the supplemental enzymes, is reported in **Table 1**. All diets were produced at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC, USA).

Bird Husbandry and Tissue Sampling

Four hundred thirty-two Nicholas hen poults were obtained from a commercial hatchery (Prestage Farms Hatchery, Clinton, NC, USA) and randomly assigned to one of 48 wire-floored cages with nine poults per cage (Alternative Design cages, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR, USA). Each bird was identified with a numbered neck-tag in sequence for each replicate cage group. Four experimental treatment groups were randomly assigned among 48 cages. Feed and water was available *ad libitum*. Birds were reared until 28 days of age. From 1 to 7 days, the birds were provided 23 h of light to 1 h darkness, and 14 L:10 D after 7 days. On 7, 14, and 21 days, a 3-cm segment of the jejunum, from the end of the duodenal loop toward the Meckel's diverticulum, was sampled from each of four poults per cage, gently flushed with saline, and fixed in 10% formalin solution for subsequent histological analysis. Another gut segment, representing 1 cm into the ileal section from the Meckel's diverticulum, was collected from each of the same four poults per cage for mucin histochemical analysis.

Experiment 2

Experimental Diets

The objective of the second experiment was to determine the effect of the dietary supplementation of an experimental blend of xylanase, amylase, and protease (XAP) and a direct-fed microbial (DFM) product (DuPont, St. Louis, MO, USA) on gut morphological development and ileal adherent mucin layer thickness in turkeys. The experiment was designed as a 2 × 4 factorial, with two inclusion levels of DDGS and four levels of different type of feed supplements [negative control (NC), 2% supplemental fat, XAP, and XAP + DFM]. Two experimental corn-soybean meal basal diets, containing 6 or 18% DDGS, were pelleted and crumbled and subsequently divided into four lots. The first lot was retained as the NC, the second lot was supplemented with 2% supplemental fat, the third lot was supplemented with the enzyme blend XAP, and the fourth lot was supplemented with the combination of XAP + DFM. The enzyme activities per gram of the XAP blend was 2000 FTU of xylanase, 200 FTU of amylase, and 5000 FTU of protease, and the XAP + DFM also

TABLE 1 | Dietary ingredient composition and nutrient composition of starter diets fed to turkey hens from 1 to 28 days of age.

Ingredients	High energy		Low energy	
	% Dietary β-mannanase supplementation			
	0	0.05	0	0.05
	(% of Diet)			
Corn	30.56	30.56	34.98	34.98
Soybean meal	44.84	44.84	44.24	44.24
Soy hulls	6.11	6.11	6.03	6.03
Poultry meal	5.00	5.00	5.00	5.00
Fat beef tallow	5.82	5.82	2.09	2.09
Dical phosphate	3.03	3.03	3.03	3.03
Limestone	0.95	0.95	0.95	0.95
DL-Methionine	0.44	0.44	0.43	0.43
Sodium chloride	0.32	0.32	0.32	0.32
L-Lysine-HCl	0.26	0.26	0.27	0.27
Choline chloride	0.22	0.22	0.22	0.22
Trace mineral[a]	0.20	0.20	0.20	0.20
Vitamin premix[b]	0.15	0.15	0.15	0.15
Selenite premix[c]	0.05	0.05	0.05	0.05
Celite™	2.00	2.00	2.00	2.00
CTCzyme[d]	0.00	0.05	0.00	0.05
Filler (vermiculite)	0.05	0.00	0.05	0.00
Calculated chemical composition				
Dry matter (%)	93.34	93.34	92.72	92.72
ME poultry (kcal/kg)	2850	2850	2700	2700
Crude protein (%)	30.06	30.06	30.19	30.19
Crude fat (%)	8.04	8.04	4.92	4.92
Crude fiber (%)	4.34	4.34	4.37	4.37
Calcium (%)	4.34	4.34	4.37	4.37
Total phosphorus (%)	1.40	1.40	1.40	1.40
Avail. phosphorus poultry (%)	1.09	1.09	1.09	1.09

[a]Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; and 1 mg Co as $CoSO_4$.
[b]Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.
[c]$NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed.
[d]Enzyme provided 0.05% of beta-mannanase in diet.

contained 75,000 cfu of *Bacillus subtilis* per gram of diet. This same experimental diet preparation procedure was used for all feed phases. All experimental diets were formulated to meet or exceed the NRC (10) nutritional recommendations for turkeys (**Table 2**). All diets were manufactured at the North Carolina State University Feed Mill Educational Unit (Raleigh, NC, USA).

Bird Husbandry and Tissue Sampling

Eight hundred sixty-four 1-day old female poults (Hybrid Converter Hens, Cold Springs Farm, Thamesford, Ontario, Canada) were randomly assigned to 48 litter floor pens containing 18 poults per pen. Six replicate pens were randomly assigned per dietary treatment. The poults had access to *ad libitum* feed and water and raised according to standard commercial practices. At 42 days of age, six birds per treatments were euthanized by cervical dislocation to collect tissue for histological assessment. A 6-cm section from the mid-portion of the jejunum was taken from each bird, gently flushed with saline, and fixed in 10% formalin solution for subsequent morphometric analysis. Ileal sections were also taken each of the euthanized birds for mucosal adherent mucin secretion as previously described.

Histological and Histochemical Analysis

Histological Analyses

The tissue samples were immediately rinsed with saline, and fixed in 10% neutral-buffered formalin solution for at least 72 h before processing. A total of four sections of about 2–3 mm in length were taken from a 3-cm fixed jejunum section collected from each sampled bird. These smaller sections were placed in tissue cassettes and submerged in 10% buffered formalin solution until processed at the Histopathology Laboratory (NC State University, College of Veterinary Medicine, Raleigh, NC, USA). The fixed jejunum sections were embedded in paraffin wax, and 5 μm thick transverse sections were cut with a microtome. The 5 μm-cut sections were placed on slides and were stained with Lilee Meyer hematoxylin and counter-stained with eosin yellow. A light-microscope (LEICA-DMR light-microscope, Leica Camera AG, Solms, Germany) was used to visualize the transverse sections placed on slides. The images were captured using a Spot-LTCR digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and analyzed using Image Tool software (UTHSCSA Image Tool Software, Version 3.0, the University of Texas, San Antonio, TX, USA). Villus height, villus apical width at the tip of the villus, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth were measured on 10 villi per sampled poult. The villi height:crypt depth ratio for each poult was calculated by dividing the average of the 10 villi heights measured per poult by the average of the 10 crypt depths measured on the same poult. The following mathematical formula was used to determine apparent villus surface area {[(villus tip + villus base)/2] × villus height}, according to Iji et al. (11).

Histochemical Analyses – Measurement of Adherent Ileal Mucin Layer Thickness

The epithelial-adherent mucin layer thickness was assessed histochemically with Alcian blue stain, based on the affinity of the stain for mucin (12). The thickness of the ileal mucus adherent layer was estimated based on the modification of Parman's method (13). A 1-cm section of ileal tissue from each sampled bird was removed and placed in 10 g/L Alcian blue dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8. After 6 h of incubation, excess dye was extracted with 250 mmol/L sucrose. The absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution overnight at room temperature. Samples were centrifuged at 700 g, plated on 96-well plate, and the optical densities were measured at 620 nm using Alcian blue solution as a standard. The amount of absorbed dye was reported as micrograms of Alcian blue per gram of ileal tissue.

TABLE 2 | Dietary ingredients and nutrients composition fed to turkey hens in experiment 2.

Ingredients	Starter		Grower 1		Grower 2	
	6% DDGS	18% DDGS	6% DDGS	18% DDGS	6% DDGS	18% DDGS
	(% of Diet)					
Soybean meal	42.65	44.37	35.59	31.07	31.99	26.65
Corn	39.25	28.74	46.43	38.83	50.44	43.74
DDGS	6.00	18.00	6.00	18.00	6.00	18.00
Poultry meal (60% CP)	5.00	3.00	5.00	5.00	5.00	5.00
Poultry fat	1.53	2.85	2.17	2.40	2.32	2.45
Dical P 18.5	2.39	2.60	1.92	1.64	1.51	1.25
Limestone	1.23	1.48	1.28	1.46	1.24	1.42
Alimet	0.55	0.53	0.41	0.37	0.38	0.35
L-Lysine HCl	0.35	0.39	0.29	0.37	0.22	0.33
Micro salt	0.32	0.33	0.30	0.26	0.27	0.24
Trace mineral premix[a]	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride 60%	0.19	0.16	0.19	0.14	0.18	0.14
Vitamin premix[b]	0.15	0.15	0.15	0.15	0.15	0.15
L-Threonine	0.11	0.13	0.02	0.02	0.02	0.02
Sodium selenite premix[c]	0.05	0.05	0.05	0.05	0.05	0.05
Feed supplement/filler[d]	0.02	0.02	0.02	0.02	0.02	0.02
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
Chemical analysis						
Dry matter* (%)	88.3	89.97	90.35	90.5	90.3	89.38
ME poultry (kcal/kg)	2900	2900	3000	3000	3050	3050
Crude protein* (%)	29.6	29.1	26.01	26.49	24.4	23.5
Crude fat* (%)	5.91	6.18	6.63	7.25	6.13	7.7
Crude fiber (%)	2.68	3.33	2.55	3.04	2.49	2.97
Arabinoxylan (%)	1.80	2.75	2.02	3.04	2.12	3.18
Calcium* (%)	1.16	0.8	1.18	1.33	1.13	1.16
Total phosphorus* (%)	0.79	10.98	0.74	0.74	0.76	0.72
Avail. phosphorus poultry	0.8	0.8	0.7	0.7	0.62	0.62
Lysine (%)	1.889	1.894	1.648	1.669	1.4917	1.5119

[a]Each kilogram of mineral premix (0.1% inclusion) supplied the following per kg of complete feed: 60 mg Zn as $ZnSO_4 \cdot H_2O$; 60 mg Mn as $MnSO_4 \cdot H_2O$; 40 mg Fe as $FeSO_4 \cdot H_2O$; 5 mg Cu as $CuSO_4$; 1.25 mg I as $Ca(IO_3)_2$; and 1 mg Co as $CoSO_4$.

[b]Each kilogram of vitamin premix (0.1% inclusion) supplied the following per kg of complete feed: vitamin A, 13,200 IU; cholecalciferol, 4000 IU; alpha-tocopherol, 66 IU; niacin, 110 mg; pantothenic acid, 22 mg; riboflavin, 13.2 mg; pyridoxine, 8 mg; menadione, 4 mg; folic acid, 2.2 mg; thiamin, 4 mg; biotin, 0.253 mg; vitamin B_{12}, 0.04 mg; ethoxyquin, 100 mg.

[c]$NaSeO_3$ premix provided 0.3 mg Se/kg of complete feed.

[d]Feed supplements include XAP, XAP + DFM, and supplemental fat. Approximately 2% supplemental fat was added to create ME difference of 150 kcal/kg. Filler was added in diets that had no feed supplement.

**As determined by chemical analysis.*

Statistical Analysis

The experiments were analyzed as completely randomized designs. Pen or cage means were respectively used as the experimental unit for the statistical analysis of adherent ileal mucin secretion. For histological analysis, 10 villi measurements were averaged per tissue sample collected, and this average number served as the experimental unit for the statistical analysis. Data were analyzed using JMP software (Version 10, SAS Institute, Cary, NC, USA). ANOVA was used to examine the main effect of dietary treatment factors, and their interaction on parameters evaluated. Means were separated using the LS Means at $P < 0.05$.

Animal Ethics

Care of the birds used in all experiments conformed to the Guide for Care and Use of Agricultural Animals in Research and Teaching (14). Moreover, all animal husbandry practices and euthanasia performed during the conduct of these experiments were conducted according to protocol # 12-014-A approved by Institutional Animal Care and Use Committee at North Carolina State University.

RESULTS

Experiment 1

Table 3 summarizes the effect of dietary energy level and β-mannanase supplementation on villi morphological characteristics and ileal adherent mucin thickness layer of turkey hens at 21 days of age.

Gut Morphological Development

There were no significant treatment effects observed on jejunum mucosa morphology at 7 and 14 days; however, there was a significant treatment effect observed at 28 days. Increasing dietary energy level was associated with an 18% decrease in the villus base width and a 21% decrease in villus surface area ($P < 0.05$). In contrast, dietary supplementation of β-mannanase increased

TABLE 3 | Effect of dietary energy level and β-mannanase supplementation on jejunum villi morphological characteristics and ileal adherent mucin thickness layer of turkey hens at 28 days of age.[c]

Main effect		Tip width	Villi height	Base width	Crypt depth	Muscularis thickness	Surface area	Villi height/crypt depth	Ileal adherent mucin thickness layer
		Micron					Micron[d]		μg/g tissue
Energy level									
High energy		173	1661	301[b]	244	256	393,523[b]	6.19	752
Low energy		207	1768	366[a]	222	266	496,505[a]	6.97	804
β-Mannanase level (%)									
0.05		219[a]	1714	367[a]	224	251	509,870[a]	7.49[a]	804[b]
0.00		161[b]	1715	300[b]	242	269	380,157[b]	5.70[b]	832[a]
Interactions									
Energy level	**β-Mannanase level (%)**								
High energy	0.05	204	1733[a,b]	335	232	266	468,246	6.97[a,b]	631[b]
High energy	0.00	143	1588[b]	266	255	243	318,800	5.43[b]	977[a]
Low energy	0.05	235	1694[a,b]	399	215	236	551,495	7.94[a]	791[a,b]
Low energy	0.00	179	1842[a]	334	228	296	441,514	5.92[a,b]	873[a,b]
Source of variations							*P*-values		
Energy		0.141	0.097	0.011	0.373	0.586	0.003	0.149	0.1616
β-Mannanase		0.017	0.983	0.009	0.470	0.383	0.001	0.006	0.0466
Energy × β-mannanase		0.909	0.030	0.928	0.831	0.060	0.501	0.025	0.0261
SEM(40)[d]		10	29	11	11	10	14,242	0.35	27.51

[a,b]Means with different letter superscripts within a column are significantly different (P < 0.05).

[c]Values are means of four replicates each treatment.

[d]SEM(40), standard error of the mean with 40 degrees of freedom.

the villus tip width by 36% and villus height/crypt depth by 32% ($P < 0.05$), while the villus base width and surface area was increased by about 22.5 and 34%, respectively ($P < 0.05$).

Ileal Adherent Mucin Layer Thickness

There was no significant main effect of dietary energy; however, poults fed the β-mannanase supplemented diet had a 4% reduction in ileal adherent mucin layer thickness as compared to the unsupplemented control diet. Furthermore, the supplementation of β-mannanase to the high-energy diet reduced the ileal adherent mucin layer thickness by about 36% as compared to the high-energy diet without supplementation ($P < 0.05$), but neither of these diets were significantly different from the results observed among birds fed the low-energy diets with or without the enzyme supplementation ($P > 0.05$).

Experiment 2

Table 4 summarizes the influence of dietary DDGS level, and XAP and DFM supplementation on the morphological measurements of jejunum villi and mucosa of turkey hens at 42 days.

Gut Morphological Development

Some effects were observed on mucosa morphology development at 42 days of age. Dietary inclusion of 18% DDGS was associated with a 15% decrease in villus tip width ($P < 0.05$) and 11% decrease in villi surface area ($P < 0.05$) relative to the 6% DDGS treatment. However, the 18% dietary inclusion of DDGS treatment had a 13% increase in muscularis thickness ($P < 0.05$) relative to the 6% DDGS inclusion. DDGS inclusion did not have significant effect on the villi height, crypt depth, villi height/crypt depth, and villi base width. The dietary supplements did not have any effect on villi tip width, height, villi height/crypt depth, villi base width, muscularis thickness, and villi surface area, although they did affect the crypt depth. Poults-fed diets supplemented with XAP alone or in combination with DFM had reduced crypt depth by about 12 and 11%, respectively, when compared with the supplemental fat and NC ($P < 0.05$).

Ileal Adherent Mucin Layer Thickness

The results of the ileal adherent mucin layer thickness are also presented in **Table 4**. Increasing the dietary level of DDGS increased the ileal adherent mucin layer thickness observed in poults at 42 days of age ($P < 0.05$). However, in comparison to the supplemental fat and NC treatments, dietary supplementation of XAP reduced the ileal adherent mucin layer thickness by 24 and 20%, respectively ($P < 0.05$); but neither response was different from XAP + DFM.

DISCUSSION

Gut Morphological Development

Dietary soluble and insoluble β-mannans and arabinoxylans have been reported to exhibit some anti-nutritional properties and adverse effects on growth performance of poultry. The high arabinoxylan content in DDGS along with its inferior amino acid digestibility and variability of other nutrients limits the dietary inclusion of DDGS in poultry feed to <6%. Likewise, the viscous nature of β-mannan in diets, that contain a lot of soybean meal, has been observed to cause physiological and morphological changes to the gastrointestinal tract in poultry, which can impede efficient nutrients utilization (15, 16).

TABLE 4 | Influence of dietary DDGS level, and XAP and DFM supplementation on the morphological measurements of jejunum villi and mucosa of turkey hens at 42 days.[c]

Main effect		Tip width	Crypt depth	Muscularis thickness	Surface area	Ileal adherent mucin thickness layer
		Micron			Micron[d]	μg/g tissue
DDGS level (%)						
6		275[a]	166	251[b]	684,224[a]	679[b]
18		233[b]	169	294[a]	606,656[b]	705[a]
Feed supplements						
Negative control (NC)		264	176[a]	285	670,514	740[a]
~2% Supplemental fat (suppl. fat)		267	180[a]	274	639,138	770[a]
XAP		240	157[c]	266	618,222	589[b]
XAP + DFM		246	159[c]	264	653,887	670[a,b]
Interactions						
DDGS level (%)	Feed supplement					
6	NC	296	177	268	735,289	737
6	Suppl. fat	295	185	255	660,544	726
6	XAP	252	145	244	630,957	576
6	XAP + DFM	260	157	238	710,104	679
18	NC	232	176	303	605,737	744
18	Suppl. fat	239	174	293	617,733	814
18	XAP	228	167	288	605,486	603
18	XAP + DFM	233	161	291	597,670	661
Source of variations						
				P-values		
DDGS		0.002	0.614	<0.01	0.037	0.045
Feed supplement		0.373	0.037	0.334	0.740	0.032
DDGS × feed supplement		0.576	0.423	0.885	0.664	0.871
SEM(40)[d]		12	6.519	13	34,110	46

[a,b]*Means with different letter superscripts within a column are significantly different (P < 0.05).*

[c]*Values are means of six replicates per treatment.*

[d]*SEM(40), standard error of the mean with 40 degrees of freedom.*

The general hypothesis tested in the two studies reported herein was that the supplementation with β-mannanase, XAP, or a combination of XAP + DFM will reduce the anti-nutritional effects and improve apparent nutrient utilization by reducing endogenous nutrient losses associated with enteric mucin secretion, enhancing gut morphological development, and improving gut health. Because dietary inclusion of the enzymes may help degrade the NSPs, their anti-nutritional effects may, thereby, be diminished and thus improve nutrient utilization, as residual enteric substrates are altered to positively favor symbiotic microflora and microbial diversity over the pro-inflammatory pathogenic ones in the hindgut ecosystem, consequently improving the gut health.

Measured changes in intestinal morphology, such as shorter villi and deeper villi crypts, have been used as indicators of gut health and enteric distress (17). Tall mucosal villi increase the surface area available for nutrients absorption (18, 19). There is correlation between the crypt depth and the rate of proliferation of the epithelial cells (20, 21). Epithelial regeneration starts from the villi crypt, so a deep crypt is an indication of rapid enterocyte turnover and increased mucosal tissue maintenance requirements (17, 22). The rapid enterocyte proliferation and the epithelial cell turnover rate greatly impacts protein and energy requirements of the small intestine mucosa (23). Diet composition may produce microscopic alterations in the intestinal mucosa, and it is possible that the change in morphology of the gastrointestinal tract may be associated with dietary NSP levels (24).

Some studies have demonstrated that enzyme treatment can influence the morphological development of intestinal villi (19, 25, 26). In the first trial, we observed that dietary inclusion of β-mannanase had a positive effect on jejunum mucosal morphology among poults sampled at 28 days: villus surface area and tip and base widths were all increased. Although there are few publications demonstrating the effect of β-mannanase on gut morphology, the work of Mehri et al. (27) corroborated our findings. There was an increase in the villi surface area and jejunum villi height/crypt depth ratio by dietary β-mannanase supplementation. Mehri et al. (27) observed an increase in villus height and crypt depth in the duodenum when β-mannanase was supplemented to corn–soy diets at 700 and 900 g/ton.

In the second experiment, the level of arabinoxylan, a NSP anti-nutritional factor (ANF), was calculated to increase by about 50% as the dietary inclusion level DDGS increased from 6 to 18% (**Table 2**). Choct and Annison (28) have shown anti-nutritional effects of depressed apparent metabolizable energy when 3% pentosans, primarily comprised of arabinoxylans, was added to broiler diets. Relative to the 6% DDGS treatment, dietary inclusion of 18% DDGS adversely affected jejunum mucosal morphology as indicated by reduced villi surface area, tip width and increased thickness of the villi muscularis. The

poor jejunum morphological development may be associated with relatively higher NSP content in the 18% DDGS diet. Iji (29) reported that NSP can negatively impact gut morphology by increasing crypt depth of both the jejunum and ileum, thus accelerating enterocyte turnover. Although we evaluated the effects of a blend of supplemental enzymes containing xylanase in the second experiment, xylanase supplementation alone has been reported to increase villus height of the duodenum, jejunum, and ileum, and the villus height/crypt depth of these three segments (19). Furthermore, the dietary inclusion of DFM may also impact the gut morphology by increasing the jejunum and ileal villi heights (30, 31). Dietary supplementation of the enzyme blend of XAP and XAP + DFM combination had a beneficial effect on gut morphology development. The addition of the enzyme blend with or without the DFM reduced the crypt depth, indicating reduced mucosal distress, in comparison to the NC or fat-supplemented diet.

Apparently, the effects observed on enteric mucosa morphology in both trials may have been associated with the changes in the substrate characteristics within the enteric ecosystem by the dietary supplementations (β-mannanase, XAP, and XAP + DFM), which in turn altered the fermentation of resident enteric microflora (32).

Adherent Ileal Mucin Secretion

Establishment of a symbiotic enteric ecosystem minimizes the inflammatory symptoms of enteric distress caused by the proliferation of enteric pathogens, which is associated with increased intestinal mucin secretion (33, 34). Parsaie et al. (35) reported that distressed intestinal morphology, as indicated by the shortened villi and deepened crypts, may cause increased mucosal secretions, primarily as mucin. Indeed, some research reports have shown that the dietary inclusion of enzymes reduce the mucosal blanket secretions in monogastric animals (36, 37).

In addition to the improved gut morphological development observed in this study, a reduction in the ileal adherent mucin layer thickness was observed with dietary β-mannanase supplementation, especially in the high-energy (fat) diets. Few studies have been reported on the influences of β-mannanase on mucin secretion. However, research results reported trial by Mehri et al. (27) indirectly validated our observation. They reported that dietary β-mannanase supplementation reduced the number of mucosal goblet cells per unit of epithelial surface area in broilers. Since mucin is secreted by the epithelial goblet cells, this observation agrees with our observations that dietary β-mannanase supplementation significantly reduces intestinal adherent mucin layer thickness.

We also observed that the addition of XAP reduced ileal mucin thickness layer. Bedford et al. (36) also noted that xylanase supplementation improved the apparent digestibility of threonine, which was associated with a decrease in intestinal mucin secretion. Pirgozliev et al. (37) also observed a decrease in the quantity of threonine excreted by the birds fed with a xylanase-supplemented diet, which he attributed to less secretion of intestinal mucin.

CONCLUSION

Improved gut morphological development of the mucosa villi in the jejunum and ileum and the reduced adherent ileal mucin thickness layer could likely contribute to the improved apparent nutrient utilization, which reflects in the better growth performance in poultry often observed when their diets are supplemented with exogenous enzymes. Enteric microflora profile has been demonstrated to be influenced by nutrient abundance (32). Digesta viscosity increases as dietary anti-nutritional NSPs increases, which impairs foregut digestion and absorption of fats, starches, and proteins, and causes more of these nutrients to pass to the hindgut where they "feed" competitive pathogenic bacteria. Proliferation of pathogenic bacteria, like *C. perfringens*, causes enteric tissue inflammation, mucosal leakage, and increased mucin secretion as a protective innate immune response. With protein being a major component of mucin, this increase in mucin secretion can further exacerbate the proliferation of putrefying bacterial pathogens and also contribute to the significantly reduced apparent nitrogen retention. Dietary supplementation of NSP enzymes can be an effective means to counter the adverse effects of high digesta viscosity and cause the hind gut microflora to shift toward a more symbiotic ecosystem.

The research results reported herein demonstrate that dietary supplementation of either β-mannanase, XAP, or XAP + DFM can improve gut health in turkey poults as indicated by improved morphological development of the enteric mucosa and reduction in adherent ileal mucin secretion, thereby increasing productive use of nutrients toward better growth performance. Further studies are still needed to evaluate the effect of other supplemental enzymes on gut health, especially as the trend of limiting the use of antibiotic growth promoters in animal feed continues.

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Evaluation of Gastrointestinal Leakage in Multiple Enteric Inflammation Models in Chickens

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Enteric inflammation models can help researchers' study methods to improve health and performance and evaluate various growth promoters and dietary formulations targeted to improve performance in poultry. Oral administration of fluorescein isothiocyanate-dextran (FITC-d; 3–5 kDa) and its pericellular mucosal epithelial leakage are an established marker to evaluate enteric inflammation in multiple species. The present study evaluated different methods to induce gut inflammation in poultry based on FITC-d leakage. Four independent experiments were completed with different inflammation treatment groups, and serum FITC-d and/or retention of FITC-d in GI tract were determined. In experiment 1 ($n = 10$ birds/treatment, broilers, processed at 14 days), groups included control (CON), dextran sodium sulfate (DSS; drinking water at 0.75%) and feed restriction (FRS; 24 h before processing). Experiment 2 ($n = 14$ birds/treatment, leghorns, processed at 7 days) included CON, DSS, FRS, and rye-based diet (RBD). In experiments 3 and 4 ($n = 15$ birds/treatment, broilers, processed at 7 days), groups were CON, DSS, high fat diet (HFD), FRS, and RBD. In all experiments, FRS and RBD treatments showed significantly higher serum FITC-d levels compared to the respective CON. This indicates that FRS and RBD results in disruption of the intact barrier of the gastrointestinal tract (GIT), resulting in increased gut permeability. DSS and HFD groups showed elevation of serum FITC-d levels although the magnitude of difference from respective CON was inconsistent between experiments. FRS was the only treatment which consistently showed elevated retention of FITC-d in GIT in all experiments. The results from present studies showed that FRS and RBD, based on serum FITC-d levels, can be robust models to induce gut leakage in birds in different age and species/strains.

Keywords: dextran sodium sulfate, serum FITC-d, rye-based diet, enteric inflammation, feed restriction

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INTRODUCTION

It is well known that antibiotic growth promoters (AGP) can improve production performance in birds (1) although the exact mode of action is still not completely understood. According to (2), a major hypothesis about the action of AGP is through the reduction of innate inflammatory response in the gastrointestinal tract (GIT) of birds. Now that the use of AGP is discouraged in food animals, and worldwide, it is time to identify growth promoters which are as effective or even better and at the same time safer than AGP.

In addition to its role in endocrine and paracrine hormones' production, nutrient permeability, water and electrolyte exchange, and digestion, the intestinal mucosa is an important barrier for protecting animals against both commensal and pathogenic microorganisms and other insults (3–5). The intestinal first line of defense is composed of the mucus layer and epithelium (6). It has been proposed that intestinal epithelial cell defenses are essential to prevent inflammation, for example, by offering protection against microbial pathogens and oxidative stresses (7). If the intestinal barrier is damaged and becomes non-selectively permeable, the submucosa and deeper layers will be subjected to continuous exposure to antigenic molecules from food and microorganisms, causing inflammatory conditions (8).

Dextran sodium sulfate (DSS) is a heparin-like polysaccharide (9) which can cause disruption of the epithelial lining in the GIT of poultry (10). In addition, our laboratory has already used the gut inflammation induced with DSS to identify various markers to measure gut health.

Fluorescein isothiocyanate-dextran is an established method to measure paracellular leakage in rodent enteric inflammation models (11–13). Since increased mucosal permeability is the first step in the cascade of enteric inflammation, fluorescein isothiocyanate-dextran (FITC-d) model to identify gut leakage would be an effective way to detect the efficiency of various alternative growth promoters, quite early, and predict their beneficial effect on growth and production performance. Studies in our laboratory thus far have shown that oral administration of FITC-d, its leakage to circulation through disrupted epithelial lining of GIT, and subsequent measurement of FITC-d levels in serum is effective in evaluating gut leakage in birds (14, 15). The main use of this marker is to determine the effect of various growth promoters on gut health in birds.

Although DSS produces enteric inflammation in birds, our previous studies suggest that birds were more sensitive to DSS (10) compared to rodents, and achieving acceptable levels of toxicity and lesions was very narrow. Thus, other methods of induction of mucosal permeability in poultry were tested and compared with the previously established DSS model. Alternatives for DSS were feed restriction (FRS) and dietary models, such as rye-based diet (RBD) and high fat diet (HFD). A major advantage to these alternatives was the reduced risk of toxicity and severe illness to birds. At the same time, these alternatives represented various real-world scenarios which can reduced in production performance in poultry.

Feed restriction has been historically used as a way to maintain appropriate body weight of parent stock in meat-type chickens to reduce lameness and health risks, as well as improve fertility rates (16, 17). However, FRS has also been shown to increase enteric permeability, translocation of enteric bacteria to various organs (14), and could make birds more susceptible to various disease conditions related to translocation of pathogens from the GIT to systemic circulation. In addition, some regions of the world use alternate feed stuffs such as rye in place of corn. But, high levels of non-starch polysaccharides (NSP) in rye result in increased digesta viscosity as well as other associated gut health problems, thereby increasing the chance of necrotic enteritis (18–20). Furthermore, inclusion of higher levels of fat in poultry diet has been a common practice that increases growth rate; however, some of studies in mice have shown that this could also produce enteric health problems (21). Based on these facts, the main objective of the present studies was to compare the effect of DSS, FRS, HFD, and RBD on enteric leakage in chickens, measured using serum FITC-d model.

MATERIALS AND METHODS

Experimental Design

Four independent experiments were conducted, and each used different combinations of diets (**Table 1**), as well as other treatments such as FRS (for 24 h before processing) and DSS (MW 40,000; Alfa Aesar, Ward Hill, MA, USA) administered 0.75% in drinking water for 3 days before processing, as given below. Different diets used in the study were basal, RBD, and HFD, of which all met or exceeded NRC requirements (22). In all experiments, birds were administered FITC-d (MW 3,000–5,000 Da; Sigma-Aldrich Co., St. Louis, MO, USA) by oral gavage, 2.5 h before processing, to determine levels of mucosal leakage by evaluating serum FITC-d and the level of FITC-d retained in different regions of GI tract. Birds were humanely killed by inhalation of carbon

TABLE 1 | Ingredients of the feed formulations used in the study.

	CON[a]	HFD[b]	RBD[c]
Ingredients (%)			
Corn	56.59	53.57	–
Rye	–	–	58.19
Soybean meal	35.74	35.13	31.16
Vegetable oil	3.29	6.60	6.29
Dicalcium phosphate	1.81	1.87	1.79
Calcium carbonate	1.12	0.98	1.05
Salt	0.38	0.52	0.38
DL-Methionine	0.31	0.34	0.35
Vitamin premix[d]	0.20	0.20	0.20
L-Lysine HCL	0.19	0.30	0.22
Choline chloride 60%	0.10	0.20	0.10
Mineral premix[e]	0.10	0.10	0.10
Threonine	0.06	0.16	0.08
Antioxidant[f]	0.02	0.02	0.02
Mold propionic acid	0.05	0.05	0.05
Total	100	100	100
Calculated analysis			
ME (kcal/kg)	3,035	3,191	2,850
CP (%)	21.7	22.1	22.4
Lys (%)	1.32	1.35	1.32
Met (%)	0.63	0.64	0.64
Met + Cys (%)	0.98	0.99	0.98
Thr (%)	0.86	0.91	0.86
Trp (%)	0.25	0.28	0.3
Total calcium (%)	0.9	0.9	0.9
Available phosphorus (%)	0.45	0.45	0.45
Sodium (%)	0.16	0.21	0.16

[a] *CON, control.*
[b] *HFD, high fat diet.*
[c] *RBD, rye-based diet.*
[d] *Vitamin premix (per 1,000 kg): vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO, USA).*
[e] *Mineral premix (per 1,000 kg): manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC).*
[f] *Ethoxyquin.*

dioxide gas prior to the collection of blood and GIT samples. All studies were conducted in accordance with protocols approved by the University of Arkansas Institutional Animal Care and Use Committee.

In experiment 1, 30-day-old broiler chicks were randomly assigned to one of the three treatment groups ($n = 10$/group), control (CON), FRS, or DSS. All birds were provided *ad libitum* with the basal diet (**Table 1**) until 13 days. Twenty-four hours prior to the end of the experiment, feed was removed from FRS through the end of the experiment. On day 14, all birds were given an oral gavage of FITC-d (2.2 mg/mL/bird) 2.5 h before they were processed. Blood and tissue samples from duodenum, ileum, and cecum were collected from each bird. Levels of FITC-d in serum and GI tract were determined as explained below.

Day of hatch Leghorns ($n = 56$) was used for experiment 2 and was randomly assigned to CON, DSS, FRS, and RBD ($n = 14$/treatment). In this experiment, all treatment groups except RBD were given basal diet, while RBD was given a RBD (**Table 1**), and feed removed from FRS 24 h prior to end of the experiment. All birds were processed on day 7 after oral gavage with FITC-d (2.2 mg/bird) 2.5 h before processing. Blood and GIT tissue (duodenum and cecum) samples were collected for determination of enteric inflammation.

Experiments 3 and 4 had the same experimental design but were conducted independently. For both experiment, 75-day-old broiler chicks were randomly assigned to CON, DSS, FRS, RBD, or HFD group ($n = 15$ birds/treatment). All groups except RBD and HFD were given basal diet throughout the trail while RBD and HFD were given RBD as well as HFDs, respectively (**Table 1**). As in previous experiments, feed was removed from FRS 24 h prior to the end. On day 7, all birds were given oral gavage with FITC-d (2.2 mg/bird), and serum level and GIT retention (duodenum and cecum) of FITC-d were determined.

Determination of Serum FITC-d Levels

Serum level of FITC-d, a measurement of enteric inflammation and mucosal permeability, was determined as explained by Kuttappan et al. (14). After humane slaughter of birds, femoral artery was severed to collect blood and allowed to clot under room temperature for 3 h. Then, the samples were centrifuged at $500 \times g$ for 15 min, and serum samples were collected. The serum samples were then diluted in phosphate buffer saline (1:1), and fluorescence was measured at 485 nm excitation and 528 nm emission (Synergy HT, multimode microplate reader, BioTek Instruments, Inc., VT, USA). Levels of fluorescence in the samples were converted to respective FITC-d microgram per milliliter of serum based on a calculated standard curve previously obtained from known levels of FITC-d.

Level of FITC-d Retained in Gastrointestinal Tract Tissue

Amount of FITC-d retained in different regions of GI tract were determined using the method suggested by Kuttappan et al. (14) and Vicuña et al. (15). For this, 2.5-cm-long tissue sections were collected from the descending duodenum, ileum immediately proximal to the Meckel's diverticulum, and a single entire cecum (opened at both ends). These samples were cleaned by flushing with Hanks buffered salt solution. After cleaning, samples were gently mopped to remove excess fluid, weighed, and dropped in tubes containing 10 mL Hanks buffer with glutamine (0.3 g/L) and antimicrobial agents (penicillin 100 U/mL, streptomycin 0.01 mg/mL, and amphotericin B 0.25 μg/mL). The tubes were incubated at 42°C for 2 h, and the FITC-d released to the buffer from the tissue was determined and reported as microgram per gram of the respective tissue.

Statistical Analysis

Data were analyzed using ANOVA (SAS 9.3, SAS Institute Inc., Cary, NC, USA) considering individual birds as experimental units for all experiments. Means were separated using Duncan's significant different test at $p < 0.05$. The serum FITC-d data showed occasional, but random outliers, $<+2$ SD from group mean, which were not representative of the respective groups similar to the reports by Kuttappan et al. (14) and Vicuña et al. (15). It is clear that these outliers were not related to treatments administered; however, the reasons for the occurrence of these erratic outliers are not yet clear. For the present studies, we focused on the effect of treatments, and the noise from these outliers was identified using the empirical or 68–95–99.7 rule and trimmed or truncated (23) at mean ± two SDs (14, 15).

RESULTS

Control levels of FITC-d in serum stayed consistent throughout experiments 1–3 with measured serum FITC-d at 0.18 ± 0.01, 0.18 ± 0.02, and 0.17 ± 0.02 μg/mL, respectively (**Tables 2–4**).

TABLE 2 | Serum FITC-d and FITC-d retentions in GI tract from experiment 1 using 2-week-old broiler birds.

Treatments	Serum FITC-d (μg/mL)	FITC-d retention (microgram per gram of tissue)		
		Duodenum	Ileum	Cecum
CON	$0.18^{c} \pm 0.01$	$0.27^{b} \pm 0.01$	$0.34^{a} \pm 0.02$	$0.37^{b} \pm 0.01$
DSS	$0.29^{b} \pm 0.02$	$0.28^{ab} \pm 0.01$	$0.35^{a} \pm 0.01$	$0.41^{b} \pm 0.01$
FRS	$0.36^{a} \pm 0.02$	$0.29^{a} \pm 0.01$	$0.38^{a} \pm 0.02$	$0.46^{a} \pm 0.02$

[a–c] Significant ($p < 0.05$) difference within each column.
CON, control (basal diet); DSS, dextran sodium sulfate administered at 0.75% in drinking water; FRS, feed restriction for 24 h before processing; n = 10/treatment.

TABLE 3 | Serum FITC-d and FITC-d retentions in GI tract from experiment 2 using 1-week-old leghorn birds.

Treatments	Serum FITC-d (μg/mL)	FITC-d retention (microgram per gram of tissue)	
		Duodenum	Cecum
CON	$0.18^{c} \pm 0.02$	$3.19^{b} \pm 0.33$	$26.16^{b} \pm 5.00$
DSS	$0.24^{bc} \pm 0.02$	$0.73^{c} \pm 0.24$	$12.64^{b} \pm 2.38$
FRS	$0.37^{a} \pm 0.01$	$5.16^{a} \pm 0.63$	$63.17^{a} \pm 13.84$
RBD	$0.28^{b} \pm 0.02$	$2.51^{b} \pm 0.25$	$25.74^{b} \pm 12.42$

[a–c] Significant ($p < 0.05$) difference within each column.
CON, control (basal diet); DSS, dextran sodium sulfate administrated at 0.75% in drinking water; FRS, feed restriction for 24 h before processing; RBD, rye-based diet; n = 14/treatment.

TABLE 4 | Serum FITC-d and FITC-d retentions in GI tract from experiments 3 and 4 using 1-week-old broiler birds.

	Serum (μg/mL)		Duodenum (microgram per gram of tissue)		Cecum (microgram per gram of tissue)	
	Experiment 3	Experiment 4	Experiment 3	Experiment 4	Experiment 3	Experiment 4
CON	$0.17^{c} \pm 0.02$	$0.25^{c} \pm 0.03$	$0.73^{c} \pm 0.16$	$0.48^{b} \pm 0.12$	$22.36^{b} \pm 6.47$	$7.55^{b} \pm 1.61$
DSS	$0.26^{bc} \pm 0.04$	$0.28^{bc} \pm 0.02$	$1.25^{bc} \pm 0.11$	$0.70^{ab} \pm 0.06$	$13.95^{b} \pm 3.73$	$8.65^{b} \pm 1.66$
HFD	$0.29^{abc} \pm 0.02$	$0.29^{bc} \pm 0.01$	$1.35^{bc} \pm 0.18$	$0.77^{ab} \pm 0.18$	$9.68^{b} \pm 1.48$	$5.83^{b} \pm 1.78$
FRS	$0.46^{a} \pm 0.10$	$0.32^{b} \pm 0.03$	$2.13^{a} \pm 0.22$	$1.15^{a} \pm 0.21$	$49.69^{a} \pm 16.42$	$37.33^{a} \pm 11.17$
RBD	$0.44^{ab} \pm 0.08$	$0.38^{a} \pm 0.02$	$1.63^{ab} \pm 0.32$	$1.05^{a} \pm 0.12$	$4.71^{b} \pm 0.90$	$2.59^{b} \pm 0.45$

$^{a-c}$*Significant ($p < 0.05$) difference within each column.*

CON, control (basal diet); DSS, dextran sodium sulfate administrated at 0.75% in drinking water; HFD, high fat diet; FRS, feed restriction for 24 h before processing; RBD, rye-based diet; n = 15/treatment.

Though there was a slight increase noted in experiment 4, at 0.25 ± 0.0 3 μg/mL, significant differences were still noted with FRS and RBD. Levels recovered from the various GIT tissues varied greatly. In experiment 1, serum FITC-d in both treatments was significantly higher than CON, with DSS at 0.29 ± 0.02 μg/mL and FRS at 0.36 ± 0.02 μg/mL, compared to 0.18 ± 0.01 μg/mL (**Table 2**). For FRS, this difference was repeated in experiment 2 with serum FITC-d measured at 0.37 ± 0.01 and 0.18 ± 0.02 μg/mL in CON, but DSS was not significantly higher at only 0.24 ± 0.02 μg/mL (**Table 3**). This experiment included an additional group, RBD, which had serum FITC-d levels higher than CON, but no different than DSS, and lower than FRS at 0.28 ± 0.02 μg/mL. In experiments 3 and 4, only FRS and RBD resulted in greater passage of FITC-d to serum with 0.32 ± 0.03 and 0.38 ± 0.02 μg/mL, respectively, compare to 0.25 ± 0.03 μg/mL in the CON group. Neither DSS nor HFD increased FITC-d levels in serum for those experiments.

Retention of FITC-d in GIT tissue was measured in duodenum, ileum, and cecum in experiment 1 and duodenum and cecum in the other experiments. Differences in retention were noted between FRS and CON groups for duodenum and cecum, but not ileum in the first experiment, though the level of change was not likely biologically significant (**Table 2**). In experiment 2, FRS resulted in higher retention of FITC-d in both duodenal and cecal tissue with levels measured at $5.16^{a} \pm 0.63$ and $63.17^{a} \pm 13.84$ μg/g of tissues, respectively, while DSS levels in the duodenum were lower than CON tissue, this decrease is not consistent with other experiments reported here (**Table 3**). RBD did not result in changes, compared to CON, in retention of FITC-d in either the duodenum or the cecum (**Table 3**).

Duodenal retention of FITC-d was affected by FRS (2.13 ± 0.22 and 1.15 ± 0.21 μg/g) and RBD (1.63 ± 0.32 and 1.05 ± 0.12 μg/g) in experiments 3 and 4, compared to CON (0.73 ± 0.16 and 0.48 ± 0.12 μg/g; **Table 4**). Whereas, in the cecum for experiments 3 and 4, only FRS was different from all other groups with FICT-d retained in tissue, which was 49.69 ± 16.42 and 37.33 ± 11.17 μg/g.

DISCUSSION

Serum FITC-d Levels

Determination of serum FITC-d to evaluate enteric inflammation has long been established marker in murine models (11–13). Results from these studies, conducted on chickens, using DSS-FITC-d model (14) suggest that DSS could have caused the disruption of tight junctions in GI tract, increased mucosal permeability (13, 24), ultimately resulting in increased serum FITC-d levels. In experiment 1, the DSS and FRS groups showed higher ($p < 0.05$) serum FITC-d levels when compared to the respective CON (**Table 2**). This result was in accordance with reports from the studies conducted by Kuttappan et al. (14). Furthermore, FRS group had even higher serum FITC-d level when compared to the respective DSS group (**Table 1**). Kuttappan et al. (14) showed comparable levels of serum FITC-d between the DSS and FRS groups. This could be because of the fact that Kuttappan et al. (14) used an oral gavage with DSS while the present study administered DSS at the level of 0.75% in drinking water. Thus, when DSS was administered as a high dose for a short period of time (14), it could have a greater impact when compared to low dose prolonged period of administration in the present study, though a direct comparison of the two methods was not completed. In addition, Kuttappan et al. (14) used broilers which were 1-week old while the present study used broiler birds which were 2 weeks old. In fact, the results from these two studies suggested that the serum FITC-d could be used as a marker for gut health in birds across different age groups.

In experiment 2, DSS showed elevated serum FITC-d levels when compared to the respective CON group although it was not significant ($p > 0.05$). However, FRS and RBD groups showed significantly ($p < 0.05$) higher serum FITC-d levels when compared to the respective CON group. Kuttappan et al. (14) previously reported that FRS could result in increased serum FITC-d leakage in broilers. Furthermore, van der Hulst et al. (25, 26) reported that starvation in human patients could result in lack of enough glutamine which could lead to increased gut leakage. Additionally, Tellez et al. (20, 27) reported that RBD, in comparison to corn-based diet, resulted in increased serum FITC-d levels and also significantly ($p < 0.05$) higher translocation of enteric bacteria to liver, both in broiler chicks and turkey poults. The present study was completed in leghorns, and these results confirmed that the model for measuring gut health using serum FITC-d is reliable through different species and strains of poultry.

Experiments 3 and 4 compared serum FITC-d in broilers in CON, DSS, HFD, FRS, and RBD (**Table 3**). Similar to experiment 2, DSS showed elevated serum FITC-d levels with CON although, it was not significantly different. HFD resulted in a trend similar to DSS both in experiments 3 and 4.

de Lartigue et al. (21) had reported that HFD in mice could increase intestinal permeability by altering the expression of tight junction proteins, though results in chickens were not as marked as were reported in mice. Consistent with experiment 2, both FRS and RBD showed higher serum FITC-d levels compared to the respective CON. The results from all four experiments in this trial showed that FRS and RBD could cause increased gut leakage in poultry, which are relevant to current poultry practices. FRS, most commonly skip-a-day feeding, is widely used in meat-type poultry breeder stocks to regulate weight gain and maintain fertility. Increased serum FITC-d due to FRS as reported by the present study suggested that FRS in breeders could result in increased gut leakage and could lead to translocation of enteric bacteria to other tissue organs which could result in disease conditions, such as lameness (28). Similarly, attempts to use least cost feed formulation for poultry often includes the incorporation of alternative feed grains, such as wheat and rye in poultry feed, quite often resulting in reduced performance and poor litter conditions (18, 29, 30). Rye contains high levels of NSP, comprised of highly branched arabinoxylans, which are mainly responsible for increased digesta viscosity, reduced activity of digestive enzymes, and reduced intestinal absorption, making birds susceptible to economically significant conditions, such as necrotic enteritis (19, 31, 32). Our laboratory is currently investigating the effect of various probiotics and dietary enzymes in reducing the gut leakage associated with FRS and RBD based on the serum FITC-d marker.

Retention of FITC-d in Gastrointestinal Tract Tissues

Kuttappan et al. (14) suggested that the disruption of epithelial layer in the GIT could result in increased infiltration of FITC-d in the paracellular space between cells on the mucosal surface. In all experiments, FRS showed significantly higher FITC-d levels in duodenum and cecum compared to the respective CON birds (**Tables 2**–**4**). This was in accordance with the results reported by Kuttappan et al. (14). However, Kuttappan et al. (14) also found that administration of DSS as oral gavage resulted in increased retention of FITC-d in duodenum of broiler chickens when compared to CON, although the cecum did not show any significant difference. With a prolonged administration of low dose DSS (0.75% in drinking water) in the present study, FITC-d did not significantly increase (**Tables 2**–**4**). Moreover, RBD, which consistently showed elevated serum FITC-d levels, failed to reflect any difference in GIT tissue FITC-d levels with respect to the CON (**Tables 3** and **4**). These data suggest that the retention of FITC-d could be more complex which depends upon factors, such as rate of GI passage, which could be affected by irritation on GIT wall, and viscosity of diet as in the case of rye diet. Thus, level of FITC-d retained in GIT tissue may not be a direct measurement of gut leakage as compared to serum FITC-d levels.

CONCLUSION

The results from present studies showed that FRS and RBD are consistent methods for inducing mucosal leakage that could lead to enteric inflammation in poultry. Serum FITC-d measurement was proven to be a very reliable and non-invasive marker to determine gut leakage in birds across different age and strains. Since the method involves oral administration of FITC-d and subsequent measurement of FITC-d in serum, it could possibly be used in live birds at multiple time points throughout a single experiment. Further studies will be conducted in our laboratory comparing different growth promoters in poultry and their effect on gut leakage under various conditions using serum FITC-d model.

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Identification of potential biomarkers for gut barrier failure in broiler chickens

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The objective of the present study was to identify potential biomarkers for gut barrier failure in chickens. A total of 144 day-of-hatch Ross 308 male broiler chickens were housed in 24 battery cages with six chicks per cage. Cages were randomly assigned to either a control group (CON) or gut barrier failure (GBF) group. During the first 13 days, birds in CON or GBF groups were fed a common corn–soy starter diet. On day 14, CON chickens were switched to a corn grower diet, and GBF chickens were switched to rye–wheat–barley grower diet. In addition, on day 21, GBF chickens were orally challenged with a coccidiosis vaccine. At days 21 and 28, birds were weighed by cage and feed intake was recorded to calculate feed conversion ratio. At day 28, one chicken from each cage was euthanized to collect intestinal samples for morphometric analysis, blood for serum, and intestinal mucosa scrapings for gene expression. Overall performance and feed efficiency was severely affected ($P < 0.05$) by a GBF model when compared with CON group at days 21 and 28. Duodenum of GBF birds had wider villi, longer crypt depth, and higher crypt depth/villi height ratio than CON birds. Similarly, GBF birds had longer crypt depth in jejunum and ileum when compared with CON birds. Protein levels of endotoxin and α1-acid glycoprotein (AGP) in serum, as well as mRNA levels of interleukin (IL)-8, IL-1β, transforming growth factor (TGF)-β4, and fatty acid-binding protein (FABP) 6 were increased ($P < 0.05$) in GBF birds compared to CON birds; however, mRNA levels of FABP2, occludin, and mucin 2 (MUC2) were reduced by 34% ($P < 0.05$), 24% ($P = 0.107$), and 29% ($P = 0.088$), respectively, in GBF birds compared to CON birds. The results from the present study suggest that serum endotoxin and AGP, as well as, gene expression of FABP2, FABP6, IL-8, IL-1β, TGF-β4, occludin, and MUC2 in mucosa may work as potential biomarkers for gut barrier health in chickens.

Keywords: gut barrier function, morphometric analysis, endotoxin, AGP, gene expression, biomarker

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Abbreviations: AGP, α1-acid glycoprotein; AJ, adherens junctions; BW, body weight; BWG, body weight gain; CON, control; ERK, extracellular signal-regulated kinase; FABP, fatty acid-binding protein; FCR, feed conversion ratio; FI, feed intake; GBF, gut barrier failure; GIT, gastrointestinal tract; IEC, intestinal epithelial cells; IL, interleukin; JAM, junctional adhesion molecule; MUC2, mucin 2; NSP, non-starch polysaccharide; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TGF, transforming growth factor; TJ, tight junction; TLR, toll-like receptor; TNF, tumor necrosis factor; ZO, zonula occluden.

Introduction

Barrier function is a critical aspect of gut heath. Oxidative stress, poorly digestible protein, and coccidiosis are some examples that can cause gut barrier failure (1–5). Nevertheless, as a consequence of the removal of anti-microbial growth promoters, new multifactorial diseases causing enteritis and gut disorders of unknown origin have emerged in broilers, causing negative impacts in health and performance (6–9). Among them, dysbacteriosis, defined as the presence of a qualitatively and/or quantitatively abnormal microbiota in the proximal parts of the small intestine, associated with reduced nutrient digestibility, impaired intestinal barrier function, bacterial translocation, and inflammatory responses have been reported (4, 5, 10). However, more recently, poor gut health has also been associated with bacterial chondronecrosis with osteomyelitis in broiler chickens and breeders (11–13). As the largest organ in the body, the gut serves as a selective barrier to take up nutrients and fluids into the body, while excluding undesirable molecules and pathogens (3, 14, 15). Therefore, proper gut barrier function is essential to maintain optimal health and balance throughout the body, and represents a key line of defense against foreign antigens from the environment (16). The first layer of gut barrier is the extrinsic mucus layer comprised an outer layer associated with bacteria and an inner layer with high concentrations of secretory IgA and mucin. The outer layer is loosely attached to epithelium. The inner layer is adherent to the second layer of gut barrier, the intestinal epithelial cells (IEC). IEC are a single layer of epithelial cells that separate the intestinal lumen from underlying lamina propria (17–19). These epithelial cells must be able to rapidly regenerate in the event of tissue damage (14, 20, 21). The enterocytes in the apical epithelium are responsible for absorption of nutrients. Tight junctions (TJ) seal the paracellular space between adjacent epithelial cells and regulate the permeability of intestinal barrier by preventing diffusion of microorganisms and antigens (22, 23). Since IEC are the primary cell type coming into contact with the external environment, they act as the host's first line of the defense. In spite of their non-hematopoietic derivation, IEC represent a core element of innate immunity within the gut-associated lymphoid tissue, displaying a wide array of immune functions. In fact, IEC are able to recognize pathogens through the expression of innate immune receptors, release of anti-microbial molecules, and secretion of a wide number of hormones, neuro transmitters, enzymes, as well as cytokines and chemokines that link innate and adaptive immune responses (24–26). Hence, any direct or indirect damage on IEC may cause a breakdown in gut barrier and consequently, disruption of normal mucosal immune homeostasis that can potentially lead to uncontrolled chronic intestinal and systemic inflammation (27, 28).

Several investigators have described the pathways associated with the disruption of the protein networks that connect epithelial cells by inflammatory mediators, such as hormones, oxygen free radical species, enzymes, as well as multiple proinflammatory cytokines (27, 29, 30). Feeding oxidized/unpreserved fat has been also shown to increase intestinal epithelial turnover rates and increase apoptosis at villus tips in poultry and swine (31). Non-starch polysaccharides (NSP), such as β-glucans and pentosans have been shown to have a detrimental influence on the utilization of nutrients in broilers by increasing digesta viscosity and reducing digestibility of nutrients (e.g., fat and protein) (32, 33), which could cause dysbacteriosis. Currently, no biomarkers have been described as tools to evaluate gut inflammation or gut barrier failure in broiler chickens. The objective of the present study was not to determine the individual effects of diet ingredients or coccidia challenge on gut health, rather to identify potential biomarkers for gut barrier failure. Therefore, we attempted to exacerbate gut barrier failure by feeding a high NSP diet containing rye, wheat, and barley to induce high digesta viscosity (4, 5) in combination with a 2× coccidiosis vaccination to induce gut health challenge.

Materials and Methods

Animal Source and Diets

A total of 144 day-of-hatch Ross 308 male broiler chickens were randomly housed in 24 battery cages with six chicks per cage in environmentally controlled rooms. To avoid cross contamination of coccidiosis vaccine, birds in control group (CON) and gut barrier failure (GBF) group were housed in two separate but identically controlled rooms. Temperature was maintained at 34°C for the first 5 days and then gradually reduced according to normal management practices, until a temperature of 23°C was achieved. Lighting was provided for 24 h/day. During the first 13 days, birds in CON or GBF groups were fed common corn–soy starter diet (**Table 1**). On day 14, birds in CON group were switched to a corn–soy grower diet (14–28 days) and the GBF group was switched to rye–wheat–barley diet (**Table 1**). The experimental diets were formulated to approximate the nutritional requirements of broiler chickens (34). On day 21, birds in GBF treatment were orally challenged with 2× dose of Advent™ coccidiosis vaccine, a mixture of *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* (Huvepharma Sofia, Bulgaria). All research procedures were reviewed and approved by a licensed veterinarian and also followed the protocols described previously (5, 35), which were approved by IACUC at University of Arkansas. All studies performed by Novus International, Inc. are in accordance to the standards of the Guide for the Care and Use of Agricultural Animals in Research and Teaching (35).

Experimental Design

The 144 day-of-hatch chickens were randomly allotted to one of two groups; CON or GBF on the basis of initial body weight (BW). Each treatment was comprised of 12 replicates of six chicks each ($n = 72$/group). At 21 and 28 days, BW, body weight gain (BWG), and feed intake (FI) were recorded in each cage to calculate feed conversion ratio (FCR).

Sample Collection

At 28 days of age, one chicken from each cage was euthanized by CO_2 asphyxiation for sample collection. Blood sample was taken from cardiac puncture using a syringe, kept at room temperature for 3 h to allow clotting, and centrifuged (1,000 × *g* for 15 min at 4°C) to separate serum. Following euthanasia, a 1-cm section of duodenum was collected from the middle of the descending duodenum; a 1-cm section of jejunum was collected at the Meckel's diverticulum; a 1-cm section of ileum was collected 2 cm before the ceca. All of intestinal sections were rinsed with

TABLE 1 | Ingredient composition and nutrient content of common, control, and gut barrier failure (GBF) diets, as-is basis.

Ingredient	Common starter, 0–13 days (%)	Control grower, 14–28 days (%)	GBF grower, 14–28 days (%)
Corn	60.6	60.6	0
Rye	0	0	33.95
Wheat	0	0	20
Barley	0	0	10
SBM, 47.5% CP	32.56	32.56	28.8
Soybean oil	1.08	1.08	2.96
L-lysine HCl	1.48	1.48	0.14
MHA®	0.3	0.3	0.42
L-threonine	0.01	0.01	0.05
L-tryptophan	0.14	0.14	0.11
Dicalcium phosphate, 18.5%	1.59	1.59	1.57
Limestone	1.09	1.09	1
Salt	0.25	0.25	0.25
Choline chloride, 60%	0.25	0.25	0.25
Sodium bicarbonate	0.2	0.2	0.2
Mineral premix[a]	0.2	0.2	0.2
Vitamin premix[b]	0.1	0.1	0.1
Santoquin™ Mixture 6	0.02	0.02	0
MycoCURB™	0.05	0.05	0
Coban® 90	0.05	0.05	0
BMD® 60	0.03	0.03	0
Calculated nutrients			
ME, kcal/kg	3,031	3,152	3,152
SID Lysine, %	1.27	1.1	1.1
SID TSAA, %	0.94	0.84	0.84
Total CP, %	22	20.7	21.8
Ca, %	1.05	0.9	0.9
Available P, %	0.5	0.45	0.45

[a]*Mineral premix supplied per kilogram of diet: Mn, 120 mg; Zn, 100 mg; Fe, 40 mg; Cu, 16 mg; I, 1.25 mg; Se, 0.30 mg.*
[b]*Vitamin premix supplied per kilogram of diet: retinol, 9.2 mg; cholecalciferol, 100 μg; dl-α-tocopherol, 90 mg; menadione, 6 mg; thiamine, 6.2 mg; riboflavin, 26.5 mg; pantothenic acid, 39.7 mg; niacin, 100 mg; pyridoxine, 11 mg; folic acid, 4 mg; biotin, 0.3 mg; cyanocobalamin, 0.1 mg.*

10% neutral buffered formalin and then fixed in 20× volume of 10% neutral buffered formalin. A 10-cm section of jejunum was rinsed with ice cold phosphate buffered saline (pH 7.4) and cut open to scrape mucosa using RNAse-free glass slides into 2-ml tubes with 1 ml RNAlater (Applied Biosystems, NY, USA). The mucosal scrapings were stored at 4°C for 24 h and then at −20°C until total RNA isolation.

Histological Sample Preparation and Intestinal Morphometry Measurement

Intestinal segments were trimmed, processed, and embedded in paraffin. A 5-μm section of each sample was placed on a glass slide and stained with hematoxylin and eosin for morphometry examination and measurement under Olympus light microscope using Olympus MicroSuite™ Imaging software (Center Valley, PA, USA). Five replicate measurements for each variable studied were taken from each sample, and the average values were used in statistical analyses. Villi height was measured from the top of the villi to the top of the submucosa. Crypt depth was measured from the base upwards to the region of transition between the crypt and villi. Villi width was measured at the middle of each villus, whereas crypt/villi ratio was determined as the ratio of crypt depth to villi height (36).

Serum Endotoxin and Serum α1 Acute Phase Protein Determination

Endotoxin was measured using a chicken Endotoxin Elisa kit from Amsbio (Cambridge, MA, USA). Acute phase protein, α1-acid glycoprotein (AGP) was measured using chicken α1-acid glycoprotein measurement kit from The Institute for Metabolic Ecosystem (Miyagi, Japan). The Optical Density for both kits was determined at 450 nm using a BIO-TEK ELx800 (BIO-TEK Instrument, Winooski, VT, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT–PCR)

Total RNA was isolated from mucosa scraping samples using Clontech Total RNA isolation NucleoSpin® RNA II kit (Clontech Laboratories, Inc., CA, USA). One microgram of total RNA, 11mer oligo mix from Fluoresentric, and M-MLV Reverse Transcriptase (Life Technologies, Grand Island, NY, USA) were used to synthesize cDNA according to the manufacturers' instructions. The relative mRNA levels of mucin 2 (MUC2), fatty acid-binding protein (FABP) 2, FABP6, interleukin (IL)-8, IL-1β, transforming growth factor (TGF)-β4, occludin, zonula occluden (ZO)-1, junctional adhesion molecule (JAM) 2, JAM3, catenin, tumor necrosis factor (TNF) α, Toll-like receptor (TLR) 2β, TLR4, and claudin 1 were measured by quantitative PCR using Applied Biosystems® SYBR® Green PCR Master Mix, the 7500 Fast Real-Time PCR System, and primers in **Table 2**. Results were expressed as the level relative to the corresponding housekeeping gene *actin*. All primers were verified for the efficiency and linearity of amplification.

Statistical Analyses

All data were tested for normality and subjected to one-way ANOVA as a completely randomized design using the GLM procedure of SAS (37). Each cage was used as the experimental unit for the analysis. Growth performance including BW, BWG, FI, and FCR used the average data per cage. Gut morphometric measurements, serum endotoxin, AGP, and qRT–PCR used individual measurement from one randomly chosen bird per cage. Data are expressed as mean ± SE.

Results

Growth Performance

The results of the growth performance parameters between CON and GBF groups are summarized in **Table 3**. BW, FI per bird, BWG and FCR at 21 and 28 days of age were dramatically reduced in GBF chickens when compared with CON chickens ($P < 0.05$), indicating that GBF model substantially compromised the growth performance of chickens.

Histomorphometric Analysis

The results of the histomorphometric analysis of duodenum, jejunum, and ileal tissue between CON and GBF chickens at 28 days of age are summarized in **Table 4**. The duodenum, jejunum, and ileum all showed increased ($P < 0.05$) crypt depth

TABLE 2 | List of primers used for qRT–PCR.

Genes	Forward primer	Reverse primer	Fragment size (bp)
Actin	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC	205
MUC2	GCCTGCCCAGGAAATCAAG	CGACAAGTTTGCTGGCACAT	59
FABP2	AAAGATAATGGAAAAGTACTCACAGCAT	CCTTCGTACACGTAGGTCTGTATGA	77
FABP6	CGGTCTCCCTGCTGACAAGA	CCACCTCGGTGACTATTTTGC	59
IL-8	TCCTGGTTTCAGCTGCTCTGT	CGCAGCTCATTCCCCATCT	52
TGF-β4	CGGCCGACGATGAGTGGCTC	CGGGGCCCATCTCACAGGGA	113
Occludin	GAGCCCAGACTACCAAAGCAA	GCTTGATGTGGAAGAGCTTGTTG	68
ZO1	CCGCAGTCGTTCACGATCT	GGAGAATGTCTGGAATGGTCTGA	63
JAM2	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA	59
JAM3	CCGACGGCTGTTTGTGTTT	GGCGGTGCAAAGTTTTGG	56
Catenin	CGACAACTGCTCCCTCTTTGA	GCGTTGTGTCCACATCTTCCT	63
TNFα	TGTTCTATGACCGCCCAGTTC	GACGTGTCACGATCATCTGGTT	63
TLR2β	CGCTTAGGAGAGACAATCTGTGAA	GCCTGTTTTAGGGATTTCAGAGAATTT	90
TLR4	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG	190
Claudin 1	TGGCCACGTCATGGTATGG	AACGGGTGTGAAAGGGTCATAG	62
IL-4	GCCAGCACTGCCACAAGA	GGAGCTGACGCGTGTTGAG	54
IL-6	GAGGGCCGTTCGCTATTTG	ATTGTGCCCGAACTAAAACATTC	67
IL-1β	CAGCCCGTGGGCATCA	CTTAGCTTGTAGGTGGCGATGTT	59

TABLE 3 | Performance parameters between control and gut barrier failure grower groups (GBF).

Treatments and growing phase	BW (g)	FI per bird (g)	BWG (g)	FCR during each phase
21 days				
CON	866.25 ± 11.87^{a}	617.25 ± 8.76^{a}	390.58 ± 6.21^{a}	1.58 ± 0.02^{b}
GBF	642.58 ± 10.50^{b}	480.67 ± 11.85^{b}	203.17 ± 11.27^{b}	2.42 ± 0.09^{a}
28 days				
CON	$1{,}302.75 \pm 26.45^{a}$	729.50 ± 26.17^{a}	436.50 ± 19.04^{a}	1.67 ± 0.03^{b}
GBF	895.50 ± 21.58^{b}	578.17 ± 9.5^{b}	252.92 ± 20.30^{b}	2.42 ± 0.17^{a}

Data are expressed as mean ± SE.
[a,b]Superscripts within columns indicate difference at $P < 0.05$.

(shown as * in **Figure 1**) in GBF chickens compared to CON chickens. GBF chickens also had wider villi in duodenum and jejunum, and higher crypt/villi ratio in duodenum compared to CON chickens; however, the crypt/villi ratio was not different in jejunum ($P = 0.064$) and ileum ($P = 0.208$) because the villus height in jejunum and ileum was also increased ($P < 0.03$) in GBF birds compared to CON birds. The increase of crypt depth and/or the crypt/villi ratio is an indication of greater need of cell proliferation to maintain proper gut health, which suggests that GBF model generated unhealthy gut barrier.

Serum Endotoxin and AGP

Table 5 shows the comparison of serum AGP and endotoxin levels between CON and GBF groups of broiler chickens at 28 days of age. AGP, a marker for systemic inflammation, was increased ($P < 0.05$) by 3.8-fold in GBF birds compared to CON birds (**Table 5**), suggesting that systemic inflammation was occurring in GBF birds. Endotoxin, a toxin released by gram-negative bacteria in the gut, was increased ($P < 0.05$) by 2.1-fold in serum of GBF birds compared to CON birds (**Table 5**), which suggests that greater amount of endotoxin was translocated from intestinal lumen into blood.

Gene Expression in Jejunal Mucosa by qRT–PCR

The relative mRNA levels of genes that are possibly involved in gut barrier function and inflammation in jejunal mucosa of broilers chickens at 28 days of age are shown in **Table 6**. The relative mRNA levels of IL-8, IL-1β, TGF-β4, and FABP6 were increased ($P < 0.04$) by 3-, 1.5-, 2.2-, and 7-fold, respectively, in GBF chickens compared to CON chickens. However, FABP2, occludin, and MUC2 mRNA levels were decreased by 34%

TABLE 4 | Histomorphometric analysis of duodenum, jejunum, and ileum in control (CON) and gut barrier failure (GBF) groups in chickens at 28 days of age.

Tissue	CON	GBF
Duodenum		
Villus height, μm	2324.7 ± 123.84^{a}	2649.8 ± 156.21^{a}
Villus width, μm	172.81 ± 5.24^{b}	214.08 ± 13.04^{a}
Crypt depth, μm	104.51 ± 4.76^{b}	201.74 ± 17.10^{a}
Crypt/villi ratio	0.04 ± 0.01^{b}	0.08 ± 0.01^{a}
Jejunum		
Villus height, μm	1883.40 ± 141.54^{b}	2273.80 ± 77.17^{a}
Villus width, μm	170.57 ± 9.17^{b}	190.02 ± 12.08^{a}
Crypt depth, μm	112.84 ± 9.32^{b}	172.78 ± 10.59^{a}
Crypt/villi ratio	0.06 ± 0.01^{a}	0.07 ± 0.01^{a}
Ileum		
Villus height, μm	1005.70 ± 45.77^{b}	1334.13 ± 79.61^{a}
Villus width, μm	163.80 ± 4.97^{a}	166.25 ± 7.85^{a}
Crypt depth, μm	113.63 ± 7.91^{b}	174.70 ± 14.11^{a}
Crypt/villi ratio	0.11 ± 0.01^{a}	0.13 ± 0.01^{a}

Values are expressed as means ± SE.
[a,b]Superscripts within rows indicate difference at $P < 0.05$.

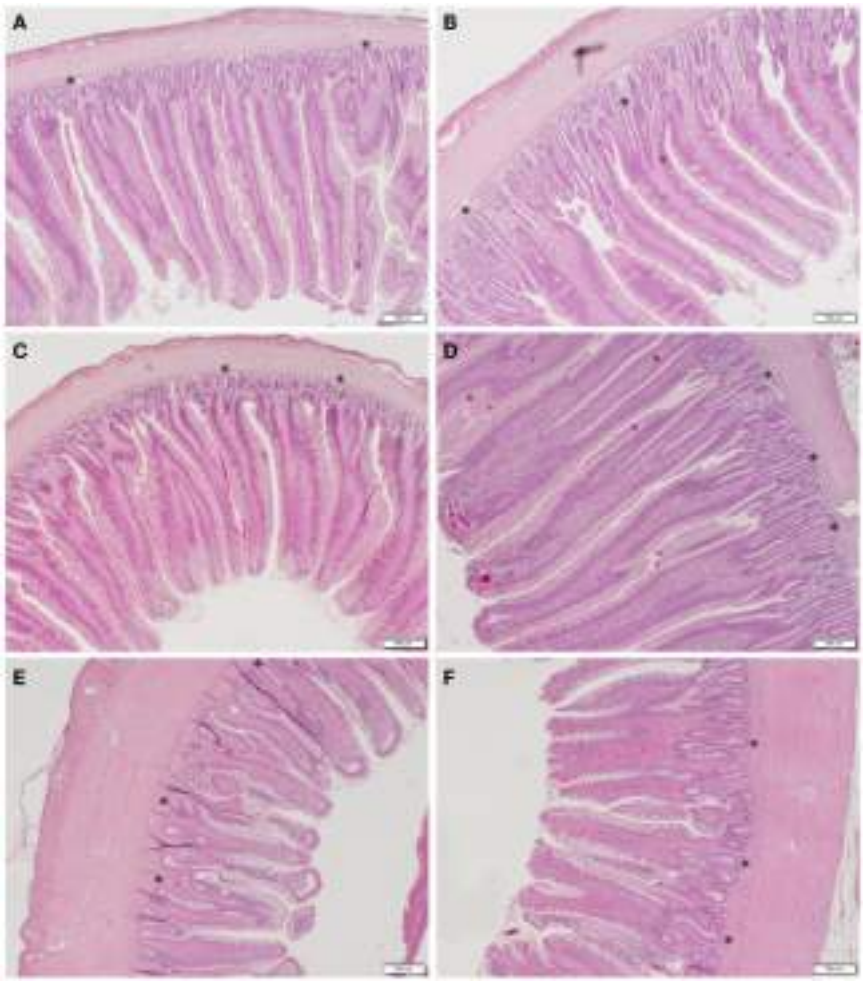

FIGURE 1 | Representative images of duodenum (A,B), jejunum (C,D), and ileum (E,F) in control (A,C,E) and gut barrier failure (B,D,F) groups of broilers chickens at 28 days of age. The representative crypts are shown as *.

TABLE 5 | Comparison of serum endotoxin and α1 acute phase protein (AGP) values between control and gut barrier failure groups in chickens at 28 days of age.

Treatment	Endotoxin pg/ml	α1 Acute phase protein (AGP) μg/ml
CON	159.03 ± 8.56^{b}	174.40 ± 28.95^{b}
GBF	331.84 ± 80.46^{a}	655.30 ± 6.38^{a}

Data are expressed as mean ± SE.
[a,b]Superscripts within columns indicate difference at $P < 0.05$.

($P = 0.005$), 24% ($P = 0.107$), and 29% ($P = 0.088$), respectively, in GBF birds compared to CON birds. The mRNA levels of catenin, claudin 1, ZO1, JAM2, JAM3, IL-4, IL-6, TLR4, TLR2β, and TNF-α were not different ($P > 0.1$) between CON and GBF chickens (data not shown).

Discussion

It is well known that poor gut health causes negative impacts in the health and growth performance of broiler chickens in poultry industry. Alternative grains, such as wheat, barley, and rye that are high in NSP, have been reported to cause a significant reduction in performance (38–40). Several mechanisms of the action of NSP on nutrient absorption have been described including an increased digesta viscosity due to reduced digestibility, thickening of the mucous layer on the intestinal mucosa, epithelial cell apoptosis, and inflammation caused by dysbacteriosis (10, 31, 39). Poultry have little or no intrinsic enzymes capable of hydrolyzing these NSP, so high concentrations of NSP in wheat, barley, or rye lead to reduced nutrient digestibility. The undigested feed ingredients in the gut provide nutrients for bacteria overgrowth in the hind gut, leading to dysbacteriosis. High NSP diets have also been associated with necrotic enteritis, a multifactorial disease caused by *Clostridium perfringens* that is probably the most important bacterial disease in terms of economic implications in broiler chickens (41). The nutritional and economic consequences of mounting an inflammatory response in poultry are inversely related to BWG and overall performance (42, 43). In the present study, a wheat–barley–rye diet in combination with a coccidia challenge was used to induce gut barrier failure in broiler chickens. The overall growth performance and feed efficiency

TABLE 6 | Relative mRNA levels of genes in jejunal mucosa between control and gut barrier failure groups of broilers chickens at 28 days of age.

Treatment	mRNA TGF-β4	mRNA IL-1β	mRNA IL-8	mRNA FABP2	mRNA FABP6	mRNA MUC2	mRNA occludin
CON	81.98 ± 4.55^{a}	25.6 ± 4.52^{a}	0.04 ± 0.01^{a}	26.72 ± 1.99^{b}	0.01 ± 0.001^{a}	123.30 ± 15.51^{b}	2.23 ± 0.26^{b}
GBF	182.03 ± 18.09^{b}	43.89 ± 6.65^{b}	0.13 ± 0.01^{b}	17.66 ± 1.89^{a}	0.07 ± 0.01^{b}	87.11 ± 12.16^{b}	1.69 ± 0.16^{b}
P-value	0.0001	0.040	<0.0001	0.0005	0.020	0.088	0.107

Data are normalized by actin mRNA and expressed as mean ± SE.
[a,b]Superscripts within columns indicate difference at $P < 0.05$.

were severely reduced by this GBF model. These results are in agreement with previous studies that high NSP diets compromised growth performance in chickens (4, 5, 44, 45).

The morphometry of duodenum, jejunum, and ileum in CON and GBF chickens at 28 days of age was measured under microscope to confirm whether the rye–barley–wheat diet and coccidia challenge generated gut barrier failure. GBF birds had longer crypt depth than CON birds in duodenum, jejunum, and ileum and also higher crypt/villi height ratio in duodenum than CON birds. Crypt depth and the ratio of crypt depth to villus height are measures of efficiency because the increase of crypt depth and/or crypt/villi ratio indicates greater need of cell proliferation to maintain gut barrier integrity (46–48). In addition to longer crypt depth, duodenum and jejunum of GBF birds also had wider villi. Narrow villi have greater nutrient absorption area. Widening of villus indicates less nutrient absorption area and probably also greater amount of gut-associated immune tissue proliferation and accumulation in the villus, which is another indication of compromised gut heath. The structural change in GBF birds confirmed that gut barrier failure was occurring in GBF birds, which may be associated with the poor performance in this study and is consistent with a previous study (49).

The gastrointestinal tract (GIT) is repeatedly challenged by foreign antigens and the intestinal mucosa must have the capability of fast restoration in the event of tissue damage (50). Impairment of this fragile barrier leads to enteritis and other inflammatory diseases (9). The intestinal mucosa contains different types of epithelial cells with specific functions. IEC control surface-associated bacterial populations without upsetting the microbiome that are vital for host health (51), and play an essential role in maintaining gut homeostasis and barrier function (52, 53). As a single-cell layer, IEC serve as a protective barrier against the external environment and maintain a defense against intraluminal toxins and antigens in addition to support nutrients and water transport (54). IEC are sealed together by adherens junctions (AJ) and TJ that are composed of cadherins, claudins, occludins, and JAM (29, 55–57). Upon injury, IEC undergo a wound healing process that is reliant on three cellular events: restitution, proliferation, and differentiation (27). Previous studies have shown that various regulatory peptides, including growth factors and cytokines, are capable of influencing the restoration of damaged IEC (58).

Gram-negative bacteria in the gut release endotoxin during growth, division, and death, and luminal endotoxin can translocate to circulation via two routes: (1) non-specific paracellular transport through TJ of epithelial cells, and (2) transcellular transport through lipid raft membrane domains and receptor-mediated endocytosis (2, 59). TLR4 is involved in the latter route (60). The lack of difference of TLR4 mRNA levels between CON and GBF birds suggests that endotoxin probably did not enter into circulation via transcellular transport. Pathogens, such as *Escherichia coli* or *C. perfringens*, as well as their elaborated toxins (e.g., endotoxin or entertoxin) have been reported to alter epithelial TJ and gut barrier function (23). Poor integrity of gut barrier or opening of TJ has been reported to facilitate paracellular transport of endotoxin, which will increase proinflammatory cytokine secretion and activate innate and adaptive immune response (61, 62). Secreted cytokines may enter the IEC through the basolateral side, resulting in further increased inflammation, disruption of TJ complexes, and increased paracellular endotoxin transport (63). Interestingly, there were detectable levels of endotoxin in CON chickens, which are actually not the background noise detected by ELISA kit. In this study, the CON chickens were much healthier than GBF chickens, the endotoxin in the serum of CON chickens could be non-specific paracellular diffusion of endotoxin from intestinal lumen into circulation. The increase of endotoxin levels in GBF birds indicates that gut barrier failure increased the transport of endotoxin from intestinal lumen into circulation, which could further negatively affect the integrity of TJ as evidenced by the decrease of occludin mRNA levels in GBF birds. Occludin, one of the major components of TJ, is involved in the regulation of inter-membrane diffusion and paracellular diffusion of small molecules (64). Occludin is down-regulated in patients with Crohn's Disease and ulcerative colitis, two common types of inflammatory bowel disease in humans (57, 64), suggesting the important role of occludin in intestinal health. However, no differences were detected between GBF and CON chickens in the expression of other TJ components, such as claudin 1, ZO1, JAM2, and JAM3. Claudin 1 is a member of multiple-span transmembrane protein called claudins, a protein family with more than 20 members, JAM2 and JAM3 are single-span transmembrane protein (51, 65, 66). ZO1 is a plaque protein that acts as adaptors to connect transmembrane proteins to the perijunctional actomyosin ring (23). These results indicate that GBF model impaired TJ integrity by reducing occludin expression, which facilitates the transport of endotoxin from intestinal lumen into blood for systemic circulation.

Endotoxin was also reported to increase satiety peptide secretion, which will reduce FI (20). The decreased growth performance in GBF birds could be partially associated with the increase of satiety peptide resulting from the elevated endotoxin levels, although satiety peptide was not measured in this study.

α1-Acid glycoprotein, an acute phase protein, has been used as a marker for systemic inflammation in poultry (67). Increase of AGP in GBF birds confirms that systemic inflammation was occurring in GBF birds, which led us to investigate the local inflammation status in the gut. Changes in the gut microbiota have

been reported to negatively affect gut barrier integrity, leading to increased leakage of endotoxin and fatty acids, which can act upon TLR4 to activate systemic inflammation (68). Activation of macrophages via TLR is important for inflammation and host defense against pathogens; however, recent studies suggest that non-pathogenic molecules are able to induce inflammation via TLR2 and TLR4 (16, 69–71). The capacity to detect tissue injury and to initiate adequate repair mechanisms is indispensable for the survival of all higher species. A common aspect of all types of injury – caused by infectious, physical, chemical, or immune processes – is a compositional change of the cellular environment leading to the presence of novel molecular patterns. These patterns are recognized by a group of receptors termed pattern recognition receptors (PRR) and trigger specific responses that promote the restoration of tissue function, including inflammation and wound healing (20, 72). Pathogen recognition is critical to survive in an essentially hostile environment that is full of potentially infective microorganisms. Detection systems for molecular patterns characteristic for pathogens (pathogen-associated molecular patterns, PAMP) develop early in evolution, and are present in most species including plants and invertebrates (69). As a group of highly conserved PRR, TLR signals the presence of various PAMP to cellular constituents of the innate and adaptive immune (69, 73), therefore acting as gatekeepers for several highly efficient response systems that regulate tissue homeostasis and protect the host after acute injury (60, 74). Upon injury, the intestinal epithelium undergoes a wound healing process (69). Recent studies have revealed the activation of TLR by the microbiota during the healing process (20). In addition, several cytokines, such as TGF-α, TGF-β, IL-1β, and IL-2, are also increased during healing process (16, 75). In this study, the mRNA levels of TGF-β and IL-1β in GBF chickens were increased, but TLR4, TLR2β, TGF-α, IL-4, and IL-6 mRNA levels were not different compared to CON chickens. These results suggest that the inflammation occurred in GBF birds in this study is likely not mediated by TLR2 or TLR4 pathway. However, TLR3 mRNA and protein levels of TLR2, TLR4, and TLR3 were not measured in this study. Therefore, we are not able to exclude the possibility that TLR pathway is involved in the inflammation in GBF birds. IL-1β is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (76). TGF-β, a key mediator of mucosal immune homeostasis, mediates IgA production, retains lymphocytes in the gut and promotes wound healing of intestinal epithelium and mucosa (75). TGF-β also promotes IEC proliferation through the activation of extracellular signal-regulated kinase (ERK) 1/ERK2 mitogen-activated protein kinase during wound healing (20). IL-8 is secreted basolaterally by intestinal epithelium in response to pathogenic bacteria or specific inflammory cytokines, and triggers neutrophil migration and inflammation in intestine (73). The increase of systemic AGP, and mucosal TGF-β, IL-8, and IL-1β in GBF birds indicate that GBF model increased intestinal inflammation and activated intestinal innate immune response and wound healing.

Mucins are large glycoproteins that cover epithelial surfaces of the intestine and form a mucus layer to protect epithelial cells from gut health challenge. There are two major types of mucins, membrane-bound and secreted (77, 78). In chickens (*Gallus gallus*), three transmembrane mucins (MUC4, MUC13, and MUC16) and four gel-forming mucins (MUC6, MUC2, MUC5ac, and MUC5b) have been identified (79). In mammals, MUC2, the mucin secreted by goblet cells, is the most abundant mucin in the intestine, and its deficiency has been reported to increase bacterial translocation and inflammation (18, 80). Evolutionary studies suggest that mucins share a common ancestor, since their domain structures are well conserved in metazoans (71, 81). All mucins (MUC) contain at least one PTS domain, a region rich in proline, threonine, and serine (18, 82). Chicken MUC2 has been reported to be remarkably similar to human and mouse outside of the central PTS domain, but is highly divergent within this central repetitive structure (82, 83). Although the physiological implications and disease associations of MUC on various mucosal surfaces are well understood, there are still many questions as to how and why the gene architecture of this family contributes to diverse protein modifications that show diverse biological effects between metazoans in health and disease (18, 84–87). MUC2 gene expression has been used as a marker for gut health in poultry and other species (85, 88, 89). For example, Li et al. found that zinc supplementation in breeder diets improved morphometry, increased the number of goblet cell per villus, and MUC2 gene expression, and reduced mRNA levels of proinflammatory cytokines, such as IL-6 and IL-1β in the jejunum of their offspring (89). In the present study, MUC2 gene expression was reduced by 29% in GBF birds compared to CON birds, suggesting that GBF model reduced mucus layer protection in jejunum.

Intracellular lipid chaperones known as FABP are a group of molecules that coordinate lipid response and metabolism in cells (90). FABP are found across species, from *Drosophila melanogaster* and *Caenorhabditis elegans* to mice and humans, demonstrating strong evolutionary conservation (90). FABP-mediated lipid metabolism is closely linked to both metabolic and inflammatory processes through modulating critical lipid-sensitive pathways in target cells, especially adipocytes and macrophages (90, 91). Nine FABP have been identified so far in intestine, liver, brain, adipose, and muscle, the organs that show high rates of lipid metabolism, in vertebrates (92, 93). Intestinal FABP, FABP2, and FABP6, are expressed at high levels in the small intestine and ileum, respectively, and in addition to mediate lipid metabolism, they are also involved in intestinal inflammatory conditions by modulating critical lipid-sensitive pathways in adipocytes and macrophages in human (94, 95). FABP2 is down-regulated in patients with ischemia/reperfusion-induced intestinal barrier injury (93), suggesting the important role of FABP2 in gut barrier health. FABP2 has been identified as a specific marker for the relative amount of epithelium in humans and pigs (96). Several FABP (FABP1, FABP2, FABP6, and FABP10) have been identified to be predominantly expressed in the digestive tract of chickens (97, 98); however, much remains to be determined regarding their expression and biological functions in poultry. FABP10 plays an important hepatic role in in response to FI in chicken (98). FABP2 is involved in lipogenesis and fatty acids transport, and plays an important role in abdominal fat content in broiler chickens (98–100). In the present study, GBF model reduced FABP2 gene expression, suggesting that, like the role of FABP2 in human intestinal barrier health, FABP2 can be used as a marker of gut barrier function

in chicken. Reduction of FABP2 expression indicates the loss of epithelial cell content and occurrence of intestinal barrier failure in GBF birds.

The ileal lipid binding protein (ILBP; human gene FABP6) was recently shown to be needed for the efficient transport of bile acids from the apical side to the basolateral side of enterocytes in the distal portion of murine intestine (101). Bile acids are synthesized by the liver and released into the lumen of the small intestine via bile, and the majority of bile acids are recovered in the distal end of the small intestine and then returned to the liver for reuse (102). Bile acid has emerged as important biological molecules that emulsify lipids and liposoluble dietary nutrients to facilitate their digestion and absorption (103, 104). It has strong anti-microbial activity and therefore is emerging as a host factor that regulates the composition of microbiota in the gut (105, 106). Reduced bile acid levels in the gut are reported to be associated with bacterial overgrowth and inflammation (106). Gut inflammation in GBF birds may have resulted in lower levels of bile acids, which unfortunately were not measured in this study. The substantial increase of FABP6 by four fold in GBF birds indicates high demand of bile acids as an anti-microbial to promote the recovery of dysbacteriosis and barrier failure in the gut of GBF birds.

In conclusion, the purpose of this study was not to determine the individual effects of diet ingredients or coccidia challenge but rather to determine the potential biomarkers that may be used to define gut barrier failure in future studies. We attempted to exacerbate gut barrier failure with the tools available for us, and the results obtained in the present study suggest that the combination of high NSP diet and a coccidia challenge induced gut barrier failure and inflammation in broilers characterized by the increase of endotoxin and AGP in serum, as well as increase of IL-8, IL-1β, TGF-β4, and FABP6 mRNA, and reduction of FABP2, MUC2, and occludin mRNA in jejunal mucosa of GBF birds compared to CON birds. These parameters may be utilized as potential biomarkers for gut barrier health in chickens. Now that we have a better understanding of what biomarkers are relevant in gut barrier failure models in chickens, further studies will be conducted to evaluate the effects of chicken enteropathogens, different dietary ingredients or feed additives, such as probiotics and prebiotics, on gut barrier function in broiler chickens.

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Selection of *Bacillus* spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and *Clostridium perfringens* proliferation using an *in vitro* digestive model in different poultry diets

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Previously, our laboratory has screened and identified *Bacillus* spp. isolates as direct-fed microbials (DFM). The purpose of the present study was to evaluate the cellulase and xylanase production of these isolates and select the most appropriate *Bacillus* spp. candidates for DFM. Furthermore, an *in vitro* digestive model, simulating different compartments of the gastrointestinal tract, was used to determine the effect of these selected candidates on digesta viscosity and *Clostridium perfringens* proliferation in different poultry diets. Production of cellulase and xylanase were based on their relative enzyme activity. Analysis of 16S rRNA sequence classified two strains as *Bacillus amyloliquefaciens* and one of the strains as *Bacillus subtilis*. The DFM was included at a concentration of 10^8 spores/g of feed in five different sterile soybean-based diets containing corn, wheat, rye, barley, or oat. After digestion time, supernatants from different diets were collected to measure viscosity, and *C. perfringens* proliferation. Additionally, from each *in vitro* simulated compartment, samples were taken to enumerate viable *Bacillus* spores using a plate count method after heat-treatment. Significant ($P < 0.05$) DFM-associated reductions in supernatant viscosity and *C. perfringens* proliferation were observed for all non-corn diets. These results suggest that antinutritional factors, such as non-starch polysaccharides from different cereals, can enhance viscosity and *C. perfringens* growth. Remarkably, dietary inclusion of the DFM that produce cellulase and xylanase reduced both viscosity and *C. perfringens* proliferation compared with control diets. Regardless of diet composition, 90% of the DFM spores germinated during the first 30 min in the crop compartment of the digestion model, followed by a noteworthy increased in the intestine compartment by ~2$\log_{10}$, suggesting a full-life cycle development. Further studies to evaluate *in vivo* necrotic enteritis effects are in progress.

Keywords: *Clostridium perfringens*, *Bacillus*-DFM, spore, enzymes, viscosity

Introduction

Necrotic enteritis (NE) in broilers is a multi-factorial disease with severe economic implications (1). It is caused by type A strains of *Clostridium perfringens* that are specific to poultry with toxin types alpha and NetB (2, 3). Coccidia infections are the most common pre-requisite for NE to occur (4), however, dysbacteriosis associated with diet ingredients, changes in feed ration, immunosuppression, *Salmonella* infections, and/or removal of the use of quimioterapeutics are known to predispose birds to NE (1–5). Antibiotic growth promoters (AGPs) are commonly used to mitigate the incidence of enteric diseases, such as NE. Nevertheless, concerns regarding the development of antibiotic-resistant microorganisms and social pressures have led to a tendency to ban AGPs in poultry production (6). In this scenario, there is an imperative necessity to find feasible alternatives for AGPs to maintain poultry health (7). In fact, the use of selected strains of various beneficial microorganisms from the genus *Bacillus* and *Lactobacillus* have shown to be a suitable option for the poultry industry (8). *Bacillus* spp. are Gram-positive, aerobe, motile, and usually found in soil and water sources, as well as in the gastrointestinal tract of animals and humans (9). Different *Bacillus* spp. have already been studied and extensively used as a source of industrial enzymes as well as antibiotics by biotechnology companies (10). However, the production of most of these enzymes depends on the intense metabolic changes associated with environmental conditions (11–13). During extreme environmental conditions, vegetative cells of *Bacillus* spp. form endospores, which are considered, the toughest way of life on Earth (14).

The use of spores from selected *Bacillus* strains, as direct-fed microbials (DFM), are shown to have the capacity to germinate and sporulate in the gastrointestinal tract of different animal species including poultry. Thus, they become metabolically active *in vivo*, imparting numerous nutritional benefits including the production of extracellular enzymes, such as protease, lipase, cellulase, xylanase, phytase, and keratinase (15, 16), and other chemical compounds beneficial for the host (17).

In most of the USA and other countries, including Brazil, broiler feed is based primarily on corn and soybean meal. However, sometimes it is difficult to formulate least-cost diets using corn. Consequently, other cereals or ethanol by-products with variable concentrations of antinutritional factors are used as alternatives. When chickens are fed alternative grains with high levels of non-starch polysaccharides (NSP), an increase in digesta viscosity, poor nutrient digestibility, reduced bone mineralization, and occurrence of enteric diseases, such as NE, have been reported (18, 19). Hence, utilization of these feedstuffs in poultry diets usually result in decreased growth performance, intestinal dysbacteriosis, and detrimental litter conditions caused by sticky droppings (20, 21). For that reason, the inclusion of enzymes, such as carbohydrases, is a routine practice in poultry diets that contain grains with elevated NSP concentration values in comparison to corn (22, 23). However, there are inconveniences related to dietary inclusion of some enzymes, due to denaturation and lost of activity under high-pelletization temperatures commonly used in poultry rations. Therefore, the objective of the present study was to perform a selection of *Bacillus* spp. for cellulase and xylanase production as DFM, and evaluate them on digesta viscosity and *C. perfringens* proliferation in different poultry diets using an *in vitro* digestive model. The practical implication of the results will be to utilize cost-effective alternative grains in poultry feed formulation, and at the same time improve digestibility as well as production performance in birds using a more thermostable DFM product.

Materials and Methods

Diets

Five mash soybean-based broiler grower diets containing different cereals, such as corn, wheat, rye, barley, or oat, were used as substrate for bacterial growth during the *in vitro* digestive model. Experimental diets were formulated to approximate the nutritional requirements of broiler chickens as recommended by the NRC (24), and adjusted to breeder's recommendations (25). No antibiotics, coccidiostats, or enzymes were added to the feed (**Table 1**). All diets were autoclaved and confirmed negative for *Bacillus* spp. spores. Later, these diets were inoculated with the respective spores (10^8 spores/g of feed) of the *Bacillus*-DFM candidate according to various treatments.

In Vitro Assessment of Cellulase and Xylanase Production

Previous research conducted in our laboratory focused on isolation of several *Bacillus* spp. from environmental and poultry sources (26, 27). Isolates were then screened for production of cellulase and xylanase. For evaluation of cellulase activity, the cellulose-Congo red agar was used and consisted of 0.50 g of K_2HPO_4 (Fisher Scientific, San Francisco, CA, USA), 0.25 g of $MgSO_4$ (Sigma Chemical Co, St. Louis, MO, USA), 1.88 g of ashed, acid-washed cellulose powder (J. T. Baker Chemical Inc, Phillipsburg, NJ, USA), 0.20 g of Congo red (J. T. Baker Chemical Inc, Phillipsburg, NJ, USA), 20 g of noble agar (Difco Laboratories, Detroit, MI, USA), and 1000 mL of distilled water (15). For evaluation of xylanase activity, the medium used to screen *Bacillus* isolates contained 3 g of $NaNO_3$, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.02 g of $MnSO_4{\cdot}H_2O$, 0.02 g of $FeSO_4{\cdot}H_2O$, 0.02 g of $CaCl_2{\cdot}2H_2O$ with 20 g of noble agar (Difco Laboratories, Detroit, MI, USA), and 1000 mL of distilled water. Besides, 1 g yeast extract and 5 g beechwood xylan (Sigma Chemical Co, St. Louis, MO, USA) were used as carbon sources (28). During the screening process, 10 µL of each *Bacillus* isolate were placed on the center of each plate containing cellulose or xylan media. After 24 h of incubation at 37°C, all plates were evaluated and the diameters of the zones of clearance were measured removing the diameter of the bacterial colony. The relative enzyme activity (REA) was calculated by using the formula: REA = diameter of zone of clearance divided by the diameter of the bacterial colony in millimeters. Based on REA test in each group, organisms were categorized in to excellent (REA $>$ 5.0), good (REA $>$ 2.0 to 5.0), or poor (REA $<$ 2.0) REA (29). Each *Bacillus* strain was evaluated by triplicate, and the average measurements are presented in **Table 2**.

DFM Culture Identification

Based on the REA results, three *Bacillus*-DFM candidates with excellent to good REA were selected. These candidates were then

TABLE 1 | Ingredient composition and nutrient content of different broiler chicken diets used for *in vitro* digestion with or without inclusion of *Bacillus*-DFM candidate spore on as-is basis[a].

Item	Corn-based diet	Wheat-based diet	Barley-based diet	Rye-based diet	Oat-based diet
INGREDIENTS (g/kg)					
Corn (80 g/kg CP)	619.6	–	–	–	–
Wheat (135 g/kg CP)	–	711.0	–	–	–
Barley (113 g/kg CP)	–	–	654.3	–	–
Rye (126 g/kg CP)	–	–	–	622.6	–
Oats (98 g/kg CP)	–	–	–	–	638.0
Soybean meal (475 g/kg CP)	298.2	203.9	241.9	264.6	260.0
Poultry oil	39.1	42.8	65.0	70.0	70.0
Dicalcium phosphate	16.9	17.1	17.0	16.6	16.4
Calcium carbonate	10.6	8.5	8.2	10.4	10.0
Salt	3.8	3.0	3.0	5.7	2.0
DL-Methionine	3.3	2.5	3.0	3.0	3.2
L-Lysine HCL	2.8	4.6	2.0	2.0	1.6
Threonine	1.2	2.1	1.1	0.6	0.6
Choline chloride 60%	2.0	2.0	2.0	2.0	2.0
Vitamin premix[b]	1.0	1.0	1.0	1.0	1.0
Mineral premix[c]	1.0	1.0	1.0	1.0	1.0
Antioxidant[d]	0.5	0.5	0.5	0.5	0.5
CALCULATED ANALYSIS					
Metabolizable energy (MJ/kg)	13.0	13.0	12.3	12.2	11.9
Crude protein (g/kg)	195.0	200.0	190.0	205.0	186.4

[a] *Inclusion of 10^8 spore/g of feed mixed with calcium carbonate.*
[b] *Vitamin premix supplied per kilogram of diet: retinol, 6 mg; cholecalciferol, 150 μg; DL-α-tocopherol, 67.5 mg; menadione, 9 mg; thiamine, 3 mg; riboflavin, 12 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.3 mg; cyanocobalamin, 0.4 mg.*
[c] *Mineral premix supplied per kg of diet: Mn, 120 mg; Zn, 100 mg; Fe, 120 mg; copper, 10–15 mg; iodine, 0.7 mg; selenium, 0.2 mg; and cobalt, 0.2 mg.*
[d] *Ethoxyquin.*

TABLE 2 | Relative enzyme activity values (REA) and *Clostridium perfringens* zone of inhibition produced by different *Bacillus* spp. strains present in the *Bacillus*-DFM candidate treatment.

Measurements	AM1002	AM0938	JD17
CELLULASE ACTIVITY AT 24 h			
Colony size (mm)	5.7 ± 0.33^{a}	6.0 ± 0.58^{a}	6.3 ± 0.33^{a}
Zone of clearance (mm)	35.2 ± 1.76^{a}	$30.7 \pm 0.67^{a,b}$	29.3 ± 2.19^{b}
REA[c]	6.2 ± 0.12^{a}	$5.1 \pm 0.49^{a,b}$	4.7 ± 0.29^{b}
XYLANASE ACTIVITY AT 24 h			
Colony size (mm)	5.0 ± 0.58^{b}	$6.7 \pm 0.33^{a,b}$	7.3 ± 0.67^{a}
Zone of clearance (mm)	31.7 ± 0.88^{a}	32.0 ± 1.15^{a}	29.0 ± 1.53^{a}
REA[c]	6.3 ± 0.87^{a}	$4.9 \pm 0.43^{a,b}$	4.0 ± 0.15^{b}
C. perfringens *AT 24 h*			
Zone of inhibition (mm)[d]	12.3 ± 1.45^{a}	14.0 ± 1.00^{a}	8.0 ± 1.15^{b}

[a,b] *Superscripts within a row with no common superscript differ significantly $P < 0.05$.*
[c] *Relative enzyme activity values (REA) reflect the capacity to produce cellulase and xylanase enzymes by Bacillus spp. REA was calculated dividing the diameter of area of clearance by the diameter of the Bacillus colony. Based on REA test, organism can be categorized into three groups showing excellent (REA > 5.0), good (REA > 2.0–5.0), or poor (REA < 2.0) relative enzyme activity. All Bacillus spp. isolates were tested by triplicate. Data expressed as mean ± SE.*
[d] *Represents the diameter of the zone of inhibition observed at 24 h of incubation without the diameter of the bacterial colony. All Bacillus spp. isolates were tested by triplicate. Data expressed as mean ± SE.*

identified and characterized using a bioMerieux API 50 CHB test kit (bioMerieux, Marcy l'Etoile, FRA). Individual strain was also subjected to 16S rRNA sequence analysis to a specialized laboratory (Midi labs, Newark, DE, USA). Generally recognized as safe (GRAS) status of these three isolates was affirmed, as described by Wolfenden et al. (30). One of the three *Bacillus* strains (AM1002) was identified as *Bacillus subtilis*, and the other two isolates (AM0938 and JD17) were identified as *B. amyloliquefaciens* (**Table 3**). Following the identification, all three *Bacillus* candidate strains were sporulated and mixed in equal amounts during the *Bacillus*-DFM preparation process as described below and incorporated to the experimental diets.

Preparation of Spore-Based DFM

In an effort to grow high numbers of viable spores, modified version of a solid state fermentation media (SS) developed by Zhao et al. (31) was used. Briefly, to prepare the SS fermentation media, ammonia broth was added to a mixture of 70% rice straw and 30% wheat bran at the rate of 40% by weight. Then, the SS fermentation media was added to 250 mL Erlenmeyer flasks and sterilized by autoclaving for 30 min at 121°C. Each of the three *Bacillus* strains candidates was grown, individually, overnight at 37°C in test tubes containing 10 mL of tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA). After incubation, 2 mL of each candidate culture were added separately to the previously prepared SS fermentation media flasks. The inoculated flasks were incubated for 24 h at 37°C to promote growth of the *Bacillus* spp. candidates, and incubated for another 72 h at 30°C to trigger the initiation of the sporulation process. Following this, the inoculated SS fermentation media was removed from the Erlenmeyer flasks, placed onto Petri dishes, and dried at 60°C for 18 h. Then, the SS fermentation media was aseptically ground into a fine powder that contained stable *Bacillus* spores (~10^{11} spores/g). One gram of spores from each isolate (1:1:1) was combined to produce the *Bacillus*-DFM candidate final product containing ~3×10^{11} spores/g. *Bacillus*-DFM candidate was included into each experimental diet to reach a final concentration of 10^8 spores/g using a rotary mixer for 15 min.

TABLE 3 | Identification of *Bacillus* spp. isolates by bioMerieux API 50 CHB[a] and 16S rRNA sequence analyses[b] present in the *Bacillus*-DFM candidate treatment.

Isolate	API50 CHB		16S rRNA sequence analysis	
	Taxon	% ID	Closest match	% ID
AM1002	*Bacillus subtilis/amyloliquefaciens*	99.2	*Bacillus subtilis*	100.0
AM0938	*Bacillus subtilis/amyloliquefaciens*	99.0	*Bacillus amyloliquefaciens*	99.7
JD17	*Bacillus subtilis/amyloliquefaciens*	99.4	*Bacillus amyloliquefaciens*	99.6

[a] *BioMerieux API 50 CHB test kit.*
[b] *16S rRNA sequence analysis.*

Samples of feed containing the DFM candidate were subjected to 100°C for 10 min to eliminate vegetative cells and validate the amount of spores per gram of feed after inclusion and mixing steps. Following heat-treatment, 10-fold dilutions of the same feed samples from the glass tubes were plated on tryptic soy agar plates (TSA, Becton Dickinson, Sparks, MD, USA); letting spores in the feed sample germinate to vegetative cells after incubation at 37°C for 24 h, hence representing the number of spores present per gram of feed.

Clostridium perfringens Strain

A strain of *C. perfringens* previously described in a NE challenge model was kindly donated by Dr. Jack. L. McReynolds, USDA-ARS, College Station, TX, USA (32). A frozen aliquot was shipped on ice to our laboratory and was amplified in TSB with sodium thioglycolate (Sigma-Aldrich, St Louis, MO, USA). The broth culture was plated on phenylethyl alcohol agar plates (PEA, Becton Dickinson, Sparks, MD, USA) with 5% sheep blood (Remel, Lenexa, KS, USA) to confirm purity, aliquots were made with 25% sterile glycerol and stored at −80°C until further use. A single aliquot was individually amplified in TSB with sodium thioglycolate overnight for the *in vitro* proliferation studies and the final dose was confirmed by plating 10-fold dilutions on TSA plates with sodium thioglycolate.

In Vitro Assessment of Antimicrobial Activity Against *Clostridium perfringens*

The three *Bacillus* isolates present in the *Bacillus*-DFM candidate treatment were individually cultured aerobically overnight on TSA and screened for *in vitro* antimicrobial activity against *C. perfringens* as reported previously (33). Briefly, 10 µL of each *Bacillus* isolate were placed on the center of TSA plates, and incubated for 24 h at 37°C. Then, the plates with visible *Bacillus* colonies were overlaid with TSA with sodium thioglycolate containing 10^6 cfu/mL of *C. perfringens*, and all plates were incubated anaerobically at 37°C. After 24 h of incubation, all plates were evaluated and the diameters of the zones of inhibition were measured removing the diameter of the bacterial colony. Each *Bacillus* strain was evaluated by triplicate, and the average measurements of antimicrobial activity against *C. perfringens* are presented in **Table 2**.

In Vitro Digestion Assay

The *in vitro* digestion model used in the present study was based on previous publications, with minor modifications (34, 35), and the assay was performed with five different experimental diets, with or without *Bacillus*-DFM candidate, in quintuplicates. Briefly, for all the gastrointestinal compartments simulated during the *in vitro* digestion model, a biochemical oxygen demand incubator (VWR, Houston, TX, USA) set at 40°C (to simulate poultry body temperature), customized with an standard orbital shaker (19 rpm; VWR, Houston, TX, USA) was used for mixing the feed content. Additionally, all tube samples were held at an angle of 30° inclination to facilitate proper blending of feed particles and the enzyme solutions in the tube. The first gastrointestinal compartment simulated was the crop, where 5 g of feed and 10 ml of 0.03M hydrochloric acid (HCL, EMD Millipore Corporation, Billerica, MA, USA) were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously reaching a pH value around 5.2. Tubes were then incubated for 30 min. Following this time, all tubes were removed from the incubator. To simulate the proventriculus as the next gastrointestinal compartment, 3000 U of pepsin per gram of feed (Sigma-Aldrich, St Louis, MO, USA) and 2.5 mL of 1.5M HCl were added to each tube to reach a pH of 1.4–2.0. All tubes were incubated for additional 45 min. The third and the final steps were intended to simulate the intestinal section of the gastrointestinal tract. For that, 6.84 mg of 8× pancreatin (Sigma-Aldrich, St Louis, MO, USA) in 6.5 mL of 1.0M sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA) were added, and the pH was adjusted to range between 6.4 and 6.8 with 1.0M sodium bicarbonate. All tube samples were further incubated for 2 h. Hence, the complete *in vitro* digestion process took 3 h and 15 min. After the digestion, supernatants from all the diets were obtained by centrifugation for 30 min at 2000 × *g*. All supernatants were then tested for viscosity and *C. perfringens* proliferation, as described below.

Viscosity

Viscosity was measured using a Brookfield digital cone-plate viscometer fitted with a CP-40 spindle (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA). From each supernatant, 0.5 mL was taken to measure viscosity at a shear rate of 42.5/s at 40°C to mimic body temperature of poultry. Viscosity was evaluated by quintuplicate per diet with or without inclusion of the *Bacillus*-DFM candidate and reported in centipoise (cP = 1/100 dyne s/cm^2).

Clostridium perfringens Proliferation

Proliferation of *C. perfringens* was performed according to previously published methods (35), with minor modifications. A suspension of 10^5 cfu/mL of *C. perfringens* was added to five replicates of each of the following groups: (1) 6 mL TSB with sodium thioglycolate as a positive control group; (2) 3 mL TSB with

sodium thioglycolate plus 3 mL supernatant from each digested control non-DFM diet; (3) 3 mL TSB with sodium thioglycolate plus 3 mL supernatant from digested diets supplemented with *Bacillus*-DFM. Samples were incubated anaerobically at 40°C, with tubes set at 30° angle with constant shaking (200 rpm) for 4 h. After incubation, 10-fold serial dilutions were made from all treatment groups in 0.85% sterile saline. Then, 10 µL was plated on TSA with sodium thioglycolate and incubated for 24 h at 40°C, anaerobically. Results were expressed as $\log_{10}$ cfu of *C. perfringens*/mL.

In Vitro Determination of Spore Persistence

Persistence of the *Bacillus*-DFM spores in the *in vitro* digestive model was also evaluated (five replicates per diet treatment). At each time point during the digestive simulation process (crop, proventriculus, and intestine), 0.2 mL was immediately loaded into 0.5 mL sterile centrifuge tubes and heat-treated (pasteurized) at 75°C for 10 min to eliminate the presence of vegetative cells (36). After pasteurization, samples were loaded into sterile 96-well flat bottom plate and 10-fold dilutions were made and plated on TSA. Plates were incubated for 24 h at 37°C on aerobic conditions to enumerate spores per gram of sample.

Statistical Analysis

Data from all measurements were subjected to One-way analysis of variance as a completely randomized design using the General Linear Models procedure of SAS (SAS version 9.1) (37). Means were separated with Duncan's multiple-range test at $P < 0.05$ considered as significant. Data were reported as mean ± SE.

Results

Isolates AM1002, AM0938, and JD17 were selected from a pooled of *Bacillus* isolates in our laboratory, based on the REA values for cellulose and xylanase, and the zone of inhibition for *C. perfringens* (**Table 2**). Isolate AM1002 showed a REA value of 6.2 and AM0938 showed a REA value of 5.1, both considered excellent REA values (>5.0) for cellulase activity (29); additionally, isolate JD17 showed a REA value of 4.7, which is considered good (>2.0–5.0) for cellulase production. A similar trend was observed for xylanase activity where isolate AM1002 showed a REA value of 6.3 (excellent); AM0938 showed a REA value of 4.8 (good), and isolate JD17 showed a REA value of 4.0 (good) for xylanase production. In the case of antimicrobial activity against *C. perfringens*, isolate AM0938 generated the largest diameter of the zone of inhibition with 14 mm, followed by isolates AM1002 and JD17 with 12 and 8 mm, respectively. Although enzyme production and antimicrobial activity were observed for all the isolates, individual differences were evident even in bacteria of the same species (**Table 2**). The API 50 CHB system characterized all three isolates as *B. subtilis/amyloliquefaciens* (**Table 3**). Analysis of 16S rRNA sequence classified two strains (AM0938, JD17) as *B. amyloliquefaciens* and one of the strains (AM1002) as *B. subtilis*, which was consistent with the results observed by the carbohydrate fermentation profile of the biochemical test.

The results of the evaluation of digesta viscosity of different diets with or without inclusion of a *Bacillus*-DFM candidate after *in vitro* digestion are summarized in **Table 4**. An evident increase in viscosity was observed in soybean-based diets containing wheat, barley, rye, and oats when compared to corn, being rye, and oat diets with the highest viscosity values. However, it was noteworthy to observe that dietary inclusion of the *Bacillus*-DFM candidate significantly ($P < 0.05$) reduced viscosity in all diets containing cereals different to corn in comparison to control diets without DFM inclusion (**Table 4**).

Table 5 summarizes the results of the proliferation of *C. perfringens* in the supernatant from different digested diets with or without inclusion of a *Bacillus*-DFM candidate. A significant increase in *C. perfringens* proliferation was observed in supernatants collected from control diets that contained wheat, barley, rye, and oat compared to the TSB positive control group. Startlingly, dietary inclusion of a *Bacillus*-DFM candidate in non-corn diets significantly reduced *C. perfringens* proliferation when compared to the control non-DFM supplemented diets. The corn-based diet showed similar cfu values of *C. perfringens* with or without inclusion of the *Bacillus*-DFM candidate.

Persistence of the *Bacillus*-DFM candidate spores in the different gastrointestinal compartments simulated in the *in vitro* digestive model is presented in **Table 6**. Regardless of diet composition, on average, a reduction of more than half of a $\log_{10}$ was observed in the crop compartment during the first 30 min of incubation, and it was followed by a further significant ~2$\log_{10}$ reduction of spore counts in the proventriculus. Remarkably, in all diets, a significant increment in spore numbers, ~2$\log_{10}$ was observed during the final digestion step simulating intestinal conditions (**Table 6**).

TABLE 4 | Evaluation of *in vitro* viscosity of different diets with or without inclusion of a *Bacillus*-DFM candidate.

Diet	Viscosity (cP)[c]	
	Control	*Bacillus*-DFM
Corn-based	0.96 ± 0.01^{a}	0.97 ± 0.01^{a}
Wheat-based	1.55 ± 0.02^{a}	1.28 ± 0.01^{b}
Barley-based	1.75 ± 0.02^{a}	1.34 ± 0.03^{b}
Rye-based	8.40 ± 0.37^{a}	2.39 ± 0.04^{b}
Oat-based	36.9 ± 2.15^{a}	1.34 ± 0.01^{b}

[a,b] *Superscripts within a row with no common superscript differ significantly $P < 0.05$.*
[c] *Viscosity was measured after 3 h and 15 min of in vitro digestion at 40°C. Data expressed as mean ± SE.*

TABLE 5 | Proliferation of *Clostridium perfringens*[d] in different digested diets with or without inclusion of *Bacillus*-DFM candidate spore[e].

Diet[c]	TSB + Thio	Control diet	*Bacillus*-DFM
Corn-based	6.38 ± 0.13^{a}	6.44 ± 0.19^{a}	6.68 ± 0.08^{a}
Wheat-based	6.12 ± 0.24^{b}	7.12 ± 0.07^{a}	5.20 ± 0.18^{b}
Barley-based	6.36 ± 0.06^{c}	7.50 ± 0.13^{a}	6.86 ± 0.11^{b}
Rye-based	6.05 ± 0.21^{c}	7.15 ± 0.09^{a}	6.68 ± 0.12^{b}
Oat-based	6.12 ± 0.07^{b}	6.96 ± 0.13^{a}	5.76 ± 0.07^{c}

[a,b] *Superscripts within a row with no common superscript differ significantly $P < 0.05$.*
[c] *Supernatant from each diet was used as part of the broth for C. perfringens growth. Data expressed as mean ± SE.*
[d] *Inoculum used 10^5 cfu of C. perfringens and 10^8 spores/g of Bacillus-DFM candidate.*
[e] *Data expressed in $\log_{10}$ cfu/mL.*

TABLE 6 | Persistence of *Bacillus*-DFM candidate[c] spore during *in vitro* digestion[f] in different diets under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry[e].

Diet[d]	Crop (30 min)	Proventriculus (45 min)	Intestine (120 min)
Corn-based	7.32 ± 0.10^{a}	5.43 ± 0.17^{b}	7.20 ± 0.09^{a}
Wheat-based	7.54 ± 0.06^{a}	5.58 ± 0.10^{b}	7.33 ± 0.19^{a}
Barley-based	7.45 ± 0.16^{a}	4.95 ± 0.21^{b}	7.27 ± 0.08^{a}
Rye-based	7.28 ± 0.10^{a}	5.60 ± 0.22^{b}	7.09 ± 0.17^{a}
Oat-based	7.66 ± 0.07^{a}	5.06 ± 0.15^{b}	7.30 ± 0.15^{a}

[a,b] *Superscripts within a row with no common superscript differ significantly $P < 0.05$.*
[c] *Inclusion of 10^8 spore/g of feed*
[d] *Heat shock was induced by placing a sample of each simulated compartment in a water bath at 75°C for 10 min. Data expressed as mean ± SE.*
[e] *Data expressed in $\log_{10}$ cfu/mL.*
[f] *pH and time of incubation varied according to the simulated organ.*

Discussion

High-energy diets have been utilized to maximize growth during starter, grower, and finisher phases of production. Consequently, the primary dietary energy sources in commercial broiler diets have been traditional cereal grains such as corn and sorghum. However, with the recent price volatility of common feed ingredients, the animal industry seeks alternative grains or industry by-products to include in diet formulations (38, 39). Wheat, barley, rye, and oat contain lower bioavailable energy, and elevated NSP levels in comparison to corn (40) are alternative options. However, these cereals have limited use in monogastric diets, because often high-inclusion results in relatively poor performance, detrimental litter conditions, and increase predisposition for NE (41–43). Hence, supplemental carbohydrases, such as NSP-degrading enzymes, have allowed to increase the utilization of these alternative ingredients by reducing their antinutritional effects (40, 44, 45). The carbohydrase market is accounted by two dominant enzymes: xylanases and cellulases. Other commercially available carbohydrases include α-amylase, α-galactosidase, β-glucanase, β-mannanase, and pectinase (46).

In the present study, the *Bacillus spp.* strains that conform the DFM candidate treatment were identified as either *B. subtilis* or *B. amyloliquefaciens* (**Table 3**), therefore being feasible for *in vivo* evaluation studies as they are GRAS candidates (12, 26–28). Furthermore, the three selected *Bacillus spp.* isolates showed a variable ability to produce cellulase and xylanase (**Table 2**), hence, in addition to the benefits that spores or vegetative cells can provide as probiotics (9, 12), they may improve the digestibility of cereals with high-soluble NSP (47).

The *Bacillus*-DFM candidate treatment also demonstrated effective antimicrobial properties against *C. perfringens*, which could be due to production of antimicrobial-like compounds and/or competition for nutrients (**Tables 2** and **5**). Little is known about the mechanisms underlying the higher incidence of NE in broilers fed diets containing cereals with elevated levels of NSP, but it could be related to a prolonged feed rate of passage and a reduction in the digestion of nutrients that later in the hind gut will be available for bacteria to growth (48). For *in vitro* evaluation of *C. perfringens* proliferation, TSB with sodium thioglycolate (positive control) groups were included. In the TSB group (positive control), the *C. perfringens* inoculum was increased ~0.5$\log_{10}$, after 4 h of incubation. However, it was interesting to observe a significant increase in *C. perfringens* proliferation in the supernatants collected from control non-DFM diets that contained wheat, barley, rye, and oat, compared with the enrichment TSB medium with sodium thioglycolate group (**Table 5**).

These results suggest that partial digestion of NSP grains and increased digesta viscosity provides a favorable nutritional environment that supports the growth of *C. perfringens*. Interestingly, dietary inclusion of a *Bacillus*-DFM candidate in non-corn diets significantly reduced both viscosity (**Table 4**) and *C. perfringens* proliferation (**Table 5**), when compared to control diets without DFM inclusion. This result shows the capacity of certain *Bacillus* isolates to inhibit the growth of pathogenic bacteria like *C. perfringens*, probably due to competition for nutrients, production of antimicrobial-like compounds, or changes in environmental conditions. Proliferation of *C. perfringens* in the corn-based diet remained constant with or without the inclusion of the *Bacillus*-DFM candidate and in the TSB positive control group (**Table 5**). This outcome could be related to the lower concentration of NSP usually found in corn grains in comparison to other cereals, which was also supported by low digesta viscosity values (**Table 4**). These results are in accordance with previous reports (35), however, it is important to mention that diet ingredients are just one of the multiple predisposing factors that could affect the incidence of NE in commercial conditions (49, 50).

Beneficial bacterial spores are popular as DFM, though little is known about their mode of action. Previous studies conducted in our laboratory, have demonstrated that ~90% of *Bacillus* spores of a selected strain germinate within 30 min under *in vitro* and *in vivo* model conditions, with relatively constant numbers of spores in each gastrointestinal compartment evaluated, hence, suggesting that full-life cycle may occurs (51). In the present study, regardless of the diet, similar *in vitro* persistence of the *Bacillus*-DFM candidate spores was observed in the different simulated compartments (**Table 6**). On average, a half $\log_{10}$ reduction in spore numbers were detected in the crop compartment suggesting spore germination. In the proventriculus compartment, a further ~2$\log_{10}$ reduction was shown, supporting our previous findings (27, 51), which suggest that further germination of spores occurs even at low pH environments. However, it was particularly interesting to observe a ~2$\log_{10}$ increment in spore counts in the intestinal simulated compartment (**Table 6**). The increment in the numbers of spores could be a response to bacterial metabolites, competition for oxygen and nutrients available, resulting in resporulation (17). The above observations also support previous reports suggesting that spore transiting through the gastrointestinal tract could potentially undergo a full-life cycle of germination and resporulation (36, 52). Moreover, it has been demonstrated that germination of spores into metabolically and functionally active vegetative cells, within a similar time frame, produced beneficial metabolic and immunological effects in different animal species (53–57).

In summary, our results confirm that poultry diets containing cereal grains with a higher content of NSP in comparison to corn can enhance viscosity and *C. perfringens* growth (18–21). Remarkably, the dietary inclusion of a selected *Bacillus*-DFM candidate in non-corn-based diets significantly reduced both viscosity and *C. perfringens* proliferation when compared

with the control non-supplemented-diets. Additionally, *Bacillus*-DFM candidate spore persisted and change their amount according to the variable biochemical conditions of the *in vitro* digestive model; therefore, supporting the hypothesis of the possible full-life cycle development in the gastrointestinal tract. The results from the present *in vitro* study encourage us to further evaluate the utilization of this *Bacillus*-DFM candidate in an *in vivo* NE model that we have developed in our laboratory (5), as well as to purify, characterize, and measure the international units of cellulase and xylanase that these *Bacillus* isolates produce. This knowledge will provide a valuable tool to use a stable DFM that produce exogenous enzymes in poultry diets.

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Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens

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The recent trend toward reduction of antibiotic growth promoters (AGP) in North American poultry diets has put tremendous pressure on the industry to look for viable alternatives. In this context, phytogenic feed additives (PFA) are researched to improve gut health and thereby performance. An experiment was conducted with the objective to evaluate the effects of PFA as an alternative to AGP on small intestinal histomorphology, cecal microbiota composition, nutrient digestibility, and growth performance in broiler chickens. A total of 432-day-old Vencobb 400 broiler chicks were randomly assigned to one of three dietary groups, each consisting of 12 replicate pens ($n = 12$ chicks/pen). The chicks were fed a corn–soybean meal-based control (CON), CON + 500 mg/kg of AGP (bacitracin methylene disalicylate containing 450 mg active BMD/g), or CON + 150 mg/kg of proprietary blend of PFA (Digestarom® Poultry) until 39 days of age when samples were collected. Birds fed either AGP or PFA had increased villus height in all three segments of the small intestine in comparison to the birds fed CON ($P \leq 0.05$). Furthermore, the PFA-fed birds had significantly increased villus height and lower crypt depth compared to AGP fed birds ($P \leq 0.05$). Birds fed either additive also had increased total tract digestibility of dry matter, crude protein, and ether extract ($P \leq 0.05$). The strong effect of the PFA on villus height in the jejunum may suggest augmented nutrient absorption in PFA-fed birds. Although both additives reduced total cecal counts of anaerobic bacteria and *Clostridium* spp., PFA alone reduced the total coliform count while increasing the *Lactobacillus* spp. count ($P \leq 0.05$). These results suggest the establishment of beneficial microbial colonies in PFA-fed birds. Overall, both PFA and AGP increased body weight gain while lowering the feed conversion ratio ($P \leq 0.05$). Hence data from this experiment demonstrate the efficacy of PFA as a substitute to AGP in poultry diets.

Keywords: digestibility, histomorphology, microbiota, performance, poultry

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Introduction

Sustaining a healthy gut environment is a prerequisite to efficient broiler performance. Antibiotic growth promoters (AGP) have been used since the mid-1940s to maintain a healthy gut environment and in turn improve the performance (1). The ban on AGP compounds from poultry diets in Europe (2) and recent moves toward reduction or termination of AGP in North America have put pressure on the poultry industry to look for viable alternatives that can improve performance, protect

animal health, and maintain profit margins (3). Phytogenic feed additives (PFA) have been gaining considerable interest lately due to their ability to improve performance by sustaining a healthy gut environment.

According to European Union legislation (EC 1831/2003) (4), PFA are categorized as sensory and flavoring compounds, which consist mainly of plant extracts (essential oils, oleoresins, and flavonoids) and their active principles (5). The essential oils present in PFA, which contain most of the active substances of the plant, have been suggested to increase the growth performance (6, 7), nutrient digestibility (8), and gut health (6, 9) in poultry species. The numerous beneficial qualities of PFA are predominantly derived from their bioactive molecules including carvacrol, thymol, capsaicin, cineole, etc. (10). It is these properties of PFA that project them as suitable alternatives to AGP.

The primary mode of action of PFA is controlling potential pathogens and beneficially modulating the intestinal microbiota. Several plant extracts are known to have antimicrobial, antiviral, anticoccidial, fungicidal, and/or antioxidant properties (11). Studies conducted in broilers have demonstrated the antimicrobial efficacy of PFA against pathogenic bacteria, such as *Escherichia coli* and *Clostridium perfringens*, potentially indicating a reduced risk for the development of colibacillosis and necrotic enteritis (8, 12). Alleviation of coccidiosis symptoms, including reduction in lesion severity and oocyst shedding, by PFA has also been documented (6). The antimicrobial effects of PFA are primarily attributed to their phenolic components and their action on pathogenic cells (13, 14).

It has been suggested that PFA augment nutrient utilization in the gastrointestinal tract (GIT) by enhancing production of digestive secretions and enzymatic activity (11, 15). Furthermore, several studies have observed positive effects of PFA on the morphology of small intestinal tissues, such as increased villus height, decreased crypt depth, and increased goblet cell counts (16–18). Such effects on gastrointestinal morphology have been postulated to increase the nutrient digestibility in poultry (19). PFA, like AGP, may also reduce mucosal thickness, thus contributing to the diffusion of nutrients to the apical surface of epithelial cells and increased absorption and feed efficiency (20).

Overall, PFA are capable of reducing microbial threat and promoting intestinal health, which is imperative for optimal bird performance and profitability. The objective of this study was to evaluate the efficacy of PFA as an alternative to AGP in broiler production by determining their effects on cecal microbiota composition, small intestinal histomorphology, nutrient digestibility, and growth performance.

Materials and Methods

Animals and Housing

All animal procedures were conducted according to the ethical norm of West Bengal University of Animal and Fishery Sciences. An experiment of 39 days duration was conducted using a total of 432-day-old Vencobb 400 broiler chicks, which were randomly assigned to one of three dietary groups at the start of experiment. The 3 groups consisted of 12 replicate pens of 12 chicks, each resulting in 144 birds per group.

Diets and Groups

The dietary groups were (i) control (CON), (ii) basal diet + AGP (AGP), and (iii) basal diet + PFA (PFA). The CON diet consisted of a corn–soybean meal-based basal diet, and was formulated to meet or exceed breeder recommendations [**Table 1**; "Nutrient Levels," Venky's Ltd. (21)]. The AGP used was bacitracin methylene disalicylate containing 450 mg active BMD/g at the inclusion rate of 500 mg/kg of diet. The PFA used was Digestarom® Poultry (Biomin Holding GmbH, Austria) at the inclusion rate of 150 mg/kg of diet. The PFA contains a combination of over 30 essential oils and phytogenic compounds. Neither exogenous polysaccharide-degrading enzymes nor anticoccidial drugs were added in the diets as these might partly mask the effects of the PFA, but a mycotoxin binder was used.

Measurements of Production Traits

Birds were monitored twice daily and any mortality was removed, weighed, and recorded. Birds were individually weighed at 7, 21, and 39 days, which designated the end of pre-starter, starter, and grower periods, respectively. Total feed intake (FI) per pen was also measured on the same days. Body weight gain (BWG), average daily feed intake (ADFI), and feed conversion rate (FCR) were calculated per pen for each period and for the overall period. Mortality BWG data were used to correct the FCR.

Determination of Apparent Total Tract Nutrient Digestibility

Two birds were randomly selected from each pen and were distributed to cages at 30 days of age (eight cages per group, three birds per cage) to provide an acclimatization period of 6 days. Total excreta collection was done for three consecutive days from

TABLE 1 | Formulation and composition (gram/kilogram) of the experimental diets.

	Starter (1–7 days)	Grower (8–21 days)	Finisher (22–39 days)
Ingredients			
Ground corn	540.7	567.2	583.4
Soybean meal (46% CP)	396.0	362.5	328.0
Soybean oil	27.0	33.7	51.1
Calcite powder	12.45	12.45	12.45
Di-calcium phosphate	16.5	16.5	17.0
D-methionine	0.55	0.95	1.35
Lysine hydrochloride	0.3	0.2	0.2
Sodium bicarbonate	2	2	2
Salt	2	2	2
Premix[a]	2	2	2
Toxin binder	0.05	0.5	0.5
Calculated composition (g/kg as-fed)			
ME_N (MJ/kg)	11.85	12.14	12.65
Crude protein	223.5	210.4	196.3
Ether extract	52.7	59.8	7.33
Crude fiber	2.5	2.4	2.4
Calcium	9.57	9.47	9.38
Available P	3.18	3.16	3.13
Digestible lysine	11.02	10.19	9.39
Digestible methionine	3.51	3.76	3.99
Digestible methionine + cysteine	6.87	6.73	6.77

[a] *Including vitamins and trace minerals.*

36 to 39 days of age at 2 h intervals. Samples were mixed, weighed, and a 10% aliquot was frozen at −20°C. The sampled excreta were again pooled by cage at the end of the collection period, and a 10% aliquot was taken and dried. Feed samples were collected daily for the same 3-day period (36–39 days) and pooled to produce a single composite of each diet. Diet and excreta samples were analyzed to determine the apparent total tract digestibility of dry matter (DM), crude protein (CP), and ether extract (EE) (22, 23).

Histomorphology of the Small Intestine

One bird per replicate pen was euthanized via carbon dioxide asphyxiation at the end of the experiment on day 39. The entire GIT tract was removed aseptically before separating into sections of duodenum, jejunum, ileum, cecum, and colon. The small intestinal segments (duodenum, jejunum, and ileum) were processed for histomorphological analysis (24). Segments measuring 2-cm in length from the mid-points of the duodenum, jejunum, and ileum were cut, flushed with cold saline, fixed in 10% buffered formalin, and stained with hematoxylin–eosin. Histological sections were examined with a phase contrast microscope coupled with a deconvolution imaging analysis system [VayTek®, Fairfield, IA, USA; (25)]. Villus height (VH, from the tip of the villus to the top of the lamina propria), crypt depth (CD, from the base to the region of transition between the crypt and villus), and the thickness of the muscularis mucosae in the duodenum, jejunum, and ileum were determined. Measurements of 10 complete villi for VH and associated crypts for CD were taken from each segment, and the average of these values was used for statistical analysis.

Caecal Microbiota Composition

The ceca with contents were stored at 4°C for determination of cecal microbiota. The cecal digesta was processed within 24 h (5). Each cecal digesta homogenate was serially diluted from 10-1 to 10-8. Coliforms were cultured using HiCrome *E. coli* HiVeg Agar (Product code: MV1295; Hi-Media Laboratories, Mumbai, India), anaerobic bacteria were cultured using Wilkins Chalgren Anaerobic Agar (Product code: M832; Hi-Media Laboratories), *Staphylococcus aureus* was cultured using HiCrome Staph Agar (Product code: M1837; Hi-Media Laboratories), *Lactobacillus* spp. were cultured using *Lactobacillus* MRS Agar (Product code: M641; Hi-Media Laboratories), *Pseudomonas aeruginosa* was cultured using Cetrimide Agar (Product code: M024; Hi-Media Laboratories), and the *Clostridium* were cultured in Reinforced Clostridial Agar (Product code: M154; Hi-Media Laboratories). Diluted digesta samples were streaked onto the agar plates and incubated at 37°C for 48 h, while the clostridia plates were incubated anaerobically at similar temperature. Visible colonies were enumerated using a colony counter and the results were expressed as $\log_{10}$ CFU/g of cecal digesta.

Statistical Analysis

The data were analyzed as a complete randomized design using General Linear Model procedure of SPSS (version 17.0). Repeated measures ANOVA was used to analyze performance parameters, which were measured over the course of the experiment. Means were separated using Tukey-WSD following ANOVA. Values were considered statistically different at $P \leq 0.05$. The replicate pens were used as experimental units for all parameters except apparent total tract nutrient digestibility where the cages were used as experimental units.

Results

Production Performance

Body Weight Gain

Mortalities for the groups were statistically insignificant at 4.22, 2.22, and 2.78% for the CON, AGP, and PFA groups, respectively, for the 1–39 days period. No significant differences in BWG were noted during the 1–7 days pre-starter phase (**Table 2**). There was an effect of dietary treatment on BWG during the starter phase (8–21 days; $P = 0.03$), grower phase (22–39 days; $P < 0.01$), and during the overall period (1–39 days; $P < 0.01$). During the starter phase, birds-fed AGP had significantly increased BWG compared to CON-fed birds ($P \leq 0.05$), while the BWG for PFA-fed birds was not significantly different from either CON or AGP groups. During the grower phase, birds-fed PFA had significantly increased BWG in comparison to both CON ($P \leq 0.05$) and AGP groups ($P \leq 0.05$), which did not differ from each other. Supplementation of either AGP ($P \leq 0.05$) or PFA ($P \leq 0.05$) to the basal diet significantly increased BWG for the overall experimental period.

Feed Intake

No significant differences in FI were noted among the groups throughout the experimental period, except during the starter phase (8–21 days; $P = 0.01$) when the FI of PFA-fed birds was significantly lower in comparison to both AGP- ($P \leq 0.05$) and CON-fed birds ($P \leq 0.05$; **Table 2**).

TABLE 2 | Performance of broilers supplemented with either an antibiotic or a phytogenic feed additive during 1–39 days of age*.

	Dietary treatments†			SEM	*P* value
	Control	AGP	PFA		
Pre-starter period (1–7 days)					
Body weight gain (g)	140.5	144.3	142.3	1.20	0.44
Feed intake (g)	185.4	185.9	185.5	1.03	0.98
Feed conversion‡	1.323	1.292	1.305	0.012	0.57
Starter period (8–21 days)					
Body weight gain (g)	823.4[b]	860.3[a]	835.1[ab]	5.93	0.03
Feed intake (g)	1004.5[a]	1007.8[a]	975.3[b]	4.96	0.01
Feed conversion‡	1.221[a]	1.173[ab]	1.169[b]	0.089	0.02
Grower period (22–39 days)					
Body weight gain (g)	1073.5[b]	1109.2[b]	1183.1[b]	12.49	<0.01
Feed intake (g)	2599.6	2606.5	2590.6	7.56	0.71
Feed conversion‡	2.431[a]	2.355[a]	2.195[b]	0.028	<0.01
Overall period (1–39 days)					
Body weight gain (g)	1896.9[b]	1969.5[a]	2018.2[a]	14.41	<0.01
Feed intake (g)	3789.5	3800.2	3751.5	9.81	0.10
Feed conversion‡	2.002[a]	1.931[ab]	1.860[b]	0.015	<0.01

Means with dissimilar letters in a row varied significantly.

**Means of 12 replicate pens (n = 12 birds per pen).*

†Supplemented with either an antibiotic growth promoter, bacitracin methylene disalicylate, 500 mg/kg (AGP), or a phytogenic feed additive (Digestarom® Poultry) 150 mg/kg (PFA). The control group received the unsupplemented basal diet.

‡Corrected for mortality.

Mortality-Corrected Feed Conversion Ratio

No significant differences in FCR were present during the 1–7 days pre-starter phase (**Table 2**). There was an effect of treatment on FCR during the 8–21 days starter phase ($P = 0.02$), 22–39 days grower phase ($P < 0.01$), and for the overall period (1–39 days; $P < 0.01$). Supplementation of PFA lowered the FCR in comparison to CON-fed birds during all time periods ($P \leq 0.05$). The FCR for birds-fed AGP did not differ significantly from either the CON or PFA groups for the starter phase as well as for the overall period. During the grower phase, however, PFA-fed birds had significantly lower FCR in comparison to the AGP-fed group ($P \leq 0.05$).

Nutrient Digestibility

There was a significant effect of dietary treatment on apparent total tract digestibility of DM ($P < 0.01$), CP ($P = 0.04$), and EE ($P = 0.02$; **Table 3**). The apparent total tract DM digestibility for the PFA-fed group was increased in comparison to the CON group ($P \leq 0.05$), while the AGP group was not different from either the CON or PFA groups. Supplementation of either AGP or PFA to the basal diet significantly increased the apparent total tract digestibility of CP and EE when compared to the CON group ($P \leq 0.05$).

Histomorphology of Small Intestine

Dietary treatment had a significant effect on VH in the duodenum ($P = 0.02$), jejunum ($P < 0.01$), and ileum ($P < 0.01$; **Table 4**). The VH was significantly increased by AGP supplementation in the duodenum in comparison to the CON group ($P \leq 0.05$), while PFA was not different from either AGP or CON. In the jejunum, both AGP and PFA significantly increased the VH in comparison to CON ($P \leq 0.05$); however, the PFA group had significantly increased VH compared to AGP-fed birds ($P \leq 0.05$). Additionally, both AGP and PFA significantly increased the ileal VH in comparison to the CON group ($P \leq 0.05$). Overall, the supplementation of either AGP or PFA increased the VH across the small intestine.

No significant differences in CD were noted in any dietary group in the duodenum or ileum (**Table 4**). There was a significant effect of dietary treatment in the jejunum ($P < 0.01$), where inclusion of PFA in the basal diet lowered the CD in the jejunum when compared to both CON and AGP groups ($P \leq 0.05$).

Additionally, there was a significant effect of treatment on mucosal thickness in the duodenum ($P < 0.01$) and ileum ($P < 0.01$; **Table 4**). Both AGP and PFA significantly lowered the mucosal thickness in the duodenum and ileum in comparison to the CON group ($P \leq 0.05$), but no differences were present in the jejunal mucosal thickness.

Cecal Microbiota Composition

The composition of cecal microbiota at 39 days of age in $\log_{10}$ CFU/g of wet cecal digesta is provided in **Table 5**. There was a significant effect of dietary treatment on total coliform ($P < 0.01$), anaerobic bacteria ($P < 0.01$), *Lactobacillus* spp. ($P < 0.01$), and *Clostridium* spp. ($P = 0.01$) counts. Supplementation of PFA significantly decreased the total coliform count in comparison to the CON and the AGP groups ($P \leq 0.05$). The CFU of total anaerobic bacteria and total *Clostridium* were decreased with the supplementation of either AGP or PFA ($P \leq 0.05$). Supplementation of either AGP or PFA tended to decrease *Staphylococcus aureus* in comparison to the CON group ($P = 0.06$). No effect of dietary treatment on the cecal population of *Pseudomonas aeruginosa* was noted; however, supplementation of PFA significantly increased the number of cecal *Lactobacillus* in comparison to the CON and AGP groups ($P \leq 0.05$).

TABLE 3 | Nutrient digestibility of broilers supplemented with either an antibiotic or a phytogenic feed additive during 1–39 days of age*.

	Dietary treatments†			SEM	*P* value
	Control	AGP	PFA		
Nutrient digestibility (g/g intake)					
Dry matter	0.674[b]	0.711[ab]	0.744[a]	0.007	<0.01
Crude protein	0.761[b]	0.784[a]	0.794[a]	0.005	0.04
Ether extract	0.736[b]	0.781[a]	0.782[a]	0.005	0.02

Means with dissimilar letters in a row varied significantly.
**Digestibility trial was conducted at 36 days of age for three consecutive days. Randomly selected birds were placed in metabolism cages. There were eight cages per treatment with three birds in each cage.*
†Supplemented with either an antibiotic growth promoter, bacitracin methylene disalicylate, 500 mg/kg (AGP), or a phytogenic feed additive (Digestarom® Poultry) 150 mg/kg (PFA). The control group received the unsupplemented basal diet.

TABLE 4 | Villus height, crypt depth, and thickness of muscularis mucosae (micrometer) of broilers supplemented with either an antibiotic or a phytogenic feed additive during 1–39 days of age*.

	Dietary treatments†			SEM	*P* value
	Control	AGP	PFA		
Duodenum					
Villus height	2549.1[b]	3481.1[a]	2903.4[ab]	140.23	0.02
Crypt depth	45.3	42.7	32.8	2.55	0.10
Mucosa thickness	387.1[a]	183.9[b]	230.4[b]	22.69	<0.01
Jejunum					
Villus height	2583.6[c]	2969.9[b]	3290.1[a]	280.51	<0.01
Crypt depth	29.8[a]	31.1[a]	20.2[b]	1.31	<0.01
Mucosa thickness	206.8	215.6	212.9	6.85	0.88
Ileum					
Villus height	2050.1[b]	2736.4[a]	2839.9[a]	94.03	<0.01
Crypt depth	34.1	30.9	31.6	1.04	0.45
Mucosa thickness	320.3[a]	233.9[b]	211.8[b]	14.31	<0.01

Means with dissimilar letters in a row varied significantly.
**Means of 12 birds per treatment. Birds were randomly selected and euthanized at 39 days of age.*
†Supplemented with either an antibiotic growth promoter, bacitracin methylene disalicylate, 500 mg/kg (AGP), or a phytogenic feed additive (Digestarom® Poultry) 150 mg/kg (PFA). The control group received the unsupplemented basal diet.

Discussion

Supplementation of PFA significantly lowered the overall FCR by 3.6% (7 points) and 7.0% (14 points) in comparison to the AGP and CON groups, respectively. This was in agreement with earlier reports (5), which indicated improvement in final BW and FCR with PFA supplementation without any effect on the FI. The growth promoting effects of the PFA and the AGP in this experiment may be correlated with the significant increase in apparent total tract digestibility of nutrients. Furthermore, a significant

TABLE 5 | Cecal microbiota composition (log_{10} CFU/g wet cecal digesta) of broilers supplemented with either an antibiotic or a phytogenic feed additive during 1–39 days of age*.

	Dietary treatments†			SEM	*P* value
	Control	AGP	PFA		
Coliforms	5.54[a]	5.62[a]	5.10[b]	0.07	<0.01
Anaerobic bacteria	5.91[a]	5.64[b]	5.53[b]	0.04	<0.01
Staphylococcus aureus	2.61	1.24	1.14	0.49	0.06
Pseudomonas aeruginosa	4.81	4.74	4.84	0.06	0.27
Lactobacillus spp.	4.96[b]	5.01[b]	5.35[a]	0.04	<0.01
Clostridium spp.	5.17[a]	4.95[b]	4.97[b]	0.03	0.01

Means with dissimilar letters in a row varied significantly.

**Means of 12 birds per treatment. Birds were randomly selected and euthanized at 39 days of age.*

†*Supplemented with either an antibiotic growth promoter, bacitracin methylene disalicylate, 500 mg/kg (AGP), or a phytogenic feed additive (Digestarom® Poultry) 150 mg/kg (PFA). The control group received the unsupplemented basal diet.*

decrease in FI in the PFA group was noted during the starter phase, perhaps suggesting that the bird's nutrient requirements are being satisfied with a smaller quantity of feed (26). It has been well documented that AGP-increased nutrient digestibility is mostly due to their effects on the intestinal microbiota (27). Increased nutrient digestibility with the addition of PFA and growth performance similar to Avilamycin was reported by Hernandez et al. (19). It has been noted that extracts from spices and herbs may stimulate digestive secretions and enzymatic activity, thus exerting beneficial actions within the digestive tract (28). The present findings further support the idea that PFA favorably modulate gut functions and digestive activities to stimulate growth in broilers.

Morphological changes in the GIT caused by PFA may provide further information on possible benefits to the digestive tract. In general, PFA and AGP significantly increased the VH across the small intestine. In the absence of any inflammation, this ought to increase absorptive surface area and efficiency of digestion and absorption (29). A similar effect of PFA on VH has been reported by Namkung et al. (17). The positive effect of PFA in the present experiment in increasing the VH in the jejunum could increase the efficiency of absorptive process considering the fact that the majority of absorption occurs in the jejunum. Furthermore, greater VH increases the activities of mucosal digestive enzymes, resulting in improved digestibility (30, 31). As intestinal crypts are the source of epithelial cells for villi and CD is directly correlated with epithelial cell turnover, the shallower crypts in the jejunum due to PFA supplementation may be indicative of decreased cellular turnover and improved intestinal health. Moreover, cellular turnover is an energy consuming process that uses resources that might otherwise be utilized toward growth; thus, shallower crypts are also related to improved performance (32). In the current study, mucosa thickness was significantly reduced in the duodenums and ileums of AGP and PFA supplemented birds. Gordon and Bruckner-Kardoss (33) reported that germ-free birds had thinner muscularis mucosa than the birds reared under conventional management systems, indicating that thinning of the mucosa might spare nutrients for productive processes and this was reflected in the BWG of the birds.

Literature depicting antimicrobial role of PFA is ample (8, 12, 14, 34, 35), while very few explore the mode of action by which the PFA may facilitate the proliferation of beneficial bacteria, such as *Lactobacillus* spp. (5, 8). In the present experiment, PFA significantly reduced the cecal population of coliforms and fortified the gut microbiota with beneficial bacteria, such as *Lactobacillus* spp. Once the *Lactobacillus* spp. are established, they might selectively exclude the pathogens from adhering due to their fast colonization, proliferation, and acidification properties in the GIT (9). The inhibitory effect of PFA on *Clostridium* spp. is encouraging and paves the way for removal of the AGP from poultry diets. The essential oils present in PFA have been shown to inhibit the growth of *Clostridia* (34). Mitsch et al. (12) opined that PFA stabilizes the gut microbiota and thereby reduces the colonization of *Clostridia* in gut. Evaluation of the cecal microbiota population in this study has revealed that AGP and PFA alike reduced total bacterial load in the gut. This inhibitory effect of PFA on bacterial load may alleviate pressure on the immune system, thus allowing the reallocation of energy toward improving performance. Overall from the gut perspective, the PFA purportedly favored a healthy gut, which in turn could be concomitant with the growth enhancement.

In conclusion, supplementation of either AGP or PFA increased the apparent total tract nutrient digestibility by increasing the VH throughout the small intestine. In comparison to the AGP, the PFA supported establishment of a favorable gut microbiota composed of higher numbers of *Lactobacillus* spp. and less *Clostridium* spp. Furthermore, supplementation of PFA to a corn–soybean meal-based coccidiostat free diet increased the BWG and lowered the FCR in 39 days, which was comparable to the AGP used in this experiment. Overall, the present work demonstrated the efficacy of PFA utilization and confirms the importance of considering the inclusion of PFA in poultry diets as an alternative to AGP.

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Corrigendum: Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens

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Keywords: digestibility, histomorphology, microbiota, performance, poultry

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There is a typographical error in Table 2 of this article. Under the "Grower period (22–39 days)" section, the body weight gain for the PFA group should have the superscript "a," instead of "b," indicating that it is significantly different from the Control and AGP groups.

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Corrigendum II: Phytogenic Feed Additives as an Alternative to Antibiotic Growth Promoters in Broiler Chickens

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REVISED CONFLICT OF INTEREST STATEMENT

The authors declare that MICRO-PLUS Konzentrate GmbH, now a subsidiary of BIOMIN Holding GmbH, financially sponsored this project. The authors GM and CP are affiliated with BIOMIN, which produces the phytogenic feed additive used in this trial. BS, affiliated with MICRO-PLUS Konzentrate GmbH at the time the experiment was conducted, is currently affiliated with BIOMIN following the acquisition.

ETHICS DECLARATION

At the time this experiment was conducted, the Institutional Animal Ethics Committee standards for conducting research on poultry species were not established, thus no committee approval was required, and the experiment was conducted according to the ethical norms of the University. The principal investigator of the study has provided the journal's Editorial Office a retrospective statement that approval was not needed.

AUTHOR CONTRIBUTIONS

GM: final approval of the version to be submitted, drafting the article, or revising it critically for important intellectual content; BS: contributions to conception and design of the experiment and analysis and interpretation of data; SH: contributions to conception and design of the experiment, acquisition of data, and analysis and interpretation of data; CP: final approval of the version to be submitted, drafting the article, or revising it critically for important intellectual content.

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