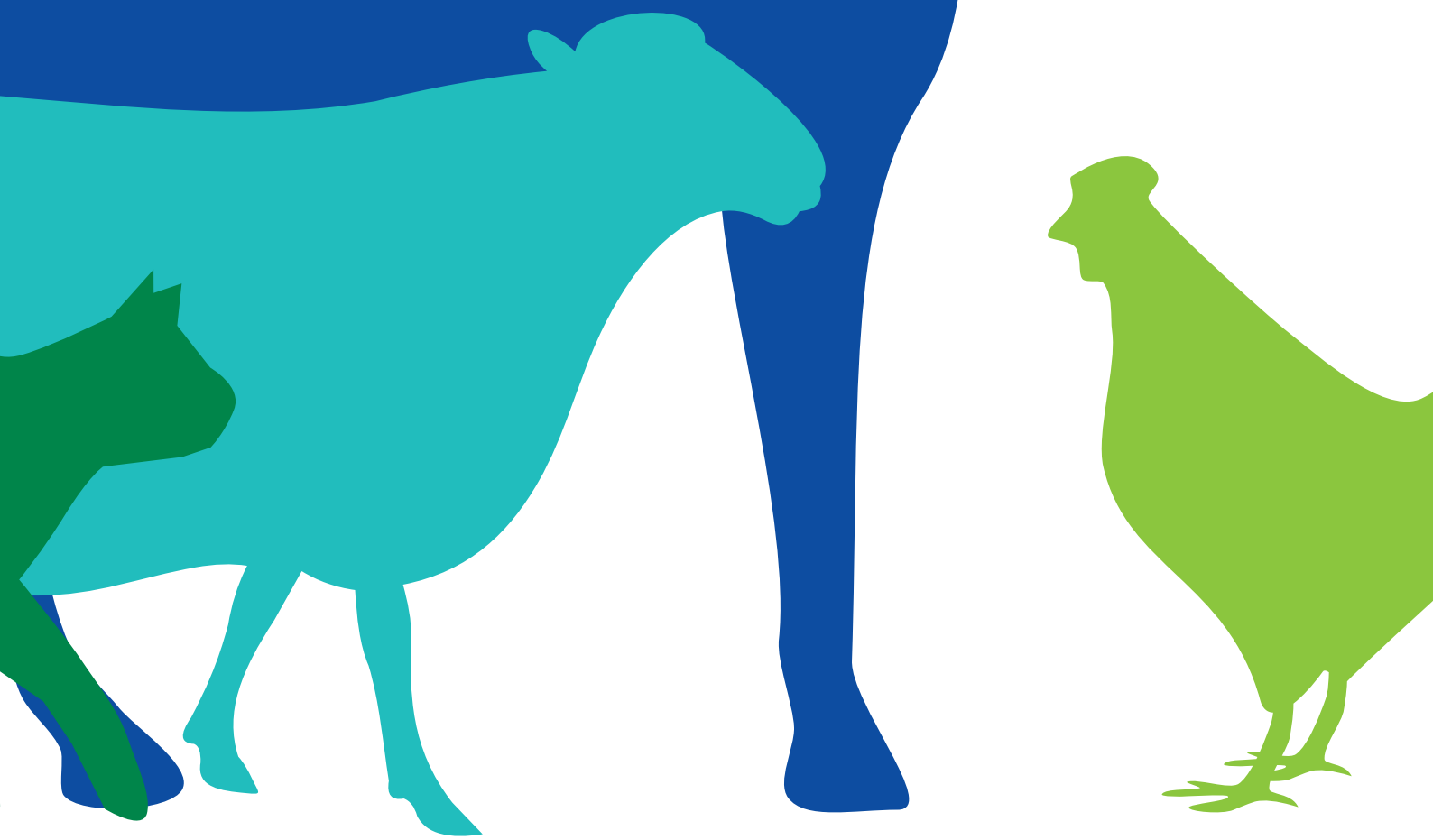
NUTRITIONAL INTERVENTION FOR THE INTESTINAL HEALTH OF YOUNG MONOGASTRIC ANIMALS

EDITED BY: Sung Woo Kim and Rajesh Jha
PUBLISHED IN: Frontiers in Veterinary Science



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ISSN 1664-8714
ISBN 978-2-88966-730-7
DOI 10.3389/978-2-88966-730-7

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NUTRITIONAL INTERVENTION FOR THE INTESTINAL HEALTH OF YOUNG MONOGASTRIC ANIMALS

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Citation: Kim, S. W., Jha, R., eds. (2021). Nutritional Intervention for the Intestinal Health of Young Monogastric Animals. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-730-7

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frontiers in Veterinary Science

EDITORIAL
published: 18 March 2021
doi: 10.3389/fvets.2021.668563

Editorial: Nutritional Intervention for the Intestinal Health of Young Monogastric Animals

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Keywords: antimicrobial resistance, gut health, prebiotics, probiotics, pig, poultry

Editorial on the Research Topic

Nutritional Intervention for the Intestinal Health of Young Monogastric Animals

INTRODUCTION

Poultry and pig production have increased at a faster rate than any other livestock production globally (1). Among others, nutritionally balanced-feeding programs, along with antibiotic growth promoters (AGP) in feeds, played a significant role in achieving this success (2, 3). The animal industry, however, aims to redefine its nutrition program to grow safe and quality meat in the light of public health concerns due to the use of AGP in diets (1). Maintenance or improvement of intestinal health is essential for optimum growth, better feed efficiency, and the overall health of pigs and poultry (4–6). Keeping a healthy intestine is also critically important for nutrient digestion and utilization, thereby ensuring better growth performance of pigs and poultry (7–9).

Intestinal health covers efficient nutrient utilization, macro- and micro-structural integrity of the gut, the stability of the microbiota, and the status of the immune system (4, 10, 11). Moreover, intestinal health is a complex field combining the nutrition, microbiology, immunology, and physiology of animals. Challenges in intestinal health directly influence nutrient digestion and absorption (4, 12, 13), which in turn reduces feed efficiency and increases susceptibility to enteric diseases (14, 15).

Recent regulatory changes on the use of AGP and selected feedstuffs have challenged the optimal growth and health of modern pigs and poultry, that have been extensively selected for growth efficiency and lean gain. Highly lean and fast-growing pigs and poultry highlight the need for a better understanding of the gut function and overall gut health. Understanding and improving the intestinal health of animals is a key essential trend needed for the success of animal production in this era of AGP free production (1, 4, 16). This Research Topic eBook covers nutritional aspects of improving the intestinal health of monogastric animals, including current challenges and potential solutions. The papers have been presented under two sections: (1) Importance and understanding intestinal health of monogastric animals and (2) Nutritional intervention for intestinal health.

IMPORTANCE AND UNDERSTANDING THE INTESTINAL HEALTH OF YOUNG MONOGASTRIC ANIMALS

It is well-established that effective modulation of the gut health parameters depends not only on feedstuffs but also on the methods and timing of the nutrients available to host animals. Furthermore, early growth and development of GIT are of critical importance in enhancing

OPEN ACCESS

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *16 February 2021*
Accepted: *24 February 2021*
Published: *18 March 2021*

Citation:
Jha R and Kim SW (2021) Editorial: Nutritional Intervention for the Intestinal Health of Young Monogastric Animals. Front. Vet. Sci. 8:668563. doi: 10.3389/fvets.2021.668563

nutrient utilization and optimizing the growth of poultry. Early nutrition programming using both *in ovo* and post-hatch feeding has been used as a means to modulate the early growth and development of GIT and has been found to be an effective strategy [(17); (Jha, Singh et al.)]. Similarly, the weaning phase of pigs is an incredibly stressful period as it causes morphological and functional changes in the gut and induces post-weaning growth depression. Different nutritional strategies, including the addition of functional feed additives in the weaner pig's diet have been proposed to minimize these effects (Zheng et al.). Similarly, different nutrients and feed additives have been used to optimize the gastrointestinal integrity and immune system of young animals (Adedokun and Olojede). However, gut microbiota plays a significant role in managing the gut environment by producing fermentation metabolites and influencing nutrient utilization pathways (18). Thus, it is important to understand the gut microbial ecology in-depth, including their taxonomic composition and biochemical functions. However, the gut microbiota is primarily influenced by diet, age, species, and location in the digestive tract (5, 18, 19). Different techniques have been used to characterize the gut microbiota, but those have different strengths and limitations. Modern techniques like 16S rRNA based next-generation sequencing and others are powerful tools to investigate the biological and ecological roles of the gut microbiota [(19); (Shang et al.)]. In the commercial animal production system, different nutritional and environmental stresses and pathological factors create oxidative stress in animals, leading to imbalances in the intestinal homeostasis due to the generation of reactive oxygen species (ROS) and reactive nitrogen species. It can be mitigated by supplementing exogenous vitamins, antioxidants, and plant extracts that have antioxidant properties that scavenge ROS [(9); (Mishra and Jha)]. Thus, it is crucial to understand the involvement of oxidative stress in the gastrointestinal functionality of animals and the potential intervention strategies available to maintain redox balance in the GIT.

NUTRITIONAL INTERVENTION FOR INTESTINAL HEALTH

It is not only the type of feedstuffs, but also their forms that have been found to affect gut health and function. A finer feed particle size enables optimal nutrient utilization and enhances animal performance due to increased surface area, allowing for better contact with digestive enzymes. Moreover, adequate diminution of feedstuffs is beneficial to feed manufacturing processes such as mixing and hydrothermal treatments, including pelleting, extrusion, and expansion. Thus, feed processing techniques, along with the type of feedstuffs, need to be considered when formulating diets for animals considering their impact on intestinal health (Kiarie and Mills).

As various feedstuffs, their components, and feed additives behave and function differently in the GIT of animals, different feeding strategies have been tested, with some success, to improve intestinal health and functionality. Furthermore, there is also a need to evaluate potential alternatives to AGPs in animal diets in the post-antibiotic era [(3); (Yang et al.)]. As potential alternatives to AGPs, different dietary fibers (DF), prebiotics, probiotics, post-biotics, enzymes, and others have been evaluated and found to have promising outcomes (Zheng et al.). Although DF are not well-digested and are often considered as anti-nutritional factors in monogastric animals, as it reduces nutrient utilization (20), it has been widely used in recent years to modulate the intestinal environment (4). DFs are fermented in the intestine and become short-chain fatty acids, stimulating the growth of health-promoting gut bacteria, and boosting the immune system [(21); (Jha, Fouhse et al.)]. In addition, specific nutrients such as functional amino acids like arginine, cysteine, glutamine, or glutamate, may enhance intestinal mucosa immunity, reduce oxidative damage, stimulate proliferation of enterocytes, and enhance the gut barrier function of weaned pigs (Xiong et al.). Amino acids, which are major nutrients for monogastric animals, are not only obligatory for maintaining the intestinal mucosal mass and integrity, but also for supporting the growth of microorganisms in the gut. Dietary amino acids are the major fuel of the small intestinal mucosa. Particularly, glutamate, glutamine, and aspartate are the primary oxidative fuel of the intestine (Yang and Liao). Trace minerals like copper, zinc, iron, and manganese have also been found to influence gut health parameters (Shannon and Hill). For example, pharmacological concentrations of copper have been shown to enhance growth, while high concentrations of zinc fed to newly weaned nursery pigs reduced the incidence of diarrhea from the proliferation of enterotoxigenic *Escherichia coli* and *Clostridium* and improved gut morphology. As a potential alternative to AGPs, prebiotics including mannan oligosaccharides, b-glucans, and fructans, are gaining more attention to be used in monogastric feeding program as prebiotics have been found to modulate microbial communities and regulate the production of cytokines and antibodies, improving gut development and the overall health of animals (Teng and Kim). Similar to prebiotics, different probiotics alone or in combination with other additives like enzymes have also been tried to improve intestinal health and nutrient utilization (3, 22). Duarte et al. evaluated the symbiotic effect of prebiotic (*Bacillus* sp.) and xylanase enzyme in *E. coli* F18+ challenged weaned pigs. The study found that the feed additives were able to mitigate the negative effects of *E. coli* F18+ infection in pigs fed an antibiotic-free diet and enhanced the growth performance by reducing diarrhea, boosting immune response, and managing oxidative stress in the jejunum. In addition, Ma et al. found that synbiotic supplementation in the maternal diet positively affects the gut health of piglets, including improving nutrient metabolism, reducing oxidative stress, and improving intestinal barrier permeability function. Similarly, Grosu et al. used grapeseed meal (GSM) with bioactive compounds (such as polyphenols, PUFA, DF, minerals, etc.) in pig diets. They found that the grapeseed meal had a selective modulatory effect on several bacterial genera in the colon of pigs challenged with dextran sodium sulfate, as a model for inflammatory bowel diseases, suggesting that the GSM can be used as a potential anti-inflammatory additive in weaned piglets.

In conclusion, there are different dietary components and feed additives that can be used to modulate the intestinal health

and functions of young monogastric animals. It can be a tool for nutritionists to develop a feeding program in the post-antibiotic era. However, types, forms, and dose levels of these dietary components and additives need to be considered to obtain optimum benefits.

AUTHOR CONTRIBUTIONS

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

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

frontiers in Veterinary Science

REVIEW
published: 23 October 2018
doi: 10.3389/fvets.2018.00254

Chicken Gut Microbiota: Importance and Detection Technology

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Sustainable poultry meat and egg production is important to provide safe and quality protein sources in human nutrition worldwide. The gastrointestinal (GI) tract of chickens harbor a diverse and complex microbiota that plays a vital role in digestion and absorption of nutrients, immune system development and pathogen exclusion. However, the integrity, functionality, and health of the chicken gut depends on many factors including the environment, feed, and the GI microbiota. The symbiotic interactions between host and microbe is fundamental to poultry health and production. The diversity of the chicken GI microbiota is largely influenced by the age of the birds, location in the digestive tract and diet. Until recently, research on the poultry GI microbiota relied on conventional microbiological techniques that can only culture a small proportion of the complex community comprising the GI microbiota. 16S rRNA based next generation sequencing is a powerful tool to investigate the biological and ecological roles of the GI microbiota in chicken. Although several challenges remain in understanding the chicken GI microbiome, optimizing the taxonomic composition and biochemical functions of the GI microbiome is an attainable goal in the post-genomic era. This article reviews the current knowledge on the chicken GI function and factors that influence the diversity of gut microbiota. Further, this review compares past and current approaches that are used in chicken GI microbiota research. A better understanding of the chicken gut function and microbiology will provide us new opportunities for the improvement of poultry health and production.

Keywords: chicken, gut function, microbiome, prebiotics, DNA sequencing

OPEN ACCESS

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *23 August 2018*
Accepted: *24 September 2018*
Published: *23 October 2018*

Citation:
Shang Y, Kumar S, Oakley B and Kim WK (2018) Chicken Gut Microbiota: Importance and Detection Technology. Front. Vet. Sci. 5:254. doi: 10.3389/fvets.2018.00254

INTRODUCTION

The integrity of the gastrointestinal tract (GIT) and the gut microbial community play vital roles in nutrition absorption, development of immunity, and disease resistance. Alterations in the GIT microbial community may have adverse effects on feed efficiency, productivity, and health of chickens (1–3). Understanding the roles of the chicken GI microbiota and understanding the current methods used in microbiome research is essential for improving the poultry GI microbiome. Historically, selective culture-based techniques have been used to identify and characterize the microbial diversity of the avian gut. In the last decade, the use of bacterial 16S ribosomal RNA (rRNA) gene sequencing has dramatically improved our understanding of the composition and diversity of the chicken GI microbiota. Modern high-throughput sequencing approaches are capable of rapidly obtaining a complete census of a bacterial community and are a powerful tool that has led to important new insights into the biological and ecological roles of the

GI microbiota. This review aims to summarize avian gut function as well as factors that influence the diversity of the chicken GI microbiota. Furthermore, we have also compared and reviewed past and current approaches used in chicken gut microbiological research.

THE ROLE OF CHICKEN GASTROINTESTINAL MICROBIOTA

The gastrointestinal compartments of chickens are densely populated with complex microbial communities (Bacteria, fungi, Archaea, protozoa, and virus) that are dominated by Bacteria (4). The interactions between the host and the chicken GI bacterial microbiome have been extensively studied and reviewed by many research groups (5–9) and are now considered to play important roles in bird nutrition, physiology and gut development (10, 11).

The gut microbiota can form a protective barrier by attaching to the epithelial walls of the enterocyte and thus reduce the opportunity for the colonization of pathogenic bacteria (12). These bacteria produces vitamins (e.g., vitamin K and vitamin B groups), short chain fatty acids (acetic acid, butyric acid and propionic acid), organic acids (e.g., lactic acid) and antimicrobial compounds (e.g., bacteriocins), lower triglyceride, and induce non-pathogenic immune responses, which provide both nutrition and protection for the animal (2, 12–14). On the other hand, the GI microbiome can also be a source of bacterial pathogens such as *Salmonella* and *Campylobacter* which can disseminate to humans or act as a pool for antibiotic resistance and transmission and therefore may pose a serious threat to public health (5, 8, 15).

A normal gut microbial community has benefits and costs to the host (1, 13). The primary benefits that are provided by commensal microbiota are competitive exclusion of pathogens or non-indigenous microbes (13), immune stimulation and programming, and contributions to host nutrition. Earlier reports have established that conventionally raised animals are far less susceptible to pathogens when compared with germ-free animals (16). Furthermore, commensal microbiota can stimulate the development of immune system including the mucus layer, epithelial monolayer, the intestinal immune cells (e.g., cytotoxic and helper T cells, immunoglobulin producing cells and phagocytic cells), and the lamina propria (13, 17, 18). These tissues build barriers between the host and the microbes and combat undesirable gut microorganisms. In the distal gut (i.e., ceca and colon), the microbiota also produces energy and nutrients such as vitamins, amino acids, and short chain fatty acids (SCFA) from the undigested feed, which eventually become available for the host (1, 13). These SCFA have bacteriostatic properties that are capable of eliminating foodborne pathogens, such as *Salmonella* spp. (19). The SCFA are also a source of energy to the animals and can further stimulate gut epithelial cell proliferation, thus increasing the gastrointestinal absorption surface (13). It has also been established that SCFA production lowers the pH of colon, which inhibits conversion of bile to secondary bile products (20). In addition, gut microbiota also contributes to metabolism of host nitrogenous compounds. For example, cecal bacteria can convert uric acid to ammonia, which is subsequently absorbed by the bird and further used to produce amino-acids such as glutamine (21). Furthermore, some of the nitrogen from the diet gets incorporated into bacterial cellular protein and therefore, bacteria themselves can be a source of proteins/amino-acids (22).

In contrast, commensal microbiota also incurs cost to the host. In the proximal gut (gizzard and small intestine), microbes compete with the host for energy and protein. In both the proximal and distal gut, microbes produce toxic metabolites (e.g., amino acid catabolites) and catabolize bile acids, which may depress growth and decrease fat digestibility of the birds, respectively (1). In the presence of microbiota, the gut mucus layer increases mucin secretion and epithelial cell turnover rate, thereby keeping the GI tract lubricated while preventing microorganisms from invading intestinal epithelial cells of the host. The intestinal immune system is also more developed and secretes IgA, which specifically binds to bacterial epitopes, helps in regulating bacterial composition in the gut (23, 24). While generally beneficial, these processes do increase the demand for energy and protein from the host and therefore have an influence on the growth performance of the birds.

An imbalanced gut microbiota is often referred to as dysbiosis. Dysbiosis can been defined as qualitative and/or quantitative imbalance of normal microbiota in the small intestine, which may lead to a sequential reaction in the GIT, including reduced intestinal barrier function (e.g., thinning of intestinal wall) and poor nutrient digestibility, and therefore, increasing the risk of bacterial translocation and inflammatory responses (25). Both non-infectious and infectious stressors can lead to dysbacteriosis. The non-infectious factors include environmental stressors, nutritional imbalances, dietary changes, mycotoxins, poor management, enzymatic dysfunction, or host genetics (25). Infectious factors include viral or bacterial challenge, coccidiosis, or toxic metabolites produced by harmful microorganisms such as *Clostridium perfringens*.

The gastrointestinal microbiota can further be classified as the luminal microbiota and the mucosal microbiota (2). The composition of the luminal microbiota is determined by available nutrients, presence of antimicrobial substance and the feed passage rate. The composition of the mucosal-attached microbiota is affected by several host factors, such as expression of specific adhesion sites on the enterocyte membrane, secretion of secretory immunoglobulins, and mucus production rate. The luminal microbiota and the mucosal-associated microbiota of course also influence each other (2) and therefore, it is important to recognize that diet can alter both luminal and mucosal-attached microbiota to influence gut health. To our knowledge, there is no study to date which has compared the taxonomic composition or metabolic functions of these two microbial habitats. However, it would be interesting to study and analyse the variations between the bacterial communities of the mucosa and lumen throughout the different GI sections. Furthermore, studying the mucosal-associated bacterial community will be important to understand the host mucosal responses as any alterations in mucosal immunity may have serious implications on bird's health (26).

THE DIVERSITY OF CHICKEN GUT MICROBIOTA

The GI tract of the chicken harbors a diverse bacterial community in which each bacterium is adapted to its own ecological niche and synergistically lives with other bacterial species in the same community. The composition and function of these communities has been shown to vary depending on the age of the birds, location in the GI tract and on the dietary components (6, 18, 27–29).

BIRD AGE

The age of the birds is one of the most important factors that influences GI bacterial composition, cell density, and metabolic function. Significant changes in the taxonomic composition of gut microbiota have been studied using both DNA fingerprinting (30) and high-throughput sequencing approaches (31) and are well-reviewed by many research groups (28, 32–34). Ballou et al. (35) and our recently published data (5) indicates that 1 day post-hatch broiler chicks already have a microbial community in their GIT. There are also successional changes in the composition of the GIT microbiome, due to the replacement and establishment of more stable bacterial taxa, as the bird advances in age (30, 36). Lu et al. (30) discovered that the GIT of chicken at 3 days of age contained *L. delbrueckii*, *C. perfringens* and *Campylobacter coli*, whereas from 7 to 21 days of age, *L. acidophilus*, *Enterococcus,* and *Streptococcus* were more common. At 28 and 49 days of age, the GI tract contains *L. crispatus*, but the composition is significantly different from other ages (30). In other work, successional changes in the gut microbial community measured with HT-NGS technology has shown that the relative abundance of *Clostridium* was higher as the bird aged, whereas lactobacilli was low throughout the growth cycle. This variability in results may be due to sample types (feces vs. cecum), and/or conventional microbiological and molecular methods that have limited coverage and accuracy compared to high-throughput NGS platforms which offer higher coverage and depth in determining microbial community. High-throughput sequencing technologies, such as targeted amplicon sequencing and shotgun metagenomic sequencing, have become more common to analyze the gut microbial composition and functions throughout the life span of broilers, but we are still at initial stage of analyses and there is a breach in knowledge regarding host morphological development, and functional properties of the gut microbiome as the bird ages.

GASTROINTESTINAL TRACT

The GI tract of the chicken includes the crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, large intestine, and cloaca (32). Each GI tract section has different metabolic functions that shape the microbial community (**Table 1**), and therefore it is important to consider sampling location and study design. The chicken crop harbors 10^8 to 10^9 cfu/g bacteria, which is usually dominated by lactobacilli (28, 37). However, large variations in microbial composition among individual broilers fed on the similar diet has been observed by Choi et al. (44) due to difference in time between feeding and sampling. In the gizzard, the concentration of bacteria is similar to the crop, but bacterial fermentation activities are low mainly because of the low pH. The majority of bacteria in the gizzard are lactobacilli, enterococci, lactose-negative enterobacteria, and coliform bacteria (28). Among the small intestinal segments, the bacterial density is the lowest in the duodenum due to short passage time and a dilution of digesta by secreted bile (45). The duodenal bacterial community mainly consists of clostridia, streptococci, enterobacteria, and lactobacilli (46). Ileum microbiota have been studied the most among the small intestine segments. Lu et al. (30) assessed the ileal bacterial community by examining 16S rRNA gene sequences and found *Lactobacillus* as the major group (70%) followed by members of the family *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%) (30). In corroboration, our recent article also showed lactobacilli as the predominant genus in the ileum (5). Compared to the ileum, the cecum harbors a more diverse, rich and stable microbial community including anaerobes (47, 48). Oakley et al. (18) have documented significant changes in cecal microbial communities from day of hatch to 6 weeks of age in commercial broilers (18, 27) and also significant differences in cecal vs. fecal samples from a single individual (27). Typically, richness and diversity in the cecum increase during these 6 weeks, and the taxonomic composition of the community quickly shifts from Proteobacteria, Bacteroides, and Firmicutes, to almost entirely Firmicutes by 3 weeks of age (18, 27). However, Kumar et al. (5) found that Firmicutes were the most abundant phylum in both ceca and ileum at all the ages (day 0 to day 42) except d 42 in the ceca where Bacteroidetes were abundant. The differences in bacterial composition can be expected due to differences in the nucleic acid extraction protocol, primers, sequencing approach, environmental factors, dietary treatment/ composition, breed, and geographical conditions. In addition to sample types, an adequate sample size is also needed for a proper study design. Higher individual variation in sample types (crop samples) results in higher sample size compared to cecal samples to find the potential differences (49).

Feed processing approaches, feed components and additives are also known to have an effect on the gut microbial community. Knarreborg et al. (50) stated that mash feed lowers the number of *Enterococcus* spp. and coliforms but increases *Lactobacillus* spp. and *C. perfringens* in the broiler ileum, when compared to pellet feed (50). Corn favors low percent G + C clostridia, enterococci and lactobacilli, whereas wheat favors higher percent G + C bifidobacteria (29). Kumar et al. (5) reported low abundance in Firmicutes and high abundance in Bacteroidetes from day 0 to day 42 as birds were shifted from starter diet to finisher diet and argued that members of the phylum Bacteroidetes are vital for fermenting starch to simple sugars. Furthermore, feed supplementation, such as fermentable sugars (prebiotics), can also have an impact on the composition and diversity of chicken gut microbiota.

TABLE 1 | Spatial distribution of most common and abundant bacterial taxa (phylum, order (o), family (f), genus) in the gastro-intestinal tract of chickens irrespective of age, diet and technique differences.

GIT location (per g of content)	Bacterial phyla	Bacteria genera	Techniques used	References
Crop (10^8-10^9/ g)	Firmicutes	*Lactobacillus*	16 S rDNA sequencing and cloning	(37)
	Actinobacteria	*Bifidobacterium*		
	Proteobacteria	*Enterobacter*		
Gizzard (10^7-10^8/ g)	Firmicutes	*Lactobacillus, Enterococcus*		
Small Intestine (most of the studies are conducted in Ileum; 10^8-10^9/ g)	Firmicutes/ Low G+C, Gram positive bacteria	Enterococcaceae (f.), *Enterococcus*, Clostridiaceae (f.), *Clostridium*, Lactobacillacae (f.) *Lactobacillus*, *Candidatus Arthomitus*, *Weisella*, *Ruminococcus*, *Eubacterium*, *Bacillus*, Stapylococcaceae (f.), *Staphylococcus*, *Streptococcus*, *Turicibacter*, *Methylobacterium*	Finger printing: T-RFLP, 16S rRNA qPCR, Cloning and sequencing and Next Generation Sequencing	(5, 30, 38–40)
	Cytophaga/ Flexibacter/ Bacteroides/ High G+C, Gram positive bacteria	Bacteroidaceae (f.), *Bacteroidetes*, *Flavibacterium*, *Fusobacterium*, *Bifidobacterium*		
	Protobacteria	*Ochrobaterium*, *Alcaligenes*, *Escherichia*, *Campylobacter*, *Hafnia*, *Shigella*,		
	Actinobacteria/ Cyanobacteria	*Corynebacterium*		
Caeca (10^{10}-10^{11}/ g)	Methanogenic Archaea (0.81%)	*Methanobrevibacter, Methanobacterium, Methanothermobacter, Methanosphaera, Methanopyrus, Methanothermus, Methanococc*	Finger printing: T-RFLP, 16S rRNA qPCR, Cloning and sequencing and Next Generation Sequencing	(5, 30, 38, 39, 41–43)
	Firmicutes/ Low G+C, Gram positive bacteria (44–56%)	*Anaerotruncus,* Ruminococcaceae (f) *Ruminoccoccus, Faecalibacterium, Lachnospirceae*, *Bacillus, Streptococcus,* Clostridiales (o), *Clostridium, Megamonas, Lactobacillus*, *Enterococcus*, *Weisella*, *Eubacterium*, *Staphylococcus*, *Streptococcus*,		
	Bacteroides/ Cytophaga/ Flexibacter/ High G+C, Gram positive bacteria (23–46%)	Rikenellaceae (f), *Bacteroidetes*, *Alistipes, Fusobacterium*, *Bifidobacterium, Flavibacterium*, *Odoribacter*,		
	Actinobacteria	*Corynebacterium*		
	Proteobacteria (1–16%)	*Ochrobaterium*, *Alcaligenes*, *Escherichia*, *Campylobacter*		
Large Intestine	Firmicutes	*Lactobacillus*	16 S rDNA sequencing and cloning	(37)
	Proteobacteria	*Escherichia*		

PREBIOTICS

The use of prebiotics as dietary modulators has been shown to have positive effects on some bacterial taxa in the colon (51). For example, Fructooligosaccharides (FOS) and Galactooligosaccharides (GOS) increased the population of *Bifidobacterium* and *Lactobacillus* (52, 53). *In vitro* studies have shown that fecal slurries which were incubated with oligofructose and inulin exhibited an increase in bifidobacteria populations in the human large intestine, whereas potential pathogens such as *Escherichia coli* and *Clostridium* spp. were maintained at lower levels (54). The majority of bifidobacteria strains (e.g., *B. fiagilk*, *B. thetaiotaomicron*, B. *vulgatus*, *B. dktasonk,* and *B. ovatus*) except *B. bifidum*, can utilize FOS as a growth and fermentation promoter (55). These bacteria secrete ß-fructosidase enzyme that can readily degrade and ferment FOS. However, microorganisms such as *E. coli* and *C. perfringens* are not able to exploit FOS as a fermentative carbohydrate source. Rats that were fed dietary FOS have shown a temporary boost in lactic acid-producing bacteria and a long-term elevation in cecal butyric acid (56). Dietary inclusion of FOS reduced *C. perfringens* and *E. coli* populations and increased the diversity of *Lactobacillus* in the broiler GIT (57). Patterson et al. (58) assessed the effects of thermal ketoses oligosaccharides on cecal microbial populations

of broiler chickens. The results showed that cecal bifidobacteria and lactobacilli concentrations were increased 24-fold and 7-fold, respectively, in ketoses supplemented diet compared to controls. Another type of prebiotics, mannooligosaccharides (MOS), are proposed to have different mechanisms of action (58). They can (1) bind to potential pathogenic Gram-negative bacteria (e.g., *E. coli* and *Salmonella*) which possess type-1 fimbriae (mannose-sensitive lectin), to prevent and dislocate the pathogens from attaching to the gut wall, (2) have immune modulatory effects based on the antigenicity features of mannan and glucan components, (3) modulate intestinal morphology, and (4) enhance the expression of mucin and reduce enterocyte turnover rate (59). The effects of prebiotics on lower GI tract include: (1) serving as food and fermentation sources for cecal and colonic microbiota, (2) production of fermentation end products (e.g., SCFAs), (3) stimulation of saccharolytic fermentation, (4) acidification of the large intestine content, (5) hyperplasia of the cecal and colonic epithelium, (6) stimulation of colonic hormonal peptides secretion, and (7) acceleration of ceco-anal transit (51).

Other than age, GIT location, and prebiotics, breed and sex of the bird can also have a large impact on the intestinal microbiota (34). In addition, it has been well-documented that environmental factors (biosecurity level, housing, litter, feed access, and climate) can also substantially influence the gut bacterial composition. Therefore, data interpretation and outcome of research largely depends on the study design. Best practices for research reporting include providing details regarding host and environmental factors that can enable researchers to do meta-analyses to better understand nutritional, microbiome, and environmental factors that can be modulated to improve bird performance and health.

DISCOVERY OF CHICKEN GUT MICROBIOTA BY MOLECULAR APPROACHES

Classical culture-based methods have historically been widely used to study the chicken gut microbiota. However, these methods are highly selective to cultivable bacteria under specific conditions (60). A majority of bacteria remain uncultured (29). Over 30 years ago, the term "the great plate count anomaly" was coined to reflect laboratory calculations that a very small minority (0.1–1%) of microbial taxa present in a given sample could be cultured (61). Similarly, over 10 years ago, it was observed that of 52 microbial phyla recognized at the time, only half of them had even a single cultivated representative, supporting the description of an "uncultivated majority" (62). Therefore, the richness (number of species) and diversity (number of species weighted by their relative abundance) of intestinal bacteria have

TABLE 2 | 16S rRNA-based molecular approaches for studying microbial ecology in the chicken gut (64–67).

Approach	Sample capacity	Applications	Challenges and confines	Advantage
SEQUENCING ANALYSIS TARGETED AMPLICONS				
16S rDNA sequencing	Limited w/ Sanger sequencing. Non-limiting w/ next-gen sequencing	16S rRNA gene sequence, wide range identification of genus/ species/ strain, as database rich	Bias in DNA extraction and Primers, PCR amplification and numbers of clones, costly, laborious	Each clone represents single molecule of rDNA, Allows precise identification of a relatively small number of OTUs
Real-time PCR (RT-PCR)	Limited	Specific gene expression in targeted groups, high in sensitivity	Bias in DNA extraction and RT-PCR, costly	
PROFILING APPROACHES				
Fingerprinting DGGE[a], TGGE[b], TTGE[c], T-RFLP[d], and SSCP[e]	Good	Amplify common 16S rDNA sequences, diversity profiles within the targeted group, rapid, comparative	Bias in DNA extraction, primers, inter and intra laboratory reproducibility remains a major challenge. Provides relatively coarse taxonomic resolution, data usually is qualitative or semi-quantitative	Amplicons may be used from sequencing
GENE QUANTIFICATION				
FISH[6]	Limited	Enumeration of the bacterial population	Laborious at the species level	Sensitivity has been improved using fluorescent probes
DNA MICROARRAY TECHNOLOGY				
Diversity arrays	High	Diversity profiles, different gene expression levels	Laborious in development, costly	
DNA microarrays	High	Transcriptional fingerprint, comparative	Bias in nucleic acids extraction and their labeling, costly	

[a]DGGE, denaturing gradient gel electrophoresis; [b]TGGE, temperature gradient gel electrophoresis; [c]TTGE, temporal temperature gradient gel electrophoresis; [d]T-RFLP, terminal restriction fragment length polymorphism; [e]SSCP, single strand conformation polymorphism; [f]FISH, fluorescence in situ hybridization.

been underestimated, and our knowledge of gut microbiota remains incomplete (63).

The development of molecular biotechnology has offered new tools to study the composition, diversity, predicted function and interaction of gut microbiota in different sections of the GI tract. Currently, a variety of molecular techniques are available, each with different strengths and weaknesses. The sample capacity, applications and limitations of some of the most common molecular techniques that can be used to study chicken GI microbial ecology are listed in **Table 2**. Among these methods, high-throughput sequencing of 16S rRNA gene amplicons has quickly become the method of choice. Although this method had been widely used in other research fields, the first report utilizing high-throughput sequencing of 16S rRNA genes for studying the population of microbial communities and their interactions in the chicken gut was published in 2013 (64).

The 16S rRNA molecule is a small subunit of the ribosome that possesses regions of sequence similarity that are highly conserved across all bacteria. To amplify these genes, microbial DNA is extracted from fecal or digesta samples, and broad-range primers, which target conserved regions of the 16S rRNA gene, are used for polymerase chain reaction (PCR) amplification (29). Sequencing of these amplified products (amplicons) can discriminate among bacteria, generally to the genus or species level (65, 68), and the relative abundance of each sequence reflects the relative abundance of that bacterium in the original sample. Thus, sequencing of 16S rRNA genes provides a true census of a bacterial community by defining the types of bacteria present in a sample and their relative abundances. Because of the high richness and diversity of intestinal bacterial communities, it has only been in the last few years that DNA sequencing technology has matured to the point where we can now completely census these complex communities. Beginning in 2008, technical advances in sequencing allowed for several orders of magnitude more sequences to be collected than was previously possible—in a single study the authors deposited as many 16S rRNA sequences in the GenBank database as had been generated historically up to that point (69). With these profound methodological advances and enormous new datasets, it is now possible to easily and accurately take a census of an intestinal sample to determine, for example, how the microbiome responds to different feed additives, husbandry conditions, or disease states (**Figure 1**).

High-throughput or next generation sequencing (NGS), is a powerful tool to investigate the biological and ecological role of gut microbiota (64). NGS has become a convenient, rapid, accurate and inexpensive method for genomic research (66, 70). Current NGS platforms offer high throughput, fast turn-around times, and low costs. Among these platforms the Illumina HiSeq and MiSeq instruments are two of the most frequently used systems in recent chicken gut microbiome and metagenomic research. Despite many advantages, these platforms suffer from limitations including short read assembly and high cost (71). Third-generation sequencing platforms such as single molecule real-time (SMRT) and nanopore sequencing require less time for DNA preparation (no PCR) and are cost effective (71). As these platforms continue to mature, their adoption will surely lead to new understanding of the poultry GI microbiome.

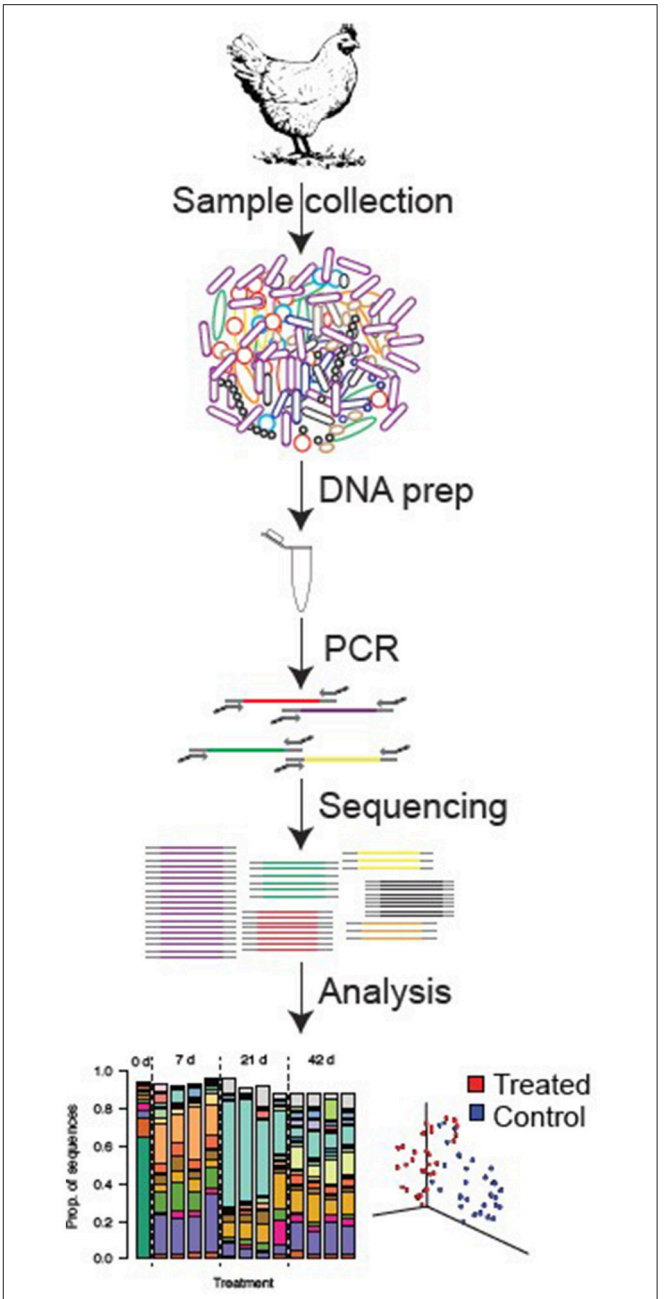

FIGURE 1 | Standard procedure from sample collection to sequencing analysis in poultry gut.

Following sequencing, bioinformatic analyses of sequence data requires open source platforms such as QIIME or mothur which utilize public databases (GreenGenes, Ribosomal Database Project and SILVA (72–76) to perform taxonomic assignment. Predictions of metabolic functions based on taxonomic identities from 16S rRNA gene sequences can be further obtained using algorithms such as PICRUSt and Tax4Fun (77, 78). To catalog the gene functions or analysis of individual genomes, metagenomic

TABLE 3 | : Different omics approaches applied in understanding gut microbial community and functions.

Omic approach	NGS platform	Research focus	Diet	Breed	Sample type	Sampling time	References
Meta-proteomics		Correlation between metagenome and proteome of a healthy chicken	Attlee's non-medicated poultry feed	White Leghorn chickens	Feces	18 wesk	(81)
		Dietary effect of mineral phosphorus and microbial phytase on protein inventory of the microbiome	3 diets with P derived from plant source (BD-), 3 diets with P supplementation (BD+), BD- and BD+ supplemented with 0, 500 and 12,500 U/kg of phytase	Ross 308	Crop, ceca	25 day	(82)
Meta-genomics	454 pyrosequencing	Role of microbial community and functional gene content in caeca	Commercial chicken feed (Eagle milling)	Ross x Ross	Ceca	28 day	(41)
	454 pyrosequencing and shotgun metagenomics	Analyze effects of subtherapeutic doses of antimicrobials and anticoccidial on bacterial popoulation	Basal diet for 7 day followed by supplementation of monensin, monensin + virginiamycin or tylosin	Ross x Ross	Ceca	0,7,14,35 day	(7)
	MiSeq 2000	Deep microbial community profiling in the caeca and functional analysis	Wheat based diet with 5% maize (no antibiotics)	Ross x Ross	Ceca	42 day	(42)
	Shotgum metagenomics	Comparing fecal microbiome of low and high FCR brids	Growers diet	Broiler strain 'MY'	Feces	49 day	(83)
	MiSeq 2000	Determining protein expression in the cecal microbiota in chickens of selected ages and in 7-day-old chickens inoculated with different cecal extracts on the day of hatching	Common mashed/granulated MINI feed	ISA Brown egg-laying hybrid	Ceca	Donor (1,3,16,28,42 week); Recipient (7 day old)	(84)
	HiSeq 2000	Metanalysis of antibiotic resistance genes and their co-occurrence with genetic elements	Commercial diet	NM	Feces	20, 80 day	(85)
	454 Genome Sequencer	Determine effect of diet on antibiotic resistance genes of gut microbiome	Basal diet with chlortetracycline and organic diet w/o antibiotic	Brown Leghorn	Feces	90 day	(9)
	HiSeq2000	Existence, diversity and abundance of antibiotic resistant genes	Commercial diet	NM	Feces	6 week broilers and 52 week laying hens	(86)
	MiSeq/ HiSeq4000	Metagenomic analysis for changes in bacterial community, antibiotic resistance genes in gut microbiota	Commercial diet with low and therapeutic dose level of chlortetracycline	NM*	Feces	0,5,10,20 day	(87)
16S rRNA targeted	454 pyrosequencing	Determine fecal microbiota subjected to repeated cycle of antimicrobial therapy	Basal diet with single cycle and repeated cycle of antibiotic therapy	Female Lohmann Brown layers	Feces	0,1,2,3,4,7,8,9,10, 11,14,14,16,17, 18,21,22 day	(48)
	MiSeq	Influence of genetic background of host on microbiome	Corn-soybean diet	NM	Feces	245 day females and males	(88)
	454 pyrosequencing	Investigate poultry-associated microbiome and food pathogens from farm to fork	Commercial diet supplemented with sub-therapeutic dose of antibiotic growth promoters	Ross x Hubbard	Feces, ceca, litter, carcass	6 week	(18)

(Continued)

TABLE 3 | Continued

Omic approach	NGS platform	Research focus	Diet	Breed	Sample type	Sampling time	References
	MiSeq	Effect of host genetic on microbiome and correlation with body weight	Corn-soybean	NM	Feces	258 d LW[$] and HW males and females	(89)
	MiSeq	Effect of age o the gut microbial dynamics	Commercial broiler diet	Cobb 500	Ilea, ceca	7,14,21,42 d	(31)
	MiSeq v2 (500 cycle)	Investigate role of prebiotics on microbiome of pasture flock raised birds	Basal diet	NM	Ceca	8 weeks	(90)
	HiSeq 2000	Determine link between variation in fatness and gut microbiota	Commercial diet	NM	Feces	37 to 40 Week, from fat and lean chickens	(91)
	HiSeq 2000	Comparison of fat and lean chickens on gut microbiota	Commercial diet		Feces	35 weeks	(92)
	454 sequencing	Evaluate effect of diet and age on gut microbiota	Wheat-based diet, Maize-based diet or maize-based concentrates supplemented with 15% or 30% crimped kernel maize silage	Ross 308	Crop, gizzard, ilea, ceca	8,15,22,25,29,36 day	(93)
	MiSeq	Effect of antibiotic withdrawal from broiler feed on gut microbial community	Commercial diet with and without Bacitracin	Cobb 500	Ceca, ilea	0,7,14,22,35,42 day	(5)
	MiSeq600	Examine the effect of age, sample type, flock and successive flock cycles on consistency and predictability of the bacterial community	NM	Cobb 500	Ceca, ilea	7,14,21,28,35,42 day	(94)

[*] *NM, not mentioned,* [$] *LW, low weight and HW, high weight.*

or metatranscriptomic approaches (in which genes or transcripts respectively are sequenced directly with no PCR) can be used to provide information on community diversity, structure and metabolic functions, or gene expression (79). Bioinformatic analyses of such datasets are more complex than 16S amplicon data and typically involve a sequence assembler such as Velvet (CLC workbench, Newbler version 3.0, Biospace) or MG-RAST. Bacterial taxa and functional groups can be assigned based on Basic Local Alignment Search Tool (BLAST), and gene functions may be analyzed using either Kyoto Encyclopedia of Genes and Genomes (KEGG) or Cluster of Orthologous genes (COG). In the chicken gut microbiome, metagenomics has been used to study the cecum functions, gut response to pathogen challenge, correlations between microbial response and performance parameters, comparison between fat and lean broiler lines, description on virulome, and antibiotic resistance genes (80). Some of the NGS based studies investigating chicken gut microbial community composition and functions in respect to the dietary responses/ antibiotic treatments are depicted in **Table 3**. However, it's difficult to compare all these studies because of variation in NGS platforms used, breed, sample type, sampling method etc. Therefore, a standard protocol is needed for studying the chicken gut microbial community, as available for human microbiome, in order to have comparable results. Currently most metagenomic approaches to studying the chicken GIT are still not affordable for most researchers or veterinarians.

To circumvent some of the confines of sequence-based analysis, proteomic methods have also recently been used to determine the metabolic and functional properties of the microbiome (81, 82). Transcriptomics measures gene transcription *in situ*, providing an accurate reflection of physiological functions even if utmost care is needed during sampling (71). Since there are limited culture collections for poultry strains, increase in bacterial cultures and proper cataloging of their biochemical and genetic properties will facilitate proteomics and other "omics" approaches.

CONCLUSION

In recent years, significant progress has been made in understanding the taxonomic composition of the GI microbiome and its contributions to gut health. It is important for future studies to apply multi-omics approaches in order to increase our understanding of the role of the microbiome in nutrition, health, disease, and productivity. Progress in this field will help us to better understand how to manage the gut microbiota based on the environment, diet and physiology changes of the birds, and will further advance our understanding on the modification of microbiota-associated metabolic pathways, thus providing new opportunities for improving overall health of the poultry.

AUTHOR CONTRIBUTIONS

YS and SK wrote this review manuscript. BO reviewed literature and the manuscript and provided critical suggestion and comments. WK decided a review topic, reviewed literature, and provided critical review and suggestion/comments.

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

frontiers in Veterinary Science

REVIEW
published: 30 October 2018
doi: 10.3389/fvets.2018.00245

Review: Roles of Prebiotics in Intestinal Ecosystem of Broilers

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In recent years, prebiotics have been considered as potential alternatives to antibiotics. Mechanisms by which prebiotics modulate the ecosystem of the gut include alternation of the intestinal microbiota, improvement of the epithelium, and stimulation of the immune system. It is suggested that the administration of prebiotics not only influences these aspects but also regulates the interaction between the host and the intestinal microbiota comprehensively. In this review, we will discuss how each prebiotic ameliorates the ecosystem by direct or indirect mechanisms. Emphasis will be placed on the effects of prebiotics, including mannan oligosaccharides, β-glucans, and fructans, on the interaction between the intestinal microbiota, gut integrity, and the immunity of broilers. We will highlight how the prebiotics modulate microbial community and regulate production of cytokines and antibodies, improving gut development and the overall broiler health. Understanding the cross talk between prebiotics and the intestinal ecosystem may provide us with novel insights and strategies for preventing pathogen invasion and improving health and productivity of broilers. However, further studies need to be conducted to identify the appropriate dosages and better resources of prebiotics for refinement of administration, as well as to elucidate the unknown mechanisms of action.

Keywords: prebiotic, broilers, immunity, microbiota, mannan oligosaccharides, β-glucans, fructans

OPEN ACCESS

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *18 May 2018*
Accepted: *17 September 2018*
Published: *30 October 2018*

Citation:
Teng P-Y and Kim WK (2018) Review: Roles of Prebiotics in Intestinal Ecosystem of Broilers. Front. Vet. Sci. 5:245. doi: 10.3389/fvets.2018.00245

INTRODUCTION

Since the use of antibiotic growth promoters was banned by the EU on January 1st, 2006, several feed additives have been studied as alternatives to antibiotics, such as probiotics, prebiotics, synbiotics, and herbal medicines (1). Among these feed additives, prebiotics have been studied and supplemented broadly into broiler diets in recent years. Gibson and Roberfroid (2) defined a prebiotic compound as a non-digestible food ingredient utilized by intestinal microbiota. It beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal tract, consequently improving gut health and hosts' intestinal microbial balance. Gibson et al. (3) revised the definition and defined a prebiotic as a selectively fermented ingredient that allows specific changes in the composition and/or activity in the intestinal microbiota that confers benefits upon the host's well-being and health. Some researchers also confined prebiotics to indigestible oligosaccharides (4). Ideal characteristics of prebiotics were described by Patterson and Burkholder (5): (1) prebiotics should not be hydrolyzed by animal gastrointestinal enzymes, (2) prebiotics cannot be absorbed directly by cells in the gastrointestinal tracks, (3) prebiotics selectively enrich one or limited numbers of beneficial bacteria, (4) prebiotics alter the intestinal microbiota and their activities, and (5) prebiotics ameliorate luminal or systemic immunity against pathogen invasion.

The ecosystem of the gut is composed of three crucial elements: (1) microbial community, (2) intestinal epithelial cells, and (3) immune system (6). Generally, prebiotics can be fermented by health-promoting bacteria in the intestine, producing lactic acid, short-chain fatty acid (SCFA), or some antibacterial substances, such as bacteriocine against pathogenic species (7). These products may not only benefit the intestinal microbial structure but also improve the integrity of intestinal epithelial cells, which further increase the absorption of nutrients and enhance the growth performance of animals (8).

Intestinal microbiota are influenced by various factors, including diet, gender, background genotype, housing environment, litter, and also age of birds (9). These factors can alter the abundance of dominant bacterial phyla and families in each part of the intestine. For instance, gut microbiota in young chickens changed rapidly with increase of age. *Clostridiaeae* and *Enterobacteriaceae* are two dominant families in the ileum of 7 day-old chickens, whereas *Lactobacillaceae* and *Clostridiacea* represent the common families in the ileum of 35 day-old birds (9). However, the balance of intestinal microbiota is alterable. Application of prebiotics in diets could establish a healthy microbial community in the intestine of young broilers by enhancing the abundance of *Lactobacilli* and *Bifidobacteria* and reducing the titers of *Coliform* (10, 11).

Furthermore, the modulation of intestinal microbiota is associated with immune responses. On the one hand, inhibiting pathogen colonization by prebiotics can decrease detrimental molecules produced by pathogenic bacteria, which have been known as exogenous signals (12). These signals are also called pathogen-associated molecular patterns (PAMPs). The PAMPs can be recognized by pattern recognition receptors (PRR), including toll-like receptors (TLRs) and NOD-like receptors (NLRs), which are expressed on the surface of sentinel cells (13). Once PRRs recognize PAMPs, sentinel cells, such as epithelial cells, macrophages, mast cells, and dendritic cells, are activated, producing cytokines for the regulation of further innate immune responses. On the other hand, prebiotics can act as non-pathogenic antigens themselves. They can be recognized by receptors of immune cells, which consequently modulate host immunity beneficially.

Various prebiotics are composed of diverse sugar units. Therefore, each prebiotic may influence the animals differently. Here, we reviewed studies of broilers that discuss the effects of prebiotics on their underlying mechanisms of action. We will discuss the direct or indirect mechanisms by which prebiotics ameliorated the ecosystem of the chicken gut. Emphasis will be placed on the impacts of mannan oligosaccharides, β-glucans, and fructans on the interaction between the intestinal microbiota, immunity, and the integrity of the epithelial cells (**Figures 1**–**3**).

MANNAN OLIGOSACCHARIDES (MOS)

Most of the mannan oligosaccharide (MOS) products are derived from yeast cell walls (*Saccharomyces cerevisiae*) and are rich in mannoproteins (12.5%), mannan (30%), and glucan (30%) (14, 15). Mannan oligosaccharides are known for their ability to bind pathogenic bacteria, which possess type-1 fimbriae, such as *E. coli* and *Salmonella* species (16). By blocking bacterial lectin, MOS could reduce colonization of these pathogens in the intestine of animals (17). Previous studies indicated that supplementation of MOS from 0.08 to 0.5% could alter cecal microbial community composition by increasing total anaerobic bacteria, *Lactobacillus* and *Bifidobacterium*, and decreasing *Salmonella*, *E. coli*, *Clostridium perfringens*, and *Campylobacter* (14, 16, 18–23). Apart from its effects on cecal microbiota, MOS also improved microbial community in other sections of the intestine, including the jejunum, the ileum, the jejunal mucosa, the ileal mucosa, and the ileocecal junction (11, 22, 24–26). It is interesting to note that MOS increased cecal *Bacteroidetes* in 7 and 35 day-old broilers (23, 27). Genus *Bacteroides* have been known for their strong metabolic activity. They can efficiently ferment indigestible polysaccharides to SCFA and, consequently, improve nutrient absorption and protect the host from pathogen infection (28). In previous studies, shown in **Table 1**, *Lactobacillus* species were the main species influenced by MOS. Mannan oligosaccharides increased the prevalence of ileal *L. acetotolerans*, *L. delbrueckii* subsp. *lactis, L. sakei* subsp. *sakei.* and cecal *L. ingluviei, L. mucosae, L. salivarius,* and *L. crispatus* (23, 29). Among these *Lactobacillus* species, *L. crispatus* was reported to have anti-*E. coli* and anti-*Salmonella* activities, whereas *L. salivarius* was mentioned to have the ability to limit *Salmonella* colonization (30, 31). The anti-pathogenic characteristics of *Lactobacillus* may be the reason why MOS reduced the numbers of *E. coli* or *Salmonella* in the intestine, ameliorating bacterial infection in pathogen-challenged broilers (14, 16, 19).

In addition, higher levels of intestinal *Lactobacillus* in birds fed with MOS may further result in the improvement of gut health status. Mannan oligosaccharides have been reported to increase villus height and surface area, decrease crypt depth, induce numbers of sulphated-acidic goblet cells, and upregulate gene expression of *MUC*, which is related to mucin secretion (10, 11, 14, 32–35) (**Table 2**). It has been reported that sulphated-acidic goblet cells are less degradable by the pathogen's glycosides (43, 44). Therefore, they can provide stronger protection against pathogens for the host. Similarly, Cheled-Shoval et al. (36) reported that *in ovo* administration of MOS enhanced villus area and proliferation of goblet cells. The greater numbers of goblet cells were able to increase the gene expression of *MUC*, synthesizing and secreting more mucin, which plays an important role as the first line of defense. Mucin can trap pathogens or impede them from invading epithelial cells (45). Thus, it is hypothesized that MOS establishes a bidirectional interaction: the increase of *Lactobacillus* counts may improve intestinal development, whereas mucin produced by goblet cells can conversely limit attachment of pathogens to epithelial cells.

The effects of MOS on immunity of broilers are presented in **Table 3**. TLR4 and TLR2 were upregulated in the ileum or cecal tonsils by 0.2% MOS supplementation (50). It indicated that MOS could be recognized by both TLR4 and TLR2. Similar to mammalian TLR4, chicken TLR4 (chTLR4) mRNA has been found in a wide range of cells, particularly in macrophages and heterophils (61). TLR4 is a receptor that recognizes

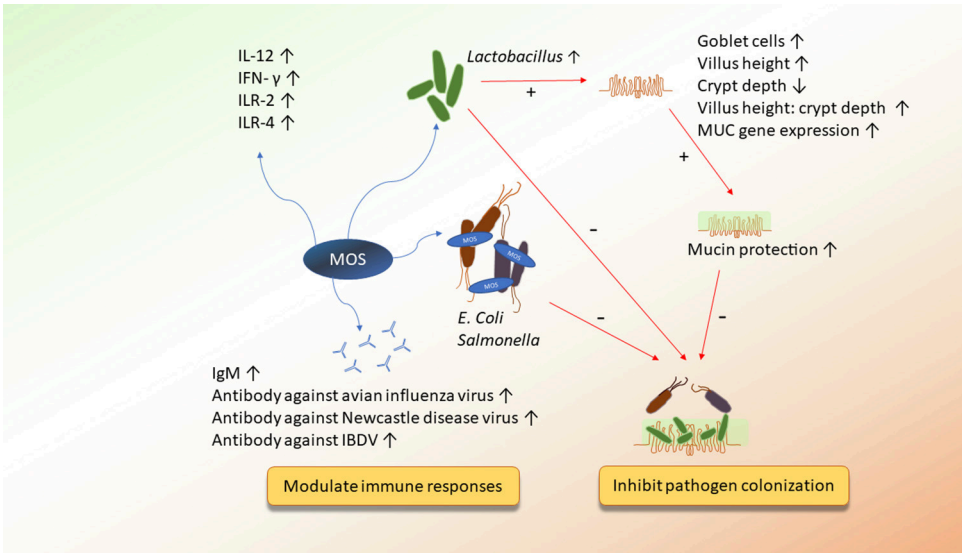

FIGURE 1 | The potential mechanisms of action of MOS on improving immunity and inhibiting pathogen colonization.

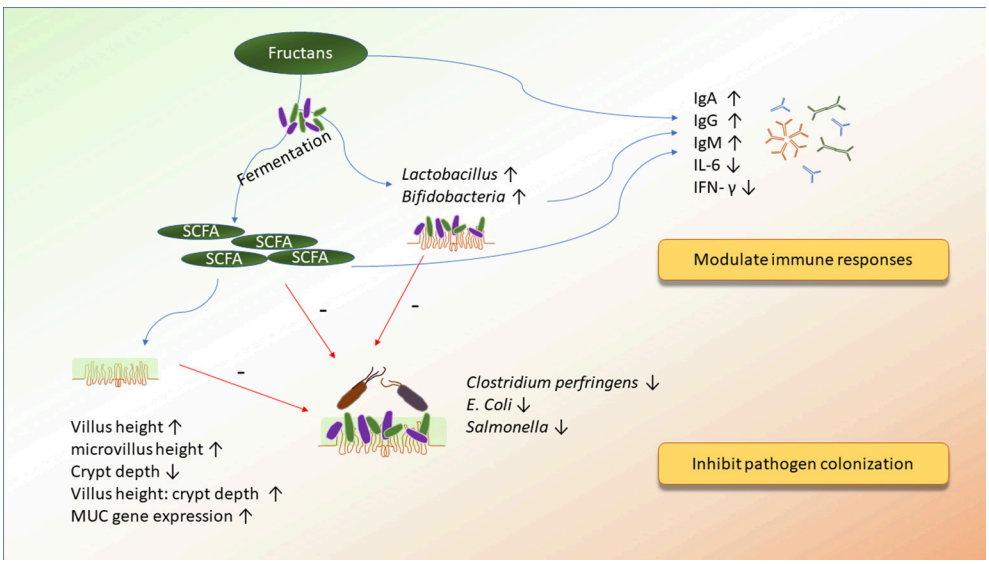

FIGURE 2 | The potential mechanisms of action of fructans on improving immunity and inhibiting pathogen colonization.

lipopolysaccharide (LPS) in mammals. After recognizing LPS, immune cells could produce high levels of nitric oxide and pro-inflammatory cytokines against pathogenic bacteria. Thus, it was suggested that reducing the exposure of LPS from *E. coli* by MOS could downregulate gene expression of chTLR4 and inhibit pro-inflammatory immunity (50). However, molecules of MOS can be recognized by TLR4 as well. It was reported that MOS may act as a pro-inflammatory factor that upregulates TLR4 gene expression and induces innate immune responses (62).

However, chicken TLR2 (chTLR2) has approximately 50% amino acid identity to mammal TLR2, which can recognize a broad variety of PAMPs, including lipoproteins, aribinomannan, and peptidoglycan fugal zymosan (61). TLR2 may recognize MOS as well, which leads to the pro-inflammatory cytokines' cascade (63). A previous study demonstrated that supplementation of 0.2% MOS in broiler diets enhances ileal gene expression of interleukin-12 (IL-12) and interferon-γ (IFN-γ) (50). Interleukin-12 is a cytokine that stimulates T-helper type-1 cells (Th1 cells) and triggers IFN-γ to induce proliferation and cytotoxicity of immune cells, such as T cells, natural killer (NK) cells, and macrophages (12). Apart from the upregulation of innate immunity, MOS can impact humoral immune responses by acting as adjuvant of vaccines to enhance antibody titers. Previous studies have shown that MOS can strengthen antibody

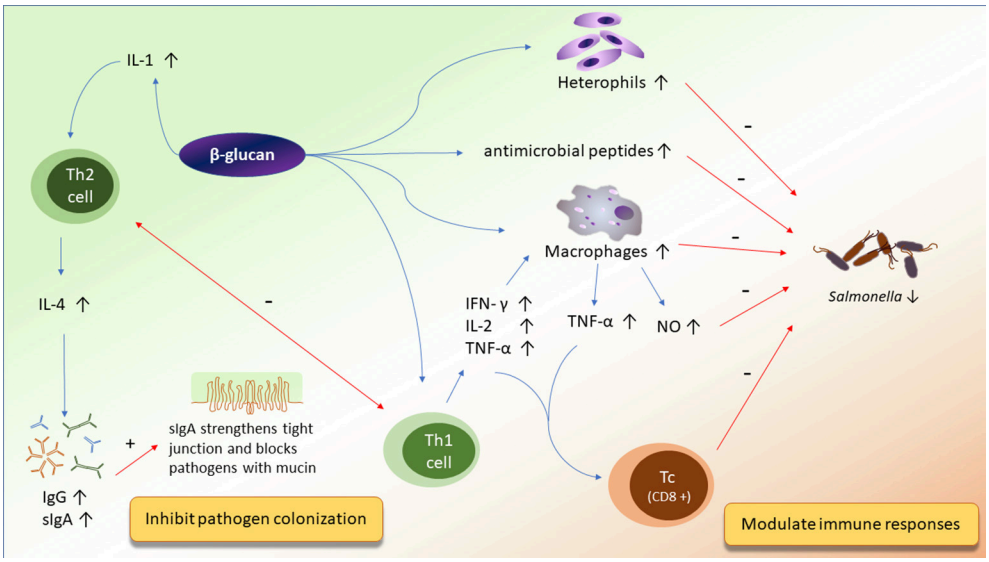

FIGURE 3 | The potential mechanisms of action of β-glucan on improving immunity and inhibiting pathogen colonization.

titers against sheep red blood cells, infectious bursal disease virus, Newcastle disease virus, and avian influenza virus (47–49). On the contrary, some reports have noted that antibody titers against Newcastle disease virus and infectious bursal disease virus failed to increase in chickens with MOS supplementation (64, 65). This discrepancy among studies may be based on whether or not broilers are infected with pathogens or the variations in MOS sources and environmental conditions (51).

The effects of MOS on intestinal microbiota have been reported broadly. Most of the MOS additions can significantly improve microbial community composition. However, there has been limited research on the impacts of MOS on mechanisms of immune responses in broilers. Although previous studies have found some auspicious results, further research is necessary to determine further antibody titers and gene expression of TLR or cytokines in order to elucidate how MOS improves the broiler's immunity.

β-GLUCAN

β-glucan is a prebiotic derived from yeast or fungal cell walls. This long-chain polysaccharide is composed of D-glucose monomers with linkages of β-glycosidic 1-3 bonds, and its side-chains are linked by the 1–6 bonds. β-glucan can be recognized by receptors on sentinel cells, triggering production of cytokines and proliferation of lymphocytes (66). Lymphocytes are classified into three major types. The first type is NK cells, which play an important role in innate immunity. The second type is T cells, which regulate adaptive immunity. The third type is B cells, which produce antibodies against antigens. All types of lymphocytes can be modulated by β-glucan. The influences on immune responses of broilers are shown in **Table 3**.

Macrophages may be one of the sentinel cells that recognize β-glucan in the animal intestine. When macrophages are activated by β-glucan, they produce inducible nitric oxide synthase (iNOS) (56), an enzyme that produces large amounts of nitric oxide. Reacting with superoxide anion, nitric oxide is oxidized to a highly-toxic nitrogen dioxide radical that can kill a wide range of invading pathogens directly or block their DNA synthesis (12, 52, 58). Moreover, β-glucan exposure also triggers macrophage proliferation, enhances macrophage phagocytic ability, and induces macrophage-modulating gene expression of interleukin-1 (IL-1), interleukin-18 (IL-18), and tumor necrosis factor-α (TNF-α) (38, 52). Increasing TNF-α in birds fed with β-glucan may stimulate the incidence of CD8+ lymphocyte, a receptor expressed only on the cytotoxic T cell (Tc) (52, 54). Thus, it is hypothesized that β-glucans can regulate innate immune response by inducing proliferation of Tc cells to attack pathogen-infected cells.

Heterophils, recruited by sentinel cells, are the major granulocytes in most birds and work in a manner similar to neutrophils in mammals. Lowry et al. (53) showed the increases of heterophil phagocytosis in broilers fed with β-glucan, including enhancing the percentage of heterophils containing *Salmonella enterica*, mean numbers of *Salmonella enterica* per heterophil, and phagocytic index. One reasonable explanation that has been proposed is that the dectin-1 receptor involved in β-glucan recognition on the surface of macrophages may also be present on the surface of heterophils (67). Furthermore, heterophils stimulated by β-glucan can release nitric oxide and kill *Salmonella enterica*, resulting in the reduction of pathogenic organ invasion (53). Apart from heterophils, β-glucan receptors are also present on NK cells in humans (68). Therefore, activating NK cells by β-glucan may be another way to improve immune responses in broilers. On the contrary, Cox et al. (56) indicated that β-glucan could be an anti-inflammatory immunomodulator inhibiting interleukin-8 (IL-8) gene expression. Interleukin-8 is a cytokine produced by macrophages, which can recruit

TABLE 1 | Effects of mannan oligosaccharides on intestinal microbiota of broilers.

Effects		Dosage, Challenge, and Diets	Day	References
Jejunum				
Alter	Community composition	0.5%	25	(24)
Jejunal mucosa				
Decrease	*Coliforms*	0.2% with *E. coli* challenge	7	(10)
Ileum				
Increase	Calculated Sorenson's similarity indices (Cs)/ intragroup	0.2%	21	(11)
Increase	Total anaerobic bacteria	0.2%	7	(10)
Decrease	*Coliforms*	0.2%	7	(11)
Decrease	*Coliforms*	0.2%	14	(10)
Decrease	*Clostridium perfringens*	0.2%	21	(11)
Increase	Diversity of *Lactobacillus*	0.2%	21	(11)
Increase	*Lactobacillus*	0.2%	7	(11)
Decrease	*Lactobacillus*	0.2%	14	(22)
Increase	*Lactobacillus*	0.017% MOS and 0.025% β-glucan	14	(26)
Increase	*L. acetotolerans*	0.2%	21	(11)
Increase	*L. delbrueckii* subsp. *lactis*	0.2%	21	(11)
Increase	*L. sakei* subsp. *Sakei*	0.2%	21	(11)
Ileal mucosa				
Increase	*Lactobacillus*	0.2%	21	(11)
Increase	*L. acetotolerans*	0.2%	21	(11)
Ileocecal junction				
Decrease	*Clostridium perfringens*	0.1%	28	(25)
Decrease	*E. coli*	0.1%	28	(25)
Decrease	*Lactobacillus*	0.1%	28	(25)
Ceca				
Alter	Community composition	0.08% in starter and 0.04% in finisher	7, 35	(23)
Alter	Community composition	0.1%	28	(29)
Alter	Community composition	0.2%	14, 28	(29)
Alter	Community composition	0.5%	25	(24)
Increase	Total anaerobic bacteria	0.2%	7	(11)
Decrease	*Firmicutes*	0.08% in starter and 0.04% in finisher	35	(23)
Decrease	*Coliforms*	0.2% in wheat diet	21	(21)
Decrease	*Salmonella*	0.4% with *Salmonella dublin* Challenge	10	(16)
Decrease	*Salmonella*	0.4% with *Salmonell typhimurium* Challenge	10	(16)
Decrease	*E. coli*	0.2% in starter and 0.1% in finisher with *E. coli* challenge	9	(19)
Decrease	*E. coli*	0.2% in starter and 0.1% in finisher	3, 28, 42	(19)
Decrease	*E. coli*	0.2% or 0.5%	34	(14)
Decrease	*Clostridium perfringens*	0.2%	21	(10)
Decrease	*Clostridium perfringens*	0.4% in wheat diet	21	(20)
Decrease	*Campylobacter*	0.2% in Dextrose-ISP diet	34	(14)
Increase	*Kitastosphora*	0.20%	21	(11)
Increase	*Bacteroides*	0.08% in starter and 0.04% in finisher	7, 35	(23)
Increase	*Bacteroides*	0.20%	7	(27)
Increase	*Bifidobacteria*	0.20%	34	(14)
Increase	*Bifidobacteria*	0.5% (MOS and β-glucan)	14, 24, 34	(14)
Decrease	*Lactobacillus*	0.10%	14	(22)
Increase	*Lactobacillus*	0.20%	24	(14)
Increase	*Lactobacillus*	0.50%	34	(14)
Increase	*Lactobacillus*	0.2% in starter and 0.1% in finisher	38, 42	(19)
Increase	*L. ingluviei*	0.20%	21	(11)
Increase	*L. mucosae*	0.20%	21	(11)

TABLE 2 | Effects of prebiotics on intestinal morphology of broilers.

Effects		Dosage, Challenge, and Diets	Day	References
MOS				
Intestine				
Increase	MUC2 gene expression	0.1% 0.6 ml *in ovo*	E20 (embryonic)	(36)
Duodenum				
Increase	Goblet cell numbers	0.2%	34	(14)
Increase	Villus height	0.5%	14	(14)
Increase	Villus height	0.2%	34	(14)
Increase	Villus height	0.2% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus height: crypt depth	0.2% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus surface area	0.2% with *Salmonella typhimurium* challenge	10	(35)
Jejunum				
Increase	Goblet cell numbers	0.2%	24, 34	(14)
Increase	Goblet cell numbers	0.5%	24, 34	(14)
Increase	Goblet cell numbers	0.1%	16, 26	(34)
Increase	Villus height	0.2%	24	(14)
Increase	Villus height	0.1%	26	(34)
Increase	Villus height	0.2% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus height: crypt depth	0.2% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus surface area	0.2% with *Salmonella typhimurium* challenge	10	(35)
Decrease	Crypt depth	0.2%	7	(10)
Decrease	Crypt depth	0.2% in wheat diet	7	(21)
Ileum				
Increase	Goblet cell numbers	0.2%	24	(14)
Increase	Goblet cell numbers	0.1%	16, 26	(34)
Increase	Villus height	0.1%	26	(34)
Increase	Villus height	0.2%	21	(11)
Increase	Villus height	0.2% with *Salmonella typhimurium* challenge	10	(35)
Increase	Cup area	0.2%	21	(11)
Increase	Goblet cell density (acidic)	0.2%	21	(11)
Increase	Goblet cell density (sulphated-acidic)	0.2%	21	(11)
Increase	Goblet cell density (total)	0.2%	21	(11)
Decrease	Goblet cell density (sialo-acidic)	0.2%	21	(11)
β-glucan				
Jejunum				
Increase	Villus height	0.01% with *Salmonella typhimurium* challenge	21	(37)
	Villus height: crypt depth	0.01% with *Salmonella typhimurium* challenge	21	(37)
	Goblet cell density	0.01% with *Salmonella typhimurium* challenge	21	(37)
Decrease	MUC2 gene expression	0.1% with *Eimeria* challenge	14	(38)
Increase	MUC2 gene expression	0.1%	14	(38)
Increase	MUC2 gene expression	0.1% with *Eimeria* challenge	21	(38)
Increase	Claudin-1	0.01% with *Salmonella typhimurium* challenge	21	(37)
Increase	Occludin	0.01% with *Salmonella typhimurium* challenge	21	(37)
Fructan				
Jejunum				
Increase	MUC gene expression	1%	21, 42	(39)
Increase	MUC gene expression	1.5%	21	(39)
Increase	Microvillus height	0.4%	49	(40)
Decrease	Crypt depth	0.4%	49	(40)
Increase	Villus height: crypt depth	0.4%	49	(40)

(Continued)

TABLE 2 | Continued

Effects		Dosage, Challenge, and Diets	Day	References
Ileum				
Increase	Villi height	0.4%	49	(40)
Increase	Microvillus height	0.2%	49	(40)
Increase	Microvillus height	0.4%	49	(40)
Decrease	Crypt depth	0.4%	49	(40)
Increase	Villus height: crypt depth	0.2%	49	(40)
Increase	villus height: crypt depth	0.4%	49	(40)
Ceca				
Increase	Villus height: crypt depth	0.1%	35	(41)
XOS				
Ileum				
Increase	Villus height	0.5%	26	(42)
GGMO				
Duodenum				
Increase	Villus height	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus height: crypt depth	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus surface area	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Jejunum				
Increase	Villus height	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus height: crypt depth	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus surface area	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Ileum				
Increase	Villus height	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus height: crypt depth	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)
Increase	Villus surface area	0.1, 0.2, or 0.3% with *Salmonella typhimurium* challenge	10	(35)

heterophiles to phagocytose pathogens at the site of inflammation (12). The inconsistent results may be attributed to whether or not the birds were challenged by pathogens. In a pathogen-challenging situation, pro-inflammatory immune responses may be enhanced by β-glucan supplementation, whereas in normal circumstances, β-glucan may be an anti-inflammatory modulator.

It was reported that the inclusion of β-glucan in diets could regulate the gene expression of antimicrobial peptides (AMPs) (57). Cathelicidins (Cath), avian β-defensins (AvBDs), and liver-expressed antimicrobial peptides (LEAP) are three major families of AMPs, which are expressed by the lung, intestine, immune, and reproductive organs in chickens (57). Antimicrobial peptides can penetrate the membrane of fungi or bacteria, leading to the death of pathogens. Among AMPs, Cath-1 and Cath-2 proteins have been shown to posses the capacity to bind to LPS, inhibiting LPS-mediated pro-inflammatory immune responses (61). On the other hand, AvBDs expressed in heterophils and the mucosal surface of the intestinal and respiratory tracts can damage pathogens, like *Staphyloccocus aureas*, *E. coli*, *Candida albicans*, *S.* Enteritidis, *S. Typhimurium*, *Listeria monocytogenes*, and *Campylobacter jejuni* (61). Shao et al. (57) reported that the gene expression of Cath-1, Cath-2, AvBD-1, AvBD-2, AvBD-4, AvBD-6, AvBD-9, and LEAP-2 were increased in *Salmonella*-challenged broilers with β-glucan addition. On the contrary, the same study showed that β-glucan reduced Cath-1, AvBD-4, and AvBD-9 in the spleen of birds without pathogen challenge. It could be concluded that if broilers were under pathogen infection, β-glucan would exhibit a strong protection against *Salmonella* and other pathogens in broilers.

After recognizing β-glucan, sentinel cells secrete cytokines that activate Th1 or Th2 cells. The Th1 cells drive the type-1 pathway attack against intracellular pathogens, whereas Th2 cells dominate the type-2 pathway triggering humoral immunity to upregulate antibody production (69). Although Th1 and Th2 cells could release cytokines to cross-inhibit each other, type-1 and type-2 pathways could both be triggered by β-glucan. In type-1 pathways, interleukin-12 (IL-12), produced by

TABLE 3 | Effects of mannan oligosaccharides, β-glucan, and fructans on immune responses of broilers.

Effects		Dosage, Challenge, and Diets	Day	References
MOS				
Blood/Serum				
Decrease	B cell	0.5%	25	(46)
Increase	IgM	0.5%	25	(46)
Increase	Antibody against Avian Influenza virus	0.1, 0.2, 0.3% with ND vaccination	42	(47)
Increase	Antibody against Avian Influenza virus	0.1, 0.2, 0.3%	42	(47)
Increase	Antibody against Newcastle disease virus	0.09%	42	(48)
Increase	Antibody against IBDV	0.5%	54 weeks	(49)
Increase	Antibody against sheep red blood cell	0.09%	28, 42	(48)
Increase	Total antibody against sheep red blood cell	0.09%	28, 42	(48)
Decrease	Basophils	0.2%	28	(25)
Decrease	Heterophil: lymphocyte	0.2%	28	(25)
Ileum				
Increase	IFN-γ	0.2%	22	(50)
Increase	IFN-γ	0.2% with *Clostridium perfringens* challenge	22	(50)
Increase	IL-12p35	0.2%	22	(50)
Increase	IL-12p35	0.2% with *Clostridium perfringens* challenge	22	(50)
Increase	TLR2b	0.2%	22	(50)
Increase	TLR2b	0.2% with *Clostridium perfringens* challenge	22	(50)
Increase	TLR4	0.2%	22	(50)
Increase	TLR4	0.2% with *Clostridium perfringens* challenge	22	(50)
Decrease	TLR2	0.1% 0.6 ml *in ovo*	1, 3	(36)
Increase	TLR4	0.1% 0.6 ml *in ovo*	1	(36)
Cecal tonsils				
Decrease	B cell	0.5%	25	(46)
Increase	IFN-γ	0.2%	22	(50)
Increase	IFN-γ	0.2% with *Clostridium perfringens* challenge	22	(50)
Decrease	TLR2b	0.2% with *Clostridium perfringens* challenge	22	(50)
Increase	TLR4	0.2%	22	(50)
Increase	TLR4	0.2% with *Clostridium perfringens* challenge	22	(50)
MOS and β-glucan				
Blood/Serum				
Increase	Antibody/ infectious bursal virus	0.1% with *Salmonella* enteritidis challenge	21, 42	(51)
Decrease	Eosinophils	0.1% with *Salmonella* enteritidis challenge	42	(51)
Increase	Monocytes	0.1% with *Salmonella* enteritidis challenge	42	(51)
β-glucan				
Abdominal exudate cell macrophages				
Increase	Nitrite	1, 2.5, 5 mg/ml	35	(52)
Increase	Phagocytic activity	0.002, 0.004%	35	(52)
Increase	IL-1	5 mg/ml	35	(52)
Increase	Total antibody responses to Sheep red blood cell	0.004%	35	(52)
Intraepithelial leukocytes				
Increase	CD4+	0.004%	16	(52)
Increase	CD8+	0.004%	16	(52)
MQ-NCSU				
Increase	Nitrite	1, 5 mg/ml	35	(52)
Increase	Macrophages	5 mg/ml	35	(52)
Organ				
Increase	Bursa weight %	0.002, 0.004%	14	(52)
Increase	Spleen weight %	0.002, 0.004%	14	(52)

(Continued)

TABLE 3 | Continued

Effects		Dosage, Challenge, and Diets	Day	References
Decrease	Liver *Salmonella* enteritidis invasion	with *Salmonella* enteritidis challenge	4	(53)
Decrease	Spleen *Salmonella* enteritidis invasion	with *Salmonella* enteritidis challenge	4	(53)
Intestine				
Increase	sIgA	0.0025, 0.005, 0.0075, 0.01, 0.0125%	21, 42	(54)
Decrease	IL-4	0.10%	21	(38)
Intestinal fluid				
Decrease	IgG	0.0001%	7, 28	(55)
Duodenum				
Decrease	IFN-γ	0.1% with *Eimeria* challenge	10	(38)
Decrease	IFN-γ	0.1%	7	(56)
Decrease	IL-4	0.02, 0.1%	7	(56)
Increase	IL-4	0.02, 0.1%	14	(56)
Decrease	IL-8	0.02, 0.1%	7, 14	(56)
Decrease	IL-13	0.1%	7	(56)
Decrease	IL-18	0.02%	14	(56)
Increase	Nitic oxide synthase	0.1%	14	(56)
Jejunum				
Decrease	IFN-γ	0.1%	14	(38)
Decrease	IFN-γ	0.1% with *Eimeria* challenge	14	(38)
Decrease	IFN-γ	0.1%	7	(56)
Decrease	IL-4	0.1%	7	(56)
Decrease	IL-8	0.1%	7	(56)
Decrease	IL-8	0.02%	14	(56)
Decrease	IL-13	0.1%	7	(56)
Decrease	IL-18	0.1%	14	(56)
Increase	IL-18	0.1%	21	(38)
Increase	IL-18	0.02%	7	(56)
Increase	*Cath-1*	0.02%	14	(57)
Increase	*Cath-2*	0.02%	14	(57)
Increase	*AvBD-1*	0.02%	22	(57)
Increase	*AvBD-4*	0.02%	22	(57)
Increase	*AvBD-10*	0.02%	22	(57)
Decrease	*AvBD-10*	0.02% with *Salmonella* enteritidis challenge	22	(57)
Increase	*LEAP-2*	0.02%	22	(57)
Decrease	Nitric oxide synthase	0.1% with *Eimeria* challenge	10	(38)
Increase	sIgA+ cell numbers	0.01% with *Salmonella typhimurium* challenge	21	(37)
Increase	sIgA	0.01% with *Salmonella typhimurium* challenge	14, 21	(37)
Increase	IgA against *Salmonella*	0.02%	22	(57)
Ileum				
Decrease	IFN-γ	0.1%	21	(38)
Decrease	IFN-γ	0.1% with *Eimeria* challenge	21	(38)
Decrease	IFN-γ	0.1%	7	(56)
Decrease	IL-4	0.1%	7	(56)
Increase	IL-4	0.1%	14	(56)
Decrease	IL-8	0.1%	7, 14	(56)
Decrease	IL-8	0.02%	14	(56)
Decrease	IL-13	0.1%	7	(56)
Decrease	nitric oxide synthase	0.1%	14	(38)
Increase	nitric oxide synthase	0.1% with *Eimeria* challenge	14	(38)
Increase	nitric oxide synthase	0.1%	14	(56)

(Continued)

TABLE 3 | Continued

Effects		Dosage, Challenge, and Diets	Day	References
Blood/Serum				
Increase	Globulin	0.0025, 0.005, 0.0075, 0.01, 0.0125%	21	(54)
Increase	Globulin	0.0025, 0.005, 0.0075, 0.01%	42	(54)
Increase	IFN-γ	0.005, 0.0075%	21	(54)
Increase	IFN-γ	0.01%	42	(54)
Increase	IgG	0.0025, 0.005, 0.0075, 0.01%	21	(54)
Increase	IgG	0.0025, 0.005, 0.0075%	42	(54)
Increase	IgG against *Salmonella*	0.02% with *Salmonella* enteritidis	14, 22	(57)
Increase	IL-1	0.0025, 0.005%	42	(54)
Increase	IL-1	0.01%	21	(54)
Increase	IL-2	0.0025, 0.005, 0.0075, 0.01, 0.0125%	21, 42	(54)
Increase	TNF-α	0.005, 0.0075, 0.01%	21, 42	(54)
Decrease	lymphocytes	0.012% and exposed to LPS	42	(58)
Decrease	lymphocytes	0.05%, and exposed to pokeweed mitogen	42	(58)
Increase	mean number of SE per heterophil	with *Salmonella* enteritidis challenge	4	(53)
Increase	percent heterophils containing SE	with *Salmonella* enteritidis challenge	4	(53)
Increase	phagocytic index	with *Salmonella* enteritidis challenge	4	(53)
Increase	SE Killing/heterophils	with *Salmonella* enteritidis challenge	4	(53)
Increase	nitric oxide/3, 6, 12 h	0.025%, and exposed to LPS	42	(58)
Fructans				
Blood/Serum				
Decrease	B cells	0.5%	25	(46)
Increase	IgG	0.5%	25	(46)
Increase	IgM	0.5%	25	(46)
Increase	Antibody against sheep red blood cells in primary response	0.05%	42	(59)
Ileum				
Increase	CD4+:CD8+	0.5%	21	(39)
Decrease	IFN-γ	0.5%	21	(39)
Decrease	IFN-γ	1%	21	(39)
Increase	IgA	1%	21, 42	(39)
Increase	IgA	1.5%	21	(39)
Increase	IgA	0.5%	42	(39)
Decrease	IL-6	0.5%	21	(39)
Cecal tonsils				
Decrease	CD80	0.2 ml (1.76 mg) *in ovo*	35	(60)
Decrease	IFN-B	0.2 ml (1.76 mg) *in ovo*	35	(60)
Decrease	IL-12p40	0.2 ml (1.76 mg) *in ovo*	35	(60)
Decrease	IL-18	0.2 ml (1.76 mg) *in ovo*	35	(60)
Decrease	IL-4	0.2 ml (1.76 mg) *in ovo*	35	(60)
Decrease	Proliferative competence of *ex vivo* leukocytes	0.5%	25	(46)
Decrease	B cells	0.5%	25	(46)

macrophages, is a key cytokine that enhances the proliferation of Th1 cells and the production of IFN-γ (12). Interferon-γ further reinforces with IL-18 in order to trigger the activation of Th1 cells and produce additional IFN-γ and IL-2 for the activation of NK cells, stimulation of macrophages and Tc cells, and inhibition against Th2 cells (12). Previous studies reported that β-glucan upregulates the gene expression of IL-2, IL-18, and IFN-γ (52, 54). Additionally, levels of the cytokines interleukin-4 (IL-4) and interleukin-13 involved in type-2 cell pathways are downregulated by β-glucan as well (56). These outcomes support the hypothesis that β-glucan can stimulate the type-1 pathway and inhibit the type-2 pathway.

However, gene expression of IL-1 involved in the type-2 pathway could also be induced by β-glucan (52). Increasing IL-1 found in abdominal exudate cell macrophages can activate Th2 cells and switch on the type-2 pathway. Once activated, Th2 cells release other cytokines to initiate the subsequent anti-inflammatory immune responses. For instance, IL-4 can suppress

Th1 cells' activation, stimulate B cells' growth and differentiation, and activate mast cells to produce immunoglobulins (12). Owing to the suppression of Th1 cells, gene expression of IFN-γ was downregulated in duodenum, jejunum, and ileumthe duodenum, the jejunum, and the ileum by β-glucan in *Eimeria*-challenged broilers (38). On the other hand, enhancing immunoglobulins, including IgG and sIgA, in broilers were found by Zhang et al. (54). This is evidence showing that the type-2 pathway can be upregulated by β-glucan. Shao et al. (57) also reported that anti-*Salmonella* specific IgA levels in the jejunum and anti-*Salmonella* specific IgG levels in the serum were increased in birds fed with β-glucan. Similarly, Shao et al. (37) demonstrated that β-glucan could protect intestinal barrier function in *Salmonella*-challenged birds by increasing the amount of goblet cells and IgA-secreting cells, which enhance the sIgA production. sIgA is an important immunoglobulin that serves as the first line of defense (70). There are three major mechanisms of sIgA to protect the integrity of gut lining from pathogenic invasion (71). Firstly, sIgA interacts with non-pathogenic bacteria and epithelium, which consequently strengthens the tight junctions between intestinal epithelial cells and inhibits nuclear translocation of NF-κB (70). A previous study also confirmed that β-glucan enhanced the production of sIgA to ameliorate the damage of tight junction in the jejunum caused by *Salmonella* (37). Secondly, immune complexes that interact with sIgA are involved in the downregulation of gene expression of pro-inflammatory cytokines that include IFN-γ, TNF-α, and interleukin-6 (IL-6) (70). Thirdly, sIgA blocks pathogens within mucin, selecting and maintaining a favorable balance of microbiota in the intestine (70). Shao et al. (37) showed that increased sIgA by β-glucan was associated with the reduction of cecal *Salmonella* colonization and liver invasion.

In summary, β-glucan affects the broiler's immunity via either the type-1 or the type-2 pathway. The conflicting results among different studies may be attributed to the different dosages offered, different ages of the birds used, different parts of the tissue examined, or numerous resources of the β-glucan supplemented. Inconsistent results have also been demonstrated in other animals. For example, cytokines involved in the type-2 pathway of immune responses were downregulated by β-glucan in humans (72) but upregulated in mice (73). Therefore, additional investigation is needed to understand fully the effects of β-glucan on immune responses of broilers.

FRUCTANS

Fructans, commonly extracted from different plants, hydrolyzed from polysaccharides, or produced by microorganism, have been administered recently in broiler diets. Fructans are classified into three distinct types: the inulin group, the levan group, and the branched group. Firstly, the inulin group, also known as fructooligosaccharides (FOS) can be divided into different categories based on degrees of polymerization (DP): Inulin, normally extracted from chicory roots (*Cichorium intybus L.*), consists of a DP of 3 to 60, and Oligofructose (OF), which can be generated by partial hydrolysis of inulin, enzymatic conversion of sucrose, or lactose, contains a DP of 2 to 10 (74, 75). Most of the inulin group can be found in plants, which comprise oligosaccharides with β-2,1 fructosyl-fructose linkage with a glucose terminal unit. Secondly, the levan group is another group of fructans, which are mostly linked by β-2,6 fructosyl-fructose bonds. Lastly, fructans, which belong to the branched group, contain both β-2,1 fructosyl-fructose and β-2,6 fructosyl-fructose bonds in fair amounts (76). It is the β-glycosidic bond in fructans that resists their breakdown by digestive enzymes in poultry and enhances the population of beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, and suppresses levels of pathogenic bacteria, such as *Clostridium pefringens* and *E. coli*, in the intestine of broilers (25, 40, 77).

Saminathan et al. (78) evaluated the utilization of different oligosaccharides by 11 *Lactobacillus* species isolated from the gastrointestinal tract of chickens. This *in vitro* report showed that FOS were utilized by *Lactobacillus* more efficiently than MOS. The high availability of FOS may be associated with specific enzymatic activity and the oligosaccharide transport system of *Lactobacillus* species (79, 80). However, the intestinal microbiota of a broiler is far more complex than those in *in vitro* trials. The prebiotics may be fermented not only by *Lactobacillus* species but also by other microorganisms in the gastrointestinal tracts of animals. Thus, it cannot be assured that the utilization of FOS and MOS in *in vitro* trials is as efficient as in *in vivo* studies.

In addition, the more DP increased, the more residual FOS remained after fermentation by *Bifidobacteria* (81). A previous study indicated that almost 55 *Bifidobacteria* preferred to grow on short-chain FOS rather than long-chain FOS (75). *Bifidobacteria* could also ferment short-chain FOS to produce more acetic acid and lactic acid compared with long-chain FOS within 24 h (81). Similarly, Perrin et al. (82) reported that the population of *Bifidobacteria* and *Lactobacilli* increased earlier in fecal cultures containing OF instead of inulin. However, an increase in the production of formic acid, acetic acid, and lactic acid and a decrease in numbers of *E. coli* group and *Cluster I clostridia* were both observed in cultures containing OF or inulin after 24-h fermentation (82). The same research group also pointed out that butyric acid might be the major product in the inulin group, whereas more acetic acid and lactate acid could be produced from OF (75).

Long-chain fructans, which are degraded slowly in the animal gut, can pass through the small intestine and be fermented in the distal regions of the intestine. Therefore, the inulin group with higher DP might not affect the microbiota in the jejunum significantly (83), but, instead it might alter microbial structure and increase the concentration of SCFA or lactic acid in the ceca of broilers. Effects of FOS on intestinal microbiota are shown in **Table 4**. Park et al. (85) demonstrated that FOS increased the Shannon diversity of intestinal microbiome compared with the control treatment. Moreover, similar to *in vitro* results, *Bifidobacteria* and *Lactobacillus* are two major beneficial bacteria that were increased in broilers and hens fed with fructans (40, 41, 76, 84, 88). *Bifidobacteria* and *Lactobacillus* not only produced extracellular enzymes to degrade FOS but also competed with other species of intestinal microorganisms and suppressed the growth of pathogenic bacteria (75). For instance, *Campylobacter* titers in the ceca and large intestine were decreased in broilers

TABLE 4 | Effects of fructans on intestinal microbiota of broilers.

Effects		Dosage, Challenge, and Diets	Day	References
Gizzard				
Decrease	*Lactobacillus*	1% inulin/female	42	(84)
Increase	*Lactobacillus*	1% oligofructose/male	42	(84)
Increase	*Lactobacillus*	1% oligofructose/female	42	(84)
Increase	*Salmonella*	1% oligofructose/male	42	(84)
Increase	*E. coli*	1% inulin/femlae	42	(84)
Small intestine				
Increase	*Bifidobacteria*	0.40%	49	(40)
Increase	*Lactobacillus*	0.40%	49	(40)
Increase	*Lactobacillus*	1% oligofructose/female	42	(84)
Decrease	*E. coli*	1% inulin/female	42	(84)
Increase	*E. coli*	0.40%	49	(40)
Ileum				
Increase	Diversity	0.25%	28	(25)
Increase	*Lactobacillus*	0.20%	35	(41)
Increase	Total anaerobic bacteria	1.00%	7	(10)
Decrease	*Coliforms*	1.00%	7	(10)
Ileocecal junction				
Increase	*Lactobacillus*	0.25%	28	(25)
Decrease	*Clostridium perfringens*	0.50%	28	(25)
Decrease	*E. coli*	0.25, 0.5%	28	(25)
Ceca				
Increase	Shannon diversity	0.1%	42	(85)
Increase	*Alistipes* genus	0.1%	42	(85)
Increase	*Bifidobacteria*	0.40%	49	(40)
Increase	*Bifidobacteria*	0.1, 0.2%	35	(41)
Increase	*Bifidobacteria*	0.25 and 0.5%	31	(76)
Decrease	*Lactobacillus*	0.30%	21, 42	(86)
Increase	*Lactobacillus*	0.2, 0.4%	49	(40)
Increase	*Lactobacillus*	0.25 and 0.5%	31	(76)
Increase	*Lactobacillus*	1% inulin/female	42	(84)
Increase	*Lactobacillus*	1% oligofructose/female	42	(84)
Increase	*Lactobacillus intestinali*	0.1%	14, 28	(85)
Increase	*Faecalibacterium prausnitzii*	0.1%	42	(85)
Decrease	Total anaerobic bacteria	0.30%	42	(86)
Increase	Total anaerobic bacteria	0.40%	49	(40)
Increase	Total anaerobic bacteria	1% inulin/female	42	(84)
Increase	Total anaerobic bacteria	1% oligofructose/female	42	(84)
Decrease	*Campylobacter*	1% oligofructose/male	42	(84)
Decrease	*Campylobacter*	1% oligofructose / male	42	(84)
Decrease	*Clostridium perfringens*	1% with *E. coli* challenge	7	(10)
Decrease	*Clostridium perfringens*	0.4% short chain FOS in dextrose-ISP diet	21	(20)
Decrease	*Clostridium perfringens*	0.25 and 0.5%	31	(76)
Decrease	*Coli bacillus*	0.30%	42	(86)
Decrease	*Salmonella*	1% inulin/female	42	(84)
Decrease	*Salmonella*	1% oligofructose/female	42	(84)
Decrease	*Salmonella Typhimurium*	1% and defined competitive exclusion with *Salmonella typhimurium* challenge	7	(87)
Decrease	*E. coli*	0.2, 0.4%	49	(40)
Decrease	*E. coli*	0.25 and 0.5%	31	(76)
Decrease	*E. coli*	1% inulin/female	42	(84)
Decrease	*E. coli*	1% oligofructose/female	42	(84)
Large intestine				
Decrease	*Campylobacter*	1% inulin/female	42	(84)
Decrease	*Campylobacter*	1% oligofructose/female	42	(84)
Decrease	*E. coli*	1% inulin/female	42	(84)
Decrease	*E. coli*	1% oligofructose/female	42	(84)

fed with FOS (84). Regardless of the supplementation of long-chain FOS or short-chain FOS, a reduction in titers of *C. perfringens* was observed in the ileocecal junction or ceca of broilers (20, 25, 76). Similarly, colonization of cecal *C. perfringens* and *Salmonella typhimurium* was decreased by FOS or FOS combined with competitive exclusion products in *E. coli* or *Salmonella- Typhimurium*-challenged birds, respectively (10, 87). Additionally, diets containing different concentrations of FOS (from 0.25 to 1%) could decrease cecal *E. coli* and *Salmonella* in broilers (25, 40, 76, 84, 86). Besides the prevention of *Salmonella* colonization in the ceca of broilers, previous reports also demonstrated that FOS-supplemented diets decreased ovary, liver, and cecal *Salmonella enteritidis* in laying hens (89, 90). The reduction of these pathogenic bacteria might be attributed to cecal SCFA and lactic acid. Same as *in vitro* results, the concentration of cecal butyric acid and lactic acid was significantly higher in broilers fed with inulin (41, 83). Donalson et al. (89) also showed that 0.75 or 0.375% of FOS combined with alfalfa molt diets could increase the concentration of cecal isobutyric acid in hens. Short-chain fatty acids are important fuels in the intestine, and butyrate is the major one that is metabolized by epithelial cells, providing energy for the growth of mucosal epithelium (91). It is suggested that higher concentrations of butyric acid are associated with the improvement of mucosal structure. Previous studies reported that microvillus height in the jejunum and ileum and the ratio of villus to crypt depth in the ceca were increased by FOS (40, 41). Bogucka et al. (92) also reported that *in ovo* injection of inulin increased villus height in broilers at the first day after hatching. In addition, the use of inulin could increase jejunal mucin mRNA expression to produce more mucin, protecting intestinal epithelial cells in broilers (39). By improving intestinal morphology, FOS could further enhance activities of protease and amylase and nutrient absorption, leading to better growth performance (40).

However, adding high levels of fructans could result in negative impacts on broilers. Rapid fermentation by microbes in the intestine could produce too much SCFA, which damage intestinal mucosal barriers and increase intestinal permeability, consequently causing pathogen invasion, diarrhea, and poor growth performance (93, 94). Xu et al. (40) demonstrated that the addition of 0.2 or 0.4% of FOS in broiler diets could improve FCR and change cecal microbiota, but the supplementation of 0.8% of FOS had no significant differences compared with control treatment. It has been suggested that the supplementation of FOS above 0.5% is excessive; a previous report mentioned that birds fed with 0.5% FOS showed poorer growth performance and less intestinal *Lactobacillus* but higher titers of *E. coli* and *C. perfringens* compared with 0.25% FOS treatment (25). Furthermore, Biggs et al. (20) even showed that ME_n and amino acid digestibility were reduced by 8% short-chain FOS or inulin addition.

Fructans improved the immune responses of gut-associated lymphoid tissue (GALT) and the systemic immune system through three major mechanisms. Firstly, increasing the levels of *Bifidobacteria* by fructans could modulate the production of cytokines or antibodies. Secondly, leukocytes could be activated after their receptors respond to fructans' metabolites, such as SCFA. Thirdly, fructans could be directly recognized by carbohydrate receptors on the surface of immune cells (95). Huang et al. (39) reported that inulin reduced the levels of IL-6 and IFN-γ, increased IgA, and tended to increase the ratio of $CD4^+/CD8^+$ cells in the ileum of broilers. Moreover, Janardhana et al. (46) found that FOS could lead to systemic immune responses by increasing the levels of plasma antibody titers of IgG and IgM. Similarly, primary antibody titers against sheep red blood cells increased in broilers fed with FOS, but antibody titers in the secondary immune response were not influenced by FOS (59). Likewise, FOS increased IgA^+ cells and upregulated TLR-4 and IFN-γ in the ileum of laying hens (90). Interestingly, there is a hypothesis that fructans might modulate the development of the immune system during embryogenesis. *In ovo* administration of inulin (d 12) downregulated the gene expression of IL-4, IL-12p40, IL-18, CD80, and interferon-β in the cecal tonsils of broilers on day 35 after hatching (60). Furthermore, *in ovo* injection of inulin had no adverse effect on GALT development but stimulated more colonization of lymphoid tissue by T cells in the cecal tonsil of broilers (96). To our knowledge, there are only a few studies that evaluated the *in ovo* administration of prebiotics. Further research is needed to understand what causes the different results between *in ovo* administration and direct-fed supplementation of fructans in broilers. It could be concluded that owing to the various fructans groups and DP, supplementation of fructans in diets might have affected broilers inconsistently. However, in a general review, fructans could modulate intestinal microorganisms, levels of intestinal SCFA, mucosal morphology, and generate immune responses.

OTHER PREBIOTICS

Besides the three major prebiotics, MOS, β-glucan, and fructans, other oligosaccharides have been evaluated and considered as potential prebiotics, including chitosan oligosaccharides (COS), galacto-oligosaccharides (GOS), galactoglucomannan oligosaccharide (GGMO), and xylo-oligosaccharides (XOS).

Chitosan Oligosaccharides (COS)

Extracted from chitin, COS contain 2–10 sugar units of *N*-acetyl glucosamine with 1–4 β-linkages. It has been reported that the supplementation of COS in broiler diets could modulate immune responses and enhance nutrient digestibility and feed efficiency. Huang et al. (97) indicated that chicken with COS supplementation had higher weight of bursa of Fabricius and thymus, higher IgG, IgA, and IgM in serum and higher antibody titers against Newcastle disease vaccines. On the other hand, 0.01% of COS improved ileal digestibility of dry matter, energy, crude protein, and most of the amino acids in broilers (21 or 42 d) (98). The improved digestibility of nutrients was associated with better growth performance in the same study (98). However, supplementation of COS above 0.01% might be excessive because chickens fed with 0.015% COS had significantly less body weight than birds fed with 0.01% COS (98).

Galacto-Oligosaccharides (GOS)

Galacto-oligosaccharides, synthetic prebiotics with galactose with 1–4 or 1–6 β-linkages, are normally produced from lactose by the enzyme lactase with high galactosyltransferase activity (99). *In ovo* injection of GOS could increase body weight of broilers 34 days after hatching (100). Administration of GOS also influenced the intestinal microbiota. Park et al. (85) reported that GOS treatment exhibited higher levels of *Alistipes* genus, *Lactobacillus intestinalis*, and *Faecalibacterium prausnitzii* in the ceca of broilers compared with the control group. Although Biggs et al. (20) demonstrated that GOS had no effects on cecal *Bifidobacteria* and *Lactobacillus* population, it has been reported that the addition of GOS in broiler diets could increase counts of *Bifidobacteria* in feces (101). Moreover, broilers that received *in ovo* GOS injection also had higher concentrations of *Bifidobacteria* and *Lactobacillus* in feces (102). The author suggested that *in ovo* administration of GOS could replace prolonged water supplementation. Owing to the inconsistent results, future studies are needed to confirm the effects of GOS in modulating intestinal microbial structures and further affecting immune responses in broilers.

Galactoglucomannan Oligosaccharides (GGMO) and Galactoglucomannan Oligosaccharides-Arabinoxylan (GGMO-AX)

Galactoglucomannan oligosaccharides and galactoglucomannan oligosaccharides-arabinoxylan (GGMO-AX) are novel prebiotics extracted and processed from the wood chips of softwood trees (103). These oligosaccharides consist of mannose, glucose, and galactose monomers. An *in vitro* investigation showed that *Lactobacillus* could grow faster on GGMO than MOS (35). The same research also indicated that the supplementation of 0.2% GGMO in broiler diets could reduce colonization of *Salmonella typhimurium* in the ileum, ceca, and liver; as a consequence of clearing *S. typhimurium* infection, GGMO ameliorates intestinal morphology and growth performance compared with a *Salmonella*-challenged control treatment (35).

The improvement might be attributed to the modulation of immune responses by GGMO. Faber et al. (104) reported that the *Eimeria acervulina*-challenged birds that received 4% GGMO-AX showed enhanced gene expression of pro-inflammatory cytokines, including IFN-γ, IL-1β, IL-6, and IL-12β, but also showed decreased levels of anti-inflammatory cytokines such as interleukin-15. Galactoglucomannan oligosaccharides-arabinoxylan might not only affect immune responses in broilers but also alter intestinal microbial population. It has been shown that the administration of 2% GGMO-AX increased counts of *Bifidobactrium* spp. in the ceca (104) and 4% GGMO-AX decreased the concentration of *C. perfringens* (105). Although the supplementation of GGMO-AX in high levels showed some positive effects on broilers, simultaneously, it could lead to poor growth performance (104). Therefore, further studies should evaluate the administration of GGMO or GGMO-AX in appropriate concentration to maintain growth performance and improve the health status of broilers at the same time.

Xylo-Oligosaccharides (XOS)

Xylo-oligosaccharides are oligosaccharides, which consist of xylose sugar units with β-linkages (42). Xylan, the main component of cereal fiber such as corn cobs, straws, hulls, and bran are the raw resources for XOS production (106). Xylan could be degraded to XOS by xylanase of fungi, steam, or diluted solutions of mineral acid (106). Similar to other prebiotics, XOS could improve growth performance, increase the intestinal villus height, increase the proportion of *Lactobacillus*, and enhance the levels of acetate, butyrate, and lactate in the ceca of broilers (42, 107, 108). It was suggested that XOS would improve humoral immunity in poultry. An increase in antibody titers against avian influenza H5N1 was observed in broilers by XOS addition (107). Furthermore, De Maesschalck et al. (42) speculated that XOS could lead to cross-feeding mechanisms between *L. crispatus* and *Anaerostipes butyraticus* in the gut of the broiler. Owing to XOS fermentation, *L. crispatus* produces lactate, which might be utilized by butyrate-producing bacteria that belong to members of *Clostridium* cluster XIVa. This hypothesis was further supported by the observation of increasing numbers of cecal *Clostridium* cluster XIVa and butyryl-CoA: acetate-CoA transferase, a marker indicating the butyrate-producing capacity of intestinal microbiota (42). As mentioned above, butyrate is a major energy source for intestinal epithelial cells. Apart from acting as an important fuel in the intestine, butyrate can stimulate *MUC-2* gene expression, exert anti-inflammatory effects, and prevent necrotic enteritis from pathogenic infection (109–111). In summary, XOS supplementation would enhance cross-feeding mechanisms and produce butyrate, consequently leading to beneficial influences on broilers.

In Ovo Injection

Direct feeding and *in ovo* injection are two main strategies for applying prebiotics. Prebiotic can be administrated by injecting 0.2 ml aqueous solution into the air chamber of eggs on day 12 of embryonic incubation (112). *In ovo* injection of prebiotics can alter microbial community in embryonic guts, improve intestinal morphology, and directly promote robustness of both cellular and humoral immune responses in the GALTs of the neonate post hatching (96, 113, 114).

The embryonic microbiota is different from the intestinal microbiota of post hatching and adult birds. The dominant bacterial phylum is *Proteobacteria*, followed by *Firmicutes, Bacteroidetes,* and *Actinobacteria* in the chicken embryos (115). In addition, the embryonic microbial community is altered during the development of the embryos. The 19-day-old embryos exhibited more microbial diversity than the 4-day-old embryos. The proportion of *Proteobacteria* decreased, whereas *Firmicutes, Bacteroidetes,* and *Actinobacteria* increased in the 19-day-old embryos compared with the 4-day-old embryos (115). Even though *Proteobacteria* decreased in the late embryonic development period, this phylum dominated in early-age birds until *Firmicutes* became prominent after 7 days post hatching (116). However, the embryonic microbiota could be contaminated by pathogens directly from the yolk, yolk membranes, albumen, shell membranes originating from the reproductive organs of laying hens, or indirectly from the egg shells. Pathogens such as *Salmonella* located in the albumen were able to migrate and penetrate the vitelline membrane and grow in the yolk (117). On the other hand, it was suggested that spore forming bacteria such as *Clostridium tertium* were capable of surviving the disinfection process and penetrating eggs, resulting in contamination (118). To avoid extensive pathogen infection, prebiotics were delivered *in ovo,* which is likely fermented by the indigenous embryonic microbiota, inhibiting pathogen proliferation and regulating gene expression of immune responses (119). Villaluenga et al. (120) reported that injection of raffinose at day 12 of embryonic incubation had the highest amounts of *Bifidobacteria* in the ceca of 2 day-old broilers. Additionally, they indicated that 8.815 mg per egg of raffinose delivered *in ovo* reduced embryo weight. A later research showed that 4.5 mg of raffinose that was delivered *in ovo* had no significant effects on body weight but enhanced gene expression of *CD3* and *ChB6,* which are associated with the activity of T cells and B cells (114). Moreover, villus height and villus height to crypt depth ratio of post hatching birds increased linearly with higher dosages of raffinose (114). *In ovo* injection of inulin and GOS also increased villus height in the jejunum of 1-day-old chickens (92). Moreover, administration of GOS *in ovo* showed differential gene expression in the ceca related to lymphocyte proliferation, activation, and differentiation and cytokine production (119). This study pointed out that *GZMA* (Granzyme A), a cytotoxic T cell-specific gene, was upregulated in the cecal tonsil of birds delivered with GOS *in ovo*. Similarly, other research has also demonstrated that GOS increased helper T cells in the cecal tonsil and B cells in the bursa of Fabricius (96). Furthermore, beta inhibin and lectin galactoside-binding soluble 3, which are related to regulation of T cell and innate immunity, were upregulated by GOS. On the other hand, GOS also downregulated the *SERPING1* gene, which could inhibit part of the complement cascade system (119). It was suggested that the *in ovo* injection of GOS might not only regulate intestinal innate and adaptive immune system but also modulate gene expression of nutrient digestion and transportation. Firstly, chicken injected with GOS *in ovo* exhibited higher levels of sodium-dependent

glucose co-transporters in the intestine, which are related to the absorption of monosaccharides (119). Secondly, birds delivered with GOS *in ovo* showed increased amylase and trypsin activity of the pancreas on embryonic day 21 and day 7 post hatching respectively (100). These studies led us to a conclusion that *in ovo* injection of prebiotics could affect the ecosystem of broilers, but, to our knowledge, little research has compared the difference between the direct-fed method and *in ovo* injection. A study reported that injection of galacto-oligosaccharides into eggs could increase *Bifidobacteria* and *Lactobacillus* in the feces of broilers. Though the author suggested that *in ovo* injection could replace prolonged supplementation via water system (102), more studies are needed to compare these two different approaches on the application of prebiotics.

CONCLUSION

The interaction between epithelium, microbiota, and immunity in animal gut is complicated. Recent data have demonstrated that prebiotics potentially alter the interaction between the host and gut microbiota and improve the health status of broilers. However, the interaction is sometimes induced by certain prebiotics or host species. Therefore, it is inevitable that prebiotics showed variable effects on animals. Still, most prebiotics can be fermented by beneficial bacteria, and the increased levels of *Lactobacillus* and *Bifidobacteria* or their metabolites may inhibit pathogen colonization and communicate with epithelial cells and immune cells. By improving gut environment or immune responses, prebiotics further provide resistance to pathogens and maintain efficient production. In addition, some prebiotics can be recognized by sentinel cells directly, triggering cytokines' cascade, which results in the upregulation of innate or humoral immunity. Although previous studies have discovered some mechanisms that participate in the cross talk between prebiotics and the ecosystem of the gut, there are still several hypotheses, which shall be confirmed in the future. In this context, administration of prebiotics presents tremendous influences on the broilers' gut health by the modulation of the gut microbial community and the interaction between the host immune system and gut microbiota. It is suggested that prebiotics delivered *in ovo* or fed directly can act as alternatives to antibiotics because of the significant improvement of microbial community, intestinal integrity, and immunity of the host.

AUTHOR CONTRIBUTIONS

P-YT reviewed papers related to the topics and wrote the manuscript. WK reviewed papers related to the topic, gave directions and ideas to P-YT, and reviewed and revised the manuscript.

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

frontiers in Veterinary Science

REVIEW
published: 31 January 2019
doi: 10.3389/fvets.2018.00348

Optimizing Gastrointestinal Integrity in Poultry: The Role of Nutrients and Feed Additives

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Immunomodulation of the immune system by stimulating or suppressing one or both arms, is an emerging concept driven by the understanding of the host defense system. In particular, the gastrointestinal tract (GIT) functions not only as a site for digestion and absorption of nutrients but also acts as a metabolic and immunological organ. This serves as a barrier against abnormal presentation of luminal constituents, caused by dysfunctional intestinal epithelial barrier, to the mucosal immune system. Invasion by pathogens in the case of disease or stress or a massive influx of commensal bacteria overcomes the defensive mechanisms, resulting in the full activation of local dendritic cells and the expression of co-stimulatory molecules and pro-inflammatory cytokines. A growing body of literature demonstrates the immune benefits of increasing the intake of specific nutrients. This strategy involves formulating diets that encompass the bioavailability and utilization of nutrients from various food sources and understanding the dynamics of the macro and micronutrients to support all physiological functions as well as maintaining the function of the immune cells. The nature and type of feed ingredients may also play some roles on the integrity of the GIT of birds. Because dietary intake or nutritional status as well as nutrient requirements may be altered as a result of disease or stress, this may eventually alter the gut microflora and intestinal mucosal integrity, resulting in a compromised barrier of the intestinal epithelium. The weakening of the intestinal integrity could result in an increase in bacterial adherence to the mucosa, bacterial translocation, susceptibility to opportunistic bacterial infection, and mis-appropriation of nutrients. In this chapter, we will discuss the role of dietary energy and nutrients as substrates that have the potential to influence GIT's health and integrity and their roles, directly or indirectly, in modulating bird's ability to be resilient or resist infection.

Keywords: exogenous enzyme, gut health, integrity, nonruminant animal, nutrition

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *11 October 2018*
Accepted: *31 December 2018*
Published: *31 January 2019*

Citation:
Adedokun SA and Olojede OC (2019) Optimizing Gastrointestinal Integrity in Poultry: The Role of Nutrients and Feed Additives. Front. Vet. Sci. 5:348. doi: 10.3389/fvets.2018.00348

INTRODUCTION

Being the continuation of the external environment, the function of the gastrointestinal tract (GIT) of a bird, like any other livestock, includes protection against insults (infectious and non-infectious), transport of ingested feed and digesta along the GIT, digestion and absorption of nutrients and energy, secretion of endogenous materials, hosting of intestinal microbiota, and excretion of undigested portion of the ingested feed and metabolic waste (1). A healthy GIT will be able to efficiently carry out these functions while a

compromised GIT may be unable to perform one or more of these functions. Although the integrity of the GIT of a bird depends on several factors, nutrients from diet play an important role in the maintenance of the integrity of the intestinal mucosa and gut microbial population (2–4). The timing of the first feed (early placement of feed), the quality (composition and physical texture) of the feed, as well as the quantity of the diet at an early age could influence the integrity of the GIT of the bird for several weeks (1, 5). In addition to the diet, efforts must be made to eradicate or minimize factors that could weaken or destroy the integrity of the GIT. Infectious agents such as bacteria (*Escherichia coli, Salmonella typhimurium* (6)*, Clostridium perfringens* (7)*, Campylobacter* etc.), intestinal parasites such as protozoan (e.g., *Eimeria species*) (8) and worms (e.g., *Ascaridia galli*), as well as stress arising from poor management (lack of adequate diets and/or water as well as sub-optimal barn or cage temperature) could compromise the integrity of the GIT (9). Toxins from mycotoxins found in feed ingredients have also been shown to be capable of negatively impacting intestinal integrity, reduce performance, and in some cases lead to high mortality (1, 10, 11).

The increasing growth of the world population and its food economy has resulted in a shift in diet and food consumption patterns toward animal products. Available data indicated that the poultry industry assumes a significant proportion of this increase in animal protein production and consumption (12, 13) which is characterized by a global increase in the production and consumption of poultry meat compared to other livestock products. Accompanying this growth, the poultry industry is faced with an enormous challenge to maintain the health and well-being of the birds. For several decades, the use of antimicrobial growth promoters (AGPs) and anticoccidia drugs became an integral part of the growing poultry industry. It was first used in non-ruminant animals' diet around the1940s (14). Antimicrobial growth promoters have been used either prophylactically to prevent an infection, therapeutically to treat an infection or sub-therapeutically as a growth promoter. According to the Center for European Agricultural Studies (15), a review of published studies from 1980 to 1989 showed increased growth performance (about 4%) and improved feed efficiency (5%) associated with the sub-therapeutic effects of AGPs. This gives antibiotics' use in livestock production an economic and health advantage. Observations from early studies on the mode of action of the growth-promoting effects of AGPs suggested that there is an interplay between AGPs and the gut microbiota (16–18). Coates et al. (16) observed that by adding AGPs to conventionally raised chick diet, body weight increased, and gut weight adjusted to constant body-weight decreased, with an apparent thinning of the gut wall compared to that of birds on the control diet. However, in the germ-free chicks, the growth-promoting effect of antibiotics was inconspicuous. Thus, several working hypotheses of the growth-promoting effects of AGPs have been governed by its ability to decrease competition for nutrients within the microflora and a subsequent decrease in growth-depressing microbial metabolites. Secondly, a thinner intestinal wall (reduced gut size or thickness) is often associated with a loss of mucosa cell proliferation during microbial fermentation, resulting in enhanced nutrient digestibility as well as a decrease in the proportion of nutrients required for gut maintenance (19, 20). However, because of the sub-therapeutic levels of AGPs administered to farm animals (at doses less than the minimum inhibitory concentration for most pathogens) and the diverse gut microbiota across various animal species, another plausible explanation has been contemplated. According to van den Broek (21), an interaction between phagocytes, microorganisms and the antibiotics cannot be overlooked. This is evident in how it exerts different inhibitory functions on inflammatory cells, chemotaxis and granuloma formation, the production of reactive oxygen species (ROS), and proinflammatory cytokine production (21–24). In this context, decreasing immunologic stress in the gut, through anti-inflammatory and immunomodulatory properties, AGPs inhibit sub-clinical infections before animals become overtly ill reducing the metabolic cost to the innate immune system (24). In view of this, unraveling the mechanisms through which AGPs improve livestock health and performance, lies in our ability to be able to piece together the role of the different activities occurring simultaneously and directing the host immune responses to interact with the intestinal microbiota. While modern-day livestock has benefitted from the use of AGPs and anti-coccidia drugs, a conundrum still exists. The re-emergence of "superbugs" that are resistant to chemotherapeutic treatment, poses a threat to public health. A widespread concern of AGPs overuse in livestock farming has resulted in its restriction and complete ban (in some cases) in livestock feed and this has led to a pressing need for an alternative to AGPs. This is important when evaluated from the welfare of the animal as well as the health implications for the consumers. The focus of this chapter is to examine the role of dietary energy, amino acids, micronutrients, and some feed additives in ameliorating the detrimental effects of stress to the bird's GIT.

BACTERIA

The most common challenge that the GIT of a bird faces is bacterial infections. In poultry, infections from *Escherichia coli, Salmonella typhimurium,* and *Clostridium perfringens* are some of the most common pathogenic bacteria that are associated with poultry production. The severity of bacteria disease will depend on factors such as the age of the bird and the load of the pathogen to which the bird is exposed to (feed, water, or the environment). This could be low grade with minimal damage to the intestine and minimal economic losses. However, in some cases, a bacterial infection could lead to significant economic loss as a result of sick birds and high mortality as seen in birds under severe necrotic enteritis (25, 26). This challenge has been effectively reduced with the inclusion of a sub-therapeutic level of AGP in the diets of poultry. However, due to concern relating to potential resistance to antibiotics (27) as well as consumers' preference, the use of AGP in poultry production is no longer desirable. Hence, there is the need to identify a new product, which must be natural (or organic) to replace AGP in birds' diet.

PROTOZOAN

In addition to the destruction or reduction in the integrity of the GIT as a result of bacterial infection, the role of intestinal protozoan, of the genus *Eimeri*a, which causes coccidiosis, has been shown to have the capacity to negatively affect the integrity of the GIT of poultry. *Eimeria* species are obligate intracellular parasites that exhibit a complex life cycle with developmental stages alternating between the external environment and intracellularly within the host (28, 29). While their virulence and pathogenicities differ among species, they cause moderate to severe intestinal lesions and induce both humoral and cell-mediated immune response. Although the incidence of *Eimeria* sp. have been drastically reduced with appropriate vaccination and the use of anti-coccidia drugs in the diets of poultry however, huge economic losses (more than US$3 billion worldwide), is still being incurred annually (26, 30). In addition to mortality that may arise from these parasites, a significant economic loss from morbidity [as a result of a reduction in feed intake, nutrient, and energy digestibility, and performance; (31–34)], the destruction of the villi and crypt (shorter and thicker villi), and a reduction in tight junction functionality have been reported. Birds are infected when the oocytes of the protozoan are ingested through water, feed, or from the litter on which they are raised. The oocysts hatch within the GIT within a few days and by day 5–7, the effects of these parasites on the bird's performance reaches its peak as revealed with a significant reduction in feed intake, oocyte shedding, and body weight gain. These parasites cause tissue damage which typically results in partial or complete destruction of villi and intestinal mucosa. Indeed, *Eimeria* sp. infection usually opens the door to secondary infections such as necrotic enteritis caused by *Clostridium perfringens*. In addition to vaccination against coccidiosis administered on the day of hatch, anti-coccidia drugs are added to the diets to prevent coccidiosis, however, with the current trend of increasing demand for organic poultry products, the use of anti-coccidia drugs in poultry diets may soon be completely phased out. By tapping into novel concepts to mitigate the effects of *Eimeria* on gut health and function, Kim et al. (35) tested the effects of epidermal growth factors (EGF) on gastrointestinal health. Epidermal growth factor, a ubiquitous polypeptide, is said to be capable of stimulating the proliferation and differentiation of epithelial cells. While EGF did not improve growth performance, they observed an improved expression of genes for nutrient transporters and tight junction proteins in *Eimeria* challenged birds (35), suggesting a cellular proliferation and rejuvenation of intestinal cells to replace damaged enterocytes during infection and inflammation. Application of molecular methods (genomics and proteomics) to provide mechanistic information on stress-induced underpinning lesions, produced in the GIT will be important in defining the role of growth factors, inflammatory cytokines, and regulatory factors in cellular proliferation, morphogenesis and tissue repair of intestinal integrity.

WORMS

Consumers of poultry products have enjoyed the supplies of healthy and wholesome meat and eggs for several decades. This is as a result of adequate veterinary care through careful use of appropriate medications to prevent or treat poultry disease(s). With an increase in demand for poultry products, especially eggs, from birds that are raised "naturally" by the consumer (including organic products), birds are increasingly being raised outdoors on pastures. This situation has led to an increase in the incidence of intestinal parasitic infections, especially by GIT helminths including nematodes and cestodes. These incidences are common in laying hens compared to broilers and this could be explained by the fact that laying hens live much longer that broiler chickens, hence they are at a higher risk of worm infection. With increasing number of organic farms being operated to cater for consumers' need for eggs from organic flocks of laying hen, the prevalence of *Ascaridia galli* is likely to increase (36). In addition to the high risk of contamination of poultry products (meat and eggs) with eggs from parasitic worms, the possibility of some of these worms finding their way into poultry eggs exists. Although there are currently data on some natural products [bioactive compound from herbs, botanicals, essential oils, and oleoresins (37)], it is extremely difficult to replicate the results from some of these studies due to factors that include experimental design, lack of enough methodological details, how the worms and eggs were treated prior to being used in the study etc. as discussed in the later part of this chapter. Additionally, the difficulty of extraction of the bioactive compound from these potential products as well as information on dose recommendation for optimal use in poultry is scarce and inconsistent.

STRESS

Stress is another factor that could predispose poultry to enteric disease including leaky gut and GIT enteritis. Stress could be caused by several factors including environmental (sub-optimal temperature), dietary (feed deprivation, unbalanced diet, sub-optimal feed, and ingredient quality, etc.), vaccination- and medication-induced stress, microflora imbalance induced stress, as well as stress as a result of pathogen or parasitic load (bacteria, protozoan, or intestinal worms). Although almost all of these could reduce the protective capability of the gut, the mechanism through which this occurs are different and hence, would require different approaches in evaluating the efficacy of an alternative product to combat this challenge. Stress to the normal functioning of the GIT will result in the disruption of the balance between the production and elimination of the ROS (38). The high level of ROS in the intestinal cells will result in the destruction of the polyunsaturated fatty acids in the membrane of cells leading to the production of peroxides which could eventually lead to the production of malondialdehyde (MDA) which has been implicated in the gradual destruction of the integrity of the cell membrane. The effect of MDA on the integrity of the GIT cell membrane includes nutrient malabsorption,

morbidity, or mortality. A compromised intestinal epithelium creates a good opportunity for opportunistic pathogens to cause an infection. In addition to this, dietary deficiencies in certain nutrients can increase the stress-induced susceptibility of poultry to oxidative stress (38–40). Loss of intestinal integrity and functionality will lead to malabsorption, a decrease in performance, bacterial translocation, product (meat and egg) contamination, morbidity, and in some cases death. The outcome of this is an economic loss to the producer.

GUT MICROBIOME AND ASSOCIATED IMMUNE SYSTEM

From birth to death, mucosal surface (including the skin, the GIT, etc.) of virtually all vertebrates are colonized by a vast array of complex and dynamic populations of microorganisms. Nobel laureate Joshua Lederberg suggested using the term "microbiome" when describing the collective genome of indigenous microbes (microflora) in the GIT (41). This microflora is composed mostly of bacteria, and to different degrees archaea, viruses, fungi, and protozoa. The GIT harbors the largest population of these organisms with over 640 different species of bacteria and more than 20 different hormones (42). These are continuously exposed to different antigens, which can be either pathogenic or nonpathogenic such as foods and commensal organisms. The list of beneficial functions attributed to intestinal bacteria continues to grow and includes nutrient processing, regulation of intestinal angiogenesis, development of gut-associated lymphoid tissue (GALT), induction of oral tolerance, mucosal immunity, and diversification of the pre-immune antibody repertoire (43). The relationship between the intestinal microbiota and the host is tightly regulated and reflects co-evolution among the inhabiting microbes, genetic, immune, and metabolic interactions with the host, and environmental influences (44). Although, the mechanisms that maintain intestinal homeostasis are just now becoming clear, evidence particularly from studies of rodents and humans has enabled the unraveling of the balance that exists between the host and its microbiota. According to Hooper and Gordon (41), these interactions can be viewed in terms of a continuum between symbiosis, commensalism, and pathogenicity. In this case, there is a fine line in the relationship between the host and microorganism from when it becomes beneficial, neutral, or detrimental to the host. This is evident in cases of intestinal epithelium damage where an opportunistic invasion of host tissue by resident bacteria can pose a serious health consequence including inflammation and sepsis. Accordingly, GALT develops in a manner that allows non-pathogenic substances, such as commensal bacteria, to survive and enables tolerance to food antigens while protecting the host from pathogenic organisms and other potentially toxic substances.

One of the main characteristics of the gut is to be sufficiently permeable to support efficient absorption of nutrients, it must avoid potentially damaging immune responses to dietary proteins and commensals. This dynamic and reciprocal interactions between the microflora, intestinal epithelium, and the immune system can be targeted to improve gut health. We know that the immune system has evolved adaptations that work together to contain the microbiota and preserve the symbiotic relationship between host and microbiota, ultimately protecting the host from pathogens and fostering complex microbial communities for their metabolic benefits. The tissues of the GIT are rich in myeloid and lymphoid cells, many of which reside in organized lymphoid tissues. The GALT is a key immunological system estimated to comprise more immune cells than any other tissue (45) with the associated structures forming a site to promote co-localization of the many immune cell types required to initiate and mediate immune function. Many of the organized GALT structures are sites of immune induction (46, 47) providing conditions necessary to induce appropriate immune responses (e.g., immunoglobulin IgA production by plasma cells). There is also considerable cellular traffic between different gut immune structures and the systemic sites including the bone marrow and spleen. Thus, the gut microbiota directs maturation of the host immune system, by eliciting antigen-specific responses which are taken up by resident dendritic cells. However, because these microbes are non-invasive, resident phagocytes are not fully activated but they stimulate a finely balanced response inducing the production of IgA which controls host-commensal interaction by both impacting commensal gene expression in the lumen and preventing adhesion of commensal bacteria to the epithelial surfaces. In the case of an infection or exposure to any variant of stress. Klasing (48) suggested that an animal susceptibility is dependent on its resistant and resilience capacity. Resistance is described as the ability to limit pathogen burden while resilience is the ability to limit the health impact caused by a given pathogen burden by maintaining productivity (e.g., growth, feed efficiency, egg production). In this context, not only do we need to be familiar with the mechanisms that are used to kill pathogens and prevent infection, a systemic understanding of how the body regulates the production, repair, and avoidance of the damage accumulated during an infection becomes imperative.

Defense against an infectious challenge requires a highly orchestrated response by the immune system. This is especially true for animals and birds that live in an environment with high pathogen load. A specific response against infection by potential pathogens, such as the production of antibodies against a particular pathogen or ROS during an immune response can be costly to the animal. By diverting the expenditures of energy and resources to the immune system surveillance, the overall performance of the bird is negatively affected. However, increasing the resilience of the animal by intentional manipulation through diet even before the occurrence of an infection, will not only confer protection but will also be advantageous in terms of productivity. Today, an interplay between diet and modulation of the immune system is a major topic of interest both in humans and livestock most of which addresses the possible maintenance or enhancement of gut health which has led to several practical applications.

THE NEXUS BETWEEN NUTRITION AND GASTROINTESTINAL HEALTH

Diet and lifestyle are crucial factors that influence the susceptibility of humans to metabolic diseases. While this can be somewhat true for livestock, the diets we provide to our birds are geared toward meeting their nutritional needs without compromising any of the desired production characteristics. The question that arises is, is the requirement set to maximize productivity in healthy birds optimal for immunocompetence and disease resistance? Using dietary protein intake as an example, at elevated temperatures, digestion and absorption are altered, favoring protein catabolism, and subsequently a reduction in protein synthesis and deposition (49). The ideal amino acid balance under high temperature remains unclear as several strategies have been invoked. Alleman and Leclerq (50) reported that low protein diets (20 vs. 16 %) impaired broiler performance at high temperature (32°C) from 21 to 42 days of age while Temim et al. (51) reported that high protein diets (28 and 33 %) compared to low protein diets (20%) slightly improved chick performance. Burkholder et al. (6) observed changes in commensal intestinal microbial populations evident by the attachment of *Salmonella enteritidis* to the ileal tissue, which increased when birds were either fasted for 24 h or exposed to high temperature (30°C) compared to the controls on the same diet.

The relationship between nutrition and immune competence has been explored over the years and more importantly, how it influences the overall health of the animal. Increasing evidence emphasizes how the nutritional value of feed is influenced in part by the structure and operations of the gut microbiome, and how that feed, in turn, shapes the microbiota. Furthermore, the nexus between nutrient metabolism and immune system as described elegantly by Klasing (48) operates through several mechanisms. These include the development of immune cells and tissues necessary for synthesizing effector cells, proliferation of certain pathogens by modifying the population of microorganisms in the GIT, providing substrates for the production of cells and molecules such as leukocytes that respond to infectious challenges, indirectly activating the endocrine system, and strengthening the intestinal epithelium against pathogenic assault. Thus, assessing immunological parameters in relation to nutritional status becomes paramount. As alluded to previously, the limit of AGPs and other drugs in livestock farming drives the need for an alternative approach to maximize productivity and to control enteric pathogens and parasites previously contained using AGPs and anticoccidia drugs in feeds. To maintain an optimum gut health in our birds, it is important to take advantage of the beneficial effect of consuming certain nutrients, beyond what is normally supplied from the diet for optimal growth and productivity. A good understanding of the aspect of gut and immunity as they relate to the maintenance of a healthy GIT flora, the modulation of the body's natural defenses systems including resistance to specific infection, improvement in diet formulation strategies to promote efficient energy and nutrient utilization is essential in order to enhance and maintain the integrity of birds. Dietary strategies including the use of major nutrients like carbohydrates, proteins (amino acids), lipids, as well as vitamins and minerals, or feed additives such as feed enzymes, pro- and pre-biotics, and antioxidants are known to play important roles in nutritional immune responses.

Dietary Strategy

Energy and Protein

Malnutrition and infection are major obstacles to survival, health, growth, and reproduction of animals and humans worldwide (52, 53). This global concern has led to the development of remarkable advances in immunology and nutrition in recent decades to shed light on the effect of various nutrients on specific GIT functions including immune response and how they influence host resistance to infection. One of the major causes of immunodeficiency globally has been attributed to protein and energy malnutrition (52, 54). The provision of diets to poultry that meets the requirements for energy and nutrient in the era of sub-therapeutic use of AGPs has been fully mastered. With partial to complete withdrawal of AGPs in the diet of swine and poultry, the ensuing challenge is how would this affect energy and nutrient utilization of poultry but more importantly, how would the nutrient requirements of poultry change in light of renewed insults on the GIT by bacteria and intestinal parasites. The former could easily be addressed while the later poses a huge challenge with the understanding that different poultry feeding operation and different species or strains of poultry experience unique challenges, and the age of the animal may also influence the severity of this challenge. Although, adequate levels of nutrients in the diets of animals play important roles in maintaining an "optimal" immune response, deficiencies, and in some cases, excessive intake, could have negative consequences on the immune status and susceptibility of the animals to a variety of pathogens. It has been shown that the strongest determinant of the gut microbial profile is the host's diet (2–4). Factors such as diet composition, nutrient density, diet physical characteristics, ingredients and diet processing method (feed processing techniques), and type of feed additives play significant roles in the dynamics of the GIT microflora. From a nutritional viewpoint, substrates (e.g., amino acids, energy, enzyme co-factors) are needed to support the clonal proliferation of antigen-driven lymphocytes, the recruitment of new monocytes and heterophils from bone marrow, the synthesis of effector molecules (e.g., immunoglobulins, nitric oxide, lysozyme, complement), and communication molecules (e.g., eicosanoids, cytokines). Recent studies indicate that dietary protein deficiency, which reduces the concentrations of most amino acids in plasma (55) and compromises the immune system, can suppress immune response by decreasing lymphocyte number, overall leukocyte count, and splenic cell proliferation stimulated with phytohemagglutinin-M (56–58). Moreover, both immune systems (innate and adaptive systems) are highly dependent upon an adequate availability of amino acids for the synthesis of these proteins and polypeptides, as well as other molecules with enormous biological importance (59). These substances include nitric oxide (NO), superoxide, hydrogen peroxide, histamine, glutathione, and anthranilic acid.

For nutritional purposes, amino acids have been divided into two groups; essential and non-essential dietary amino acids. Essential amino acids are those that cannot be synthesized endogenously, or at the rate that is sufficient to meet physiological needs (including maintenance, growth, and reproduction) of the bird and must be supplemented in the diet. Non-essential amino acids, on the other hand, are those that can be synthesized endogenously from a non-amino acid source. Approximately 90% of diets for poultry in the U.S. is comprised of corn and soybean meal. Because the cost of these key ingredients has increased markedly in recent years, keen interest exists in feeding reduced protein corn-soybean meal diets with an adequate level of supplemental crystalline amino acids. It is to be noted that metabolically, several of the amino acids defined as essential can be synthesized from precursors that are structurally similar to these amino acids. Amino acids are an important class of nutrient that is needed for gut health and the ability of the bird to fight infection. During immunological stress, a higher level of available amino acids (needed for growth) is repartitioned to produce cytokines (e.g., interleukin-1, interleukin-6, and tumor necrosis factor-α) which alter the overall protein metabolism. Based on this, a higher level of certain amino acids may be required in the diet of birds that are raised under a relatively higher pathogen load.

Individual amino acids affect immune responses either directly or indirectly through their metabolites. The role of these amino acids (glutamine, arginine, tryptophan, and cysteine) on the integrity, growth, and development of the intestinal epithelium, gene expression, cell signaling, antioxidative responses, and their associated immune functions have been investigated (8, 39, 55, 60). In particular, the role of glutamine, arginine, tryptophan, and cysteine (39, 55, 60) has been reported. Gao et al. (39) showed that *in ovo* feeding of arginine influenced the development of lymphoid organs in broiler chicks while, Tan et al. (8, 61) showed that L-arginine supplementation could regulate the immune function in challenged birds. In addition to this, Lee et al. (62) showed that arginine had a positive effect on the chicken cellular response to infectious bronchitis virus with its potential to function in the repair of damaged intestinal epithelium cells by activating the mTOR pathway (63). Glutamine, a precursor for several biosynthetic pathways, is required for growth and cell division and a principal metabolic fuel for enterocytes, lymphocytes, macrophages, and fibroblast (64, 65). Classified as a non-essential amino acid, its requirement may not be able to meet the optimum level for specific conditions such as stress, infection, or injury that birds may be predisposed to, due to extensive genetic selection (66). Dai et al. (67) reported a significant improvement in weight gain and feed efficiency in broiler chickens supplemented with glutamine. Improved meat quality and humoral immune response in poultry associated with better development of the intestinal mucosa have also been observed (68). Supplementing glutamine in the diet may be beneficial, not only in hyper-catabolic states but also in the maintenance of optimal health and maximal rates of growth in healthy animals (65). Glutamine is a good source of energy for mesenteric lymph nodes lymphocytes (59, 69) and is essential for the proliferation and function of lymphocytes (52). It enhances phagocytic activity of macrophages, and the production of cytokines and antibodies by T and B lymphocytes (59, 70), as well improving the growth of chicks (68). Threonine is another important amino acid that is abundant in the mucin that lines the entire GIT. An adequate dietary level of threonine has been shown to enhance intestinal integrity in poultry (4, 71).

In terms of quality of the different feed ingredients, it has been reported that animal protein sources, such as meat and bone meal, has the potential to enhance the proliferation of the bad bugs such as *Clostridium perfringens*, as a result of its high collagen and elastin contents that are resistant to endogenously secreted digestive enzymes in swine and poultry (72–74). Additionally, the nature of the starch crystallinity could delay amylase action on carbohydrate recovery (75–78). Proper selection coupled with an optimal level of inclusion in the diet is essential. Cereal (corn, wheat, sorghum, etc.), and legumes (soybean meal and canola meal) make up more than 80% of the diets given to poultry, all of which contain non-starch polysaccharides (NSP). Depending on the composition of the diet and the inclusion level, poultry, in general, lacks the ability to effectively break down the NSP in the midgut due to the shortage or the absence of substrate-specific endogenous enzymes capable of the breaking down NSPs, hence they exhibit a decreased nutrient digestion and absorption. An estimated 400–450 kcal of digestible energy per kg of feed remains undigested by broilers because of the NSP content present in corn-SBM diets (79). Hence, the use of NSP enzymes (carbohydrases) have been explored to ensure breakdown, degradation, and utilization of most of the components in corn and SBM to attain ideal performance and profit from these diets. This also minimizes the quantity of undigested NSP that reaches the hindgut, hence reduces the proliferation of harmful bacteria. High levels of NSP in these diets can also predispose the chickens to necrotic enteritis, a disease that has become prevalent with the removal of AGPs. The primary challenge is to minimize the exposure of birds to potentially damaging bugs as well as the insults arising from such a challenge on intestinal and mucosal integrity. This becomes paramount because, an increase in intestinal inflammation and ROS level, and a reduction in intestinal membrane integrity through a reduction in tight junction functionality is one of the signs of gastrointestinal infection in non-ruminant animals (8, 78, 80). The composition of the feed given to poultry is important. Diets that are deficient (quantitatively and qualitatively) in energy and nutrient have the tendency to limit the ability of the bird to be able to react accordingly to any developing insult in its GIT. Because of the tendency of the bird to reduce feed intake as a result of intestinal infection and inflammation, it may be necessary to increase the density of certain nutrients to equip the bird against any challenge (8, 81). Similarly, to minimize productive and economic losses as well as improving livestock welfare in the era of no AGP in the diets of non-ruminant animals, it is essential to look for a solution that is effective but also acceptable to the consumers. Based on consumer demand, the way to address this challenge may be the use of natural or organic products. Therefore, close attention should be placed on the quality of the different feed ingredients that goes into the diets of poultry.

For example, feeding a highly digestible diet has an advantage over diets that contain relatively higher indigestible components such as NSP or resistant starch. The higher the quantity of the undigested portion of the diets that reaches the hindgut, the higher the probability of such promoting the growth of microbiota that may be potentially harmful to the integrity of the GIT of the bird. Early feeding is another dietary and management approach that can enhance and strengthen intestinal integrity. It has been suggested that the sooner the birds are exposed to nutrient-rich diet the better the development of the GIT mucosal. Thus, it is important to adequately provide the much-needed nutrients to birds especially during the transition period from *in ovo* nutrient utilization from the yolk to their gradual reliance on nutrients from the diet (82). What happens during this transition period could be critical to the health and integrity of the GIT of the bird later in life. The advantage of the *in ovo* feeding of the developing embryo during the late incubation stage could be taken.

Micronutrients and Feed Additives

In a different capacity, adequate levels of vitamins and minerals are essential for the birds to efficiently utilize dietary nutrients, post-absorption, for growth, health, reproduction, and survival. Most vitamins cannot be synthesized by poultry in sufficient amounts to meet physiological demands, hence must be obtained from the diet. Vitamins are present in many feedstuffs in minute quantities and can be absorbed from the diet during the digestive process. In general, chronically severe deficiencies of these micronutrients are more debilitating to the development of the immune system than macronutrients such as energy and protein. Nutrient deficiencies that are especially damaging to the development of the immune system include linoleic acid, vitamin A, iron, selenium, and several of the B-vitamins. Adequate levels of dietary selenium, nucleotides, long-chain polyunsaturated fatty acids, and vitamins A, C, and E in modulating the host defense against infectious pathogens have been reported (52). Vitamin A deficiency (83, 84) and excess (84, 85) have been shown to depress immune responses in chicks. Most research suggests that vitamin A deficiency is associated with reduced cellular immune responses whereas vitamin A excess impairs antibody responses. Vitamin E is primarily known for its role as an antioxidant in reducing cellular free radical damage, but its deficiency could lead to a reduction in immune responses (86). Male broilers fed diets varying in DL-α-tocopherol acetate from 0 to 87 mg/kg of diet exhibited altered thymic and splenic T cell populations indicating that more helper T cells (CD4) were present with increased dietary vitamin E and thus improved responsiveness to immunologic stimuli (87). Growth performance and immunity as affected by drinking water fortified with vitamins and electrolytes were evaluated in heat stressed broilers (88). The addition of B-vitamins, fat-soluble vitamins (A, D, and E), and electrolytes to drinking water improved aspects of antibody production to the serum red blood cell (SRBC) over that of control birds, and reduced broiler mortality from the heat stress. The trace minerals that have been associated with an improvement in immunity, or functions that support immunity, are Zn, Mn, Cu, and Se. Dietary Se interacts with vitamin E in antioxidant protection of cells because it is a component of glutathione peroxidase. Dietary Se intake increases TCR signal strength through mechanisms that involve free thiol concentrations. In addition to antioxidant status, Se has been shown to impact disease resistance. For example, broilers infected with *E. tenella* had improved resistance (i.e., reduced mortality and cecal lesions) when supplemented with Se (89). The immune system is dependent on the functions of cellular metabolism with Zn being central in cellular metabolism and functions both structurally and catalytically in important biochemical pathways. It has been hypothesized that the antimicrobial effect of Zn leads to growth promotion where gut microbiota is altered to reduce fermentation loss of nutrients and to suppress gut pathogens (90). Similarly, other evidence suggests that pathogens can have a competitive advantage over the commensal microbiota under Zn-limiting conditions, thereby being promoted under an inflamed state (91). Recently, it was shown (91) that Zn competition exists in *C. jejuni* and other bacterial species in the host microbiota of conventionally-raised vs. germ-free broiler chickens (*Gallus gallus*). Under conditions of Zn deficiency, preferential growth of bacteria able to survive at low-Zn levels might ensue. Furthermore, many recent studies have shown that prophylactic doses of Zn (as Zn oxide, ZnO) in various animal models increased the presence of Gram-negative facultative anaerobic bacterial groups, the colonic concentration of short chain fatty acids (SCFAs), as well as overall species richness and diversity. Adequate levels of Zn supplementation (between 50 and 70 mg/kg) in poultry diet have been shown to reduce the production and minimize the impact of oxidative damage in the intestine of broilers under intestinal stress (38, 40). This demonstrates the need for nutrient-directed management practices to reduce the effect of pathogens on the GIT of poultry (52). The level and nature of micronutrients in the diet given to poultry could be used to control the population and diversity of hindgut microbiota.

Furthermore, about 80% of the feed ingredients in poultry diets is plant-based, hence the use of exogenous enzymes such as phytase, which liberates phytate- and phytic-acid bound phosphorus (and other nutrients as a result of extra phosphoric effects), protease to enhance protein digestion, and carbohydrases for NSP breakdown is essential (92). One of the ways through which the NSP-digesting enzymes function is by reducing digesta viscosity which subsequently allows digestive enzymes to gain better access to the digesta and hence, increase nutrient and energy digestibility and absorption. Secondly, the passage rate of the digesta is slowed down allowing for sufficient time for digestion and absorption to take place. Carbohydrase enzymes indirectly could enhance GIT health by reducing the wetness of litter which could result in a reduction in the buildup of pathogenic organisms in the litter. The combination of these actions will lead to a reduction in the quantity and quality of nutrient and energy that reach the hindgut thereby denying pathogenic organisms the needed nutritional support needed for proliferation (78, 93). By supplementing the diet with enzymes, Jia et al. (94) observed an improvement in growth performance as well as a reduction in the negative effect of *Clostridium perfringens* on birds' performance. Their rationale was that NSP-degrading enzymes might reduce microbial activity because of

substrate limitation in the ileum. Further evidence of their beneficial effect is the ability of NSP, through depolymerization, to generate galacto-, gluco-, or manno-oligomers, which can serve as prebiotics stimulating the growth and activity of lactic acid bacteria (95, 96). Another component of the diet of swine and poultry is the phytin which has been implicated in poor phosphorus digestibility in poultry. However, it has been reported that the breakdown of this structure in the gizzard could result in the formation of phytic acid that is negatively charged and could interfere with protein digestion [poor protein digestion (97–99)] in the diet which eventually becomes a rich source of nutrients to the microbiome in the hindgut. Although pro- and pre-biotics have been shown in some cases to show some potentials as alternatives to AGP, the lack of consistency in published data complicates this promise. Additionally, some of the inconsistencies or lack of significant effects could be explained, in part, by the nature of experimental design (appropriate design, and an adequate number of replicates), the age of the birds, and the availability of sufficient substrates for the enzymes.

In most cases, the inclusion of feed additives in poultry diets has resulted in improved feed intake and growth performance with a resultant improvement in feed efficiency. With the ban on AGP, phytogenics (a relatively new group of feed additives), have the potential to be embraced by the consumers as an alternative to AGP. Unlike drugs, these products are looked at as being of a natural origin. For more than a century, it has been recognized that certain plants, especially their secondary metabolites, have medicinal properties and have been used both in human and animal medicine with some products displaying antioxidative properties as well as other beneficial effects on the GIT (100–103). This group of compounds is large, and this makes their classification quite challenging and variable. Windisch and Kroismayr (37) have attempted to classify these plant products into four broad categories. These groups include herbs, botanicals, essential oils, and oleoresins. Herbs are produced from flowering and non-woody plants while botanicals are produced from roots, leaves, and bark of entire or processed plants (37). Essential oils, which is one of the most common group from this classification, are produced from hydro-distilled extracts of volatile plant compounds while oleoresins are extracts from non-aqueous solvents. Despite the potentials that these products possess, there is still a significant issue with purity, adequate description, and established dosage levels. Most of the available phytogenics have been shown in *in vitro* studies to possess antimicrobial and growth promoting effects, as well as being able to enhance the digestive process (103, 104).

LOOKING FORWARD

Efforts should be placed on developing an alternative to AGP as well as materials for the control of intestinal parasites (worms and *Eimeria* sp.) that are acceptable to the consumers, in this case, "natural product." The process of evaluating any alternative products must be cognizant of the welfare of the birds and the concerns of the consumers. With increasing number of birds raised on pasture, coupled with an increase in the level of interaction between birds (e.g., battery cage vs. aviary systems; intensive vs. semi-intensive production systems), there is the need to redefine biosecurity to take into account the latest development in how birds are currently being raised. In order to fully evaluate any product (e.g., as an alternative to APG), a lot of efforts must be placed on the design, the health status of the animals, and the products (type, dose, parameters to measure, and when such samples are to be collected).

METHODOLOGICAL CONSIDERATIONS

In order to effectively evaluate the efficacy of a product such as an alternative to AGP, it is essential to create an environment in which the birds can respond to the treatments. For instance, AGP will not be as efficacious in a healthy bird that is raised in a clean or new poultry barn compared to a bird that is raised under conditions that naturally will predispose it to a certain type of stress or infection, in this case, intestinal stress, or infection. In order to achieve this, birds must be subjected to a certain level of intestinal stress and challenge. Moreover, the design of the study must include a positive control that is unchallenged as well as a challenged positive control+AGP. Furthermore, there should be a negative control (challenged without AGP) and the negative control treatment (diet) to which the product that is being evaluated would be added. Contamination of any kind (feed, bird, water, litter, cages, etc.) should be avoided and efforts must be made to determine, quantitatively, the concentration (or activity level) of the product that is being evaluated. Furthermore, it is essential to make sure that the positive and negative control diets are similar in energy and nutrient composition as much as possible in order to make sure that we do not inadvertently create different intestinal microbiota as a result of slight differences in the composition of the experimental diets. The tendency for the challenged birds to consume less feed has been widely reported; hence, depending on the study, it may be important to pair-feed the birds to the feed intake level similar to that of birds on the negative control diet (usually the treatment in which the birds consume the least amount of feed). This will control the feed intake and the quantity of the test product(s) that birds across all the treatments will consume. If this is carefully done, any interpretation of significant effect could be strongly attributed specifically to the test material. It is understandable that birds could be different from one location to the other; however, it is essential to include in the report as much information as possible. This would allow the reader to draw his or her own conclusion based on the information provided. Information such as the age of the bird, species or strain of the bird, gender, genetics, vaccination program, medication program (if used), room temperature and humidity, mortality, and morbidity should be provided. Proper experimental design with an adequate number of replications is essential for data analysis and interpretation. Finally, as the volume of research in the area of gut health in poultry and swine in response to withdrawal of AGP increases, it may be essential for researchers to develop some important biomarker of gastrointestinal functionality against

which results from these studies may be standardized. Similar steps have been taken by nutritionists in the area of standardized ileal amino acid digestibility. According to Celi et al. (105), parameters to be measured to access the proper functioning of the gut should include, diet, effective digestion and absorption biomarkers, microbiota community, effective immune status, gut mucosa, and neuroendocrine and motor function of the gut. In line with these parameters, the development of biomarkers of gut health is imperative to gain clarity of understanding the pathophysiological events that influence the intestinal barrier, its functionality and the ecology of the GIT microbiota. Biomolecular monitoring of the GIT could offer rapid but precise disease detection and management mechanism by providing non-invasive strategies to define potential pathways behind the pathogenesis of diseases. Furthermore, this can assist in the assessment and diagnosis of various gastrointestinal conditions.

SUMMARY

Maintaining a healthy gut in our birds would continue to be a challenge for the foreseeable future. This will not be as a result of our inability to come up with products that will, to a reasonable extent, be able to fill in the gap left with the withdrawal of AGP, but rather coming up with a product that the consumers will readily accept. Based on the current trend, this product must be a "natural" product. If this trend continues, our ability to be able to accurately identify and extract products that are able to protect and enhance the development of the "good bug" and eliminate the "bad bug" will be crucial. To maintain the integrity of the GIT may require more than one product but a combination of products that could exhibit both pro- and pre-biotic effects on the GIT. Furthermore, future poultry breeding and selection program should include genes responsible for the bird's ability to resist an infection as well as the ability of the bird to be resilient in the face of high pathogen load. It is a common observation that with a group of birds that are fed the same diet, raised in the same space, and subjected to the same environmental conditions (similar level of stress, physical or biological), a few of these birds are able to completely resist an infection, some are able to cope with the infection (resilient), while others easily succumb to the infection. Evidently, in addition to the economic traits, the selection criteria should include those genes that make some of these birds resistant or resilient to gastrointestinal challenge. This means a holistic approach through novel strategies is necessary to minimize the impact of these stressors on poultry GIT health.

AUTHOR CONTRIBUTIONS

OO helped with research for some of the articles used when writing this article. OO wrote a portion of the manuscript (part of the introduction and a portion of the following section: GUT MICROBIOME AND ASSOCIATED IMMUNE SYSTEM) under direction from SA. SA wrote a significant portion of the article and reviewed and edited the manuscript before submission.

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

frontiers in Veterinary Science

REVIEW
published: 19 February 2019
doi: 10.3389/fvets.2019.00019

Role of Feed Processing on Gut Health and Function in Pigs and Poultry: Conundrum of Optimal Particle Size and Hydrothermal Regimens

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The aim is to give an overview of available literature data on the role of feed processing on gut health and function with specific focus on particle size and hydrothermal processing. In addition, influence of feed processing on efficacy of exogenous feed enzymes will be discussed. The current feed processing technologies are such that ingredient choices and diet form are refined to improve feed intake and nutrient utilization efficiency. Finer feed particle size enables optimal nutrient utilization and enhances animal performance due to increased surface area allowing better contact with digestive enzymes. Moreover, adequate diminution of feed ingredients is beneficial to feed manufacturing processes such as mixing and hydrothermal treatments including pelleting, extrusion, and expansion. However, emerging trends in consumer and regulatory demands for restriction or cessation of animal production practices such as use of antimicrobial growth promoters are challenging current approaches to feed processing. There is limit as to the fineness of the particle size, as very fine particles negatively affect gut health due to higher incidences of stomach ulceration in pigs and gizzard dysfunction in poultry. Coarse particle size increases stomach and hindgut acidification which may be beneficial in controlling proliferation of enteric pathogens such as *salmonella* and *E. coli*. Optimal particle size could be designed in the grinding process using roller or hammer mill. However, since most commercial pigs and poultry diets are subjected to hydrothermal processes, additional reduction of feed particle size is inevitable. The need to achieve high physical quality and to reduce potential levels of feed-borne pathogens such as *Salmonella* has led to the application of relatively high conditioning temperatures during conventional hydrothermal processes, a practice that does not favor high nutrient utilization and stability of heat sensitive feed additives such as feed enzymes. Therefore, with evolving pig and poultry production practices, the regimens for feed processing will no longer be appreciated only in terms of optimizing nutrients utilization, but also in terms of impact on feed hygienic status, efficacy of feed additives, animal health, and food safety.

Keywords: antibiotic-free feeding programs, gut health and function, exogenous feed enzymes, feed particle size and hydrothermal processing, pigs, poultry, nutrition

OPEN ACCESS

Edited by:
Rajesh Jha,
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Reviewed by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *01 November 2018*
Accepted: *18 January 2019*
Published: *19 February 2019*

Citation:
Kiarie EG and Mills A (2019) Role of Feed Processing on Gut Health and Function in Pigs and Poultry: Conundrum of Optimal Particle Size and Hydrothermal Regimens. Front. Vet. Sci. 6:19. doi: 10.3389/fvets.2019.00019

INTRODUCTION

Advances in genetics has certainly produced commercial strains of poultry and pig with greater performance (growth, reproduction etc.) with minimal feed input. For example, over the last 5 decades, the body weight of broilers at 42 days has increased by 25–50 g per year and the feed conversion ratio to 2 kg body weight has improved 2–3 points annually (1, 2). A review of North Carolina white leg horns performance tests from 1958 to 2011 showed that the average age at 50% production decreased by 34 days, pullet body weight at point of lay dropped from 1.61 to 1.16 kg, mature hen body weight from 2.05 to 1.68 kg, feed conversion improved from 2.90 to 1.99, while egg mass increased from 16.3 to 19.9 kg per hen housed (3). With the introduction of crosses in the early 60's, specialization in dam and sire lines have been very successful in effecting genetic improvement of economically important traits in pigs, especially daily gain, backfat thickness, feed efficiency, and litter size. An annual genetic progress for gain of +20 g/day, lean meat of +0.5% and litter size of +0.2 piglet/litter has been achieved over the last few decades (4, 5). The nutrition of these animals has also evolved overtime but not as much as genetic advances; for example genetic selection brought about by breeding companies is responsible for 85–90% of the improvements in broiler growth, and advances in nutritional management contributed only 10–15% (1). However, the necessity to achieve and sustain genetic potential has been the driving force behind continuous advances in nutrition concepts seen in modern day commercial pig and poultry enterprises. In this context, feeding, a major control point of profitability has evolved and progressed both in terms of understanding digestive physiology and metabolism, and in the more precise evaluation of the quality of dietary raw materials. Advances in monogastric nutrition is clearly exemplified by the widespread adoption of net energy, standardized ileal digestible amino acids ideal ratio and digestible phosphorous concepts enabling nutritionists to formulate cost-effective and optimal diets (6, 7). Application of these concepts have also stimulated tremendous investments in commercial research and development in speciality feed ingredients such as crystalline amino acids and feed additive technologies such as feed enzymes, probiotics, and organic acids among others to further optimize nutrition (8, 9). Feedstuffs processing and diet manufacturing have also evolved such that the composition, ingredient choices, and diet form have been refined to improve feed intake and efficiency. However, the modern-day nutritionists perceive dysfunctional gastrointestinal tract as a potential rate-limiting factor in the survival and productivity of monogastric farm animals. This perception has been fostered by the emergence of ideas and concepts concerning the development and function of the digestive tract in the light of advances in genetic improvement and restriction on the use of antibiotic growth promoters and anti-coccidial drugs. The intention of this chapter is to provide a critical overview of feed processing with emphasis on particle size and hydrothermal processing (HTP) in the context of gut health and function. Implication of feed processing on application of exogenous feed enzymes will also be discussed.

FEED PROCESSING

The principal role of feedstuffs is to provide nutrients that can be digested and utilized for maintenance and productive functions. To maximize performance, pig and poultry diets must contain the correct balance of the essential nutrients required to meet the nutritional needs of various stages of production (6, 7). However, applying accuracy and a degree of precision in diet formulation requires an intimate knowledge of the animal, its daily nutrient requirements, feed intake potential and a more comprehensive understanding of the ability of the selected feedstuffs to provide target nutrient at least/best cost (10, 11). The range of feedstuffs incorporated into modern monogastric diets is continually changing due to several factors such as price volatility, component pricing dynamics, emerging novel, and opportunity feedstuffs, government regulatory regimens among many other reasons (12–20). However, feed processing must constantly produce feed products that are palatable, safe, and meets nutritional needs of the target animals. In this context, feed technology has progressed from simple mixing of mash feed to advanced preparations that involves various physical and hydrothermal processing operations (21). Today, most pig and poultry feeds are manufactured by employing a combination of technologies including physical grinding with hammer and/or roller mills in conjunction with hydrothermal processing including pelleting, expansion, or extrusion (21, 22). Indeed, feed processing includes single or multiple manipulation of feedstuffs or complete feed prior to presentation to the animal (22). Many advantages that can be attributed to feed processing includes improved availability of nutrients, destruction of inhibitors and toxins, facilitation of the use of a wide range of raw materials in diet formulations, production of hygienic feed, and reduction of feed wastage (21, 23). However, it is well-recognized that processing parameters such as extent of particle modification, processing temperature, pressure, duration, and water determine the physical and chemical reactions in and between nutrients as well as the adhesive properties on the feed particle surfaces, the final physicochemical structure and the hygiene status of the feed (22, 24). These attributes can directly and indirectly influence the impact of the processed feed on the digestive tract ecology and thus animal health, performance, and feed cost. There is a large body of reviews on aspects of feed processing in terms engineering (21, 24–26) as well as animal performance and feed economics (23, 27, 28). Subsequent sections will focus on the impact of particle size and HTP on gut health and function.

PARTICLE SIZE

Pigs and poultry are simple stomached animals largely dependent on repertoire of endogenous enzymes for their nourishment. One of the most important factor that determines feed utilization in these animals is the particle size distribution. Cereal grains are primary energy sources in monogastric diet and they require to be processed before or after mixing with other diet components. Particle size reduction always includes grinding step with hammer or roller mill to facilitate further processing (e.g., mixing, pelleting, extrusion, expansion). There are numerous

reviews on the benefits of grinding feed ingredients in terms of milling throughput, nutrient utilization, growth performance and economics (22, 27–29). With respect to animal performance, the smaller the particle size the greater is the feed utilization because of increased specific surface of feed particles allowing better contact with digestive enzymes. The quality of grinding is assessed by factors such as homogeneity, uniformity, and size of the feed particles. One of the main challenges in monogastric feed manufacturing is uniformity and mixing homogeneity i.e., particle size distribution (22, 28). The feed industry strives to produce homogeneous feed, however, it has been reported that different factors including particle size, particle shape, density, electrostatic charge, dustiness, hygroscopicity, and flowability can significantly affect the quality of the feed mixtures (21, 29, 30). Particle characteristics, particularly particle size, are one of the most controversial issues in pig and poultry nutrition. From economic point of view, optimal particle size distribution adapted to physiological needs of animal enables optimal utilization of nutrients and enhances animal performance. However, recommendations regarding optimum particle size is contradictory as the results from feeding trials are confounded by a number of factors including feed physical form, complexity of the diet, grain type, endosperm hardness, grinding method, pellet quality, and particle size distribution (27, 28). In general, it is recognized that finer grinding increases the energy consumption at the mill and decreases capacity of grinding equipment and flowability, increases dust problems, and most importantly, too fine particles are associated with negative impact on gastrointestinal tract health and function.

IMPACT OF PARTICLE SIZE ON GUT PHYSIOLOGY

Pigs

Gastric ulcers are one of the most important causes of sudden death of market hogs and can result in large economic losses (31). Presentation of gastric ulcers is typically in non-glandular gastric mucosa (*pars esophagea*) and estimates indicate that 1–2% of growing-finishing pigs die from gastric ulcers annually (31, 32). The reasons for occurrence of gastric epithelial alterations have not been clearly elucidated but numerous reports indicates feed particle size of cereals and other feed components are risk factors (**Table 1**) (38, 40–43). The presence of high quantities of fine particles in pig feed lead to higher incidence of stomach ulceration and other negative alterations of gastric mucosa as exemplified by keratization and mucosal erosion (28). In this context, concept of optimal particle size of pig feed is a widely researched aspect. Finer particles tend to increase fluidity of the stomach content which is associated with lesions of the *pars esophagea*. Pigs fed a coarse diet have heavier stomachs than pigs fed a fine diet, which probably reflects that coarse diets require more muscular action for processing by the stomach than fine diets. However, deleterious effects of finer particle size in pigs is dependent on grain type. For example, macroscopic keratosis scores were greater for pigs fed 0.30 vs. 0. 90 mm corn and hard sorghum but lower for pigs fed 0.30 vs. 0.90 mm soft sorghum (38). The effects of feed particle size on small and large intestine is less clear than in the stomach. However, an increased crypt depth in the colon was observed in pigs fed coarse diets (44, 45). This was linked to increased flow of undigested starch in the hindgut promoting production of butyrate, a preferred substrate for the colonocytes.

Poultry

Proventriculus and gizzard are the true stomach compartments, HCl and pepsinogen are secreted in the proventriculus and mixed with contents in the gizzard via muscular movements. However, because poultry do not have teeth, the gizzard has an important additional function of grinding feed material. Peculiarity is that the gizzard contains strongly myolinated muscles and has a koilin layer that aid in the grinding process (46). Detailed overview of gizzard functionality and regulation has been described (46, 47). Experimentations indicate that proventriculus and gizzard should be considered as one compartment with respect to digestive function where material flows rather rapidly through the proventriculus but will potentially be refluxed back into the proventriculus repeatedly during gizzard contractions. Lack of structural component in poultry diets has been associated with dilated proventriculus and a non-functional gizzard consequently compromising feed utilization and intestinal health (46, 48, 49). It has been reported that the volume of the gizzard may increase substantially when structural components such as whole or coarsely ground cereals are added to the diet (**Table 1**) (33–37), sometimes increasing to more than double the original size (46). The peculiarity is that when the diet contains structural components, digestive function improves through increased retention time, lower pH, and better grinding. These mechanisms in conjunction with better synchronization of feed flow are thought to improve nutrient utilization (46). Nir et al. (50) reported that a greater coarseness of feed increased the relative gizzard weight, whereas Amerah et al. (51) suggested gizzard stimulation was due to the length of time that the coarse particles resided in it. However, the effect of feed form (discussed later) must be considered in combination with particle size. Interestingly, it has been reported that longer retention times of the digesta in a well-developed gizzard might modify dietary protein digestion dynamics through increased HCl and pepsin secretion (52). Because of gizzard grinding, particles reaching the small intestine have no relationship with feed particle size, therefore, the impact of feed particle size on small intestine and ceca physiology is minimal (27).

IMPACT ON GUT MICROBIAL ACTIVITY

Pigs

Feed particle size distribution has been associated with strong influence on the presence of enteric bacteria pathogens. Data indicate that coarse feed particle size decreases pH in the stomach content compared with fine particle size linked to changes in gastric physicochemical and microbial properties (44, 45). Mikkelsen et al. (44) showed that coarsely ground feed increased solid gastric content, anaerobic bacteria count and concentration of organic acids in pigs. Further *in vitro*

TABLE 1 | Impact of feed particle size on gastrointestinal physiology in pigs and poultry.

Species, age/BW	Particle size range, cereal	Effects, larger vs. smaller	References
BROILER CHICKENS, AGE IN DAYS			
21–42	0.34–1.12 mm, corn	Increased gizzard weight and duodenal VH and CD	(33)
1–21	0.84–1.16 mm, wheat	Increased, crop, gizzard, small intestine, and ceca weight	(34)
1–21	0.59–0.95 mm, corn	Increased gizzard weight, no effects on ceca weight	(35)
1–42	0.65–1.3 mm, corn	No effects on gizzard and small intestine weight	(36)
LAYERS, AGE IN WEEKS			
20	0.15–2.5 mm, corn, wheat	Increased gizzard and GIT weight. No effects on histomorhology	(37)
PIGS, BODY WEIGHT RANGE, KG			
5–18.0	0.30–0.90 mm, corn, hard sorghum	Reduced stomach ulcerations. No effects on intestinal histomorphology	(38)
5–18.0	0.30–0.90 mm, soft sorghum	Reduced stomach ulcerations and no effects on SI histomorphology	(38)
50–100	0.40–1.00 mm, corn	Reduced stomach keratosis	(39)
30–60	0.43–1.10 mm, barley	Reduced stomach ulcerations, no effect on SI histomorphology	(40)
60–90	0.40–1.30 mm, wheat	Reduced stomach ulceration	(41)
5–100	0.50–1.25 mm, corn	Reduced stomach ulcers	(42)

experimentation with the stomach content of pigs fed coarsely ground feed showed increased death rate of *Salmonella enterica serovar Typhimurium* DT12. This was associated with significantly higher concentration of undissociated lactic acid as exemplified by a strong correlation between the concentration of undissociated lactic acid and the death rate of *S. enterica serovar Typhimurium* DT12. These data demonstrated that pigs fed coarsely ground feed had much higher gastric microbial fermentation than pigs fed finer diets linked to slower gastric passage rate, increased gastric dry matter content and consistency (44, 45, 53). Gastric acidification in suckling pigs is mainly due to the presence of lactic acid, resulting from bacterial fermentation of lactose (54, 55). Cranwell (56) demonstrated that piglets achieved maximal gastric HCl output at the age of 5–6 weeks and that exposure to solid feed was important in this process. It follows that at weaning, the piglet not only loses lactose induced acidity but the ensuing anorexia exacerbate the ability of physiologically immature gut to produce enough HCl to keep stomach pH at an optimum of 3.5 (8, 9). Furthermore, diets fed to young pigs often have a high buffering capacity, which can further reduce stomach acidity (57–59). At low gastric pH, digestion of protein and populations of beneficial bacteria (*lactobacilli*) are maximized and harmful bacteria such as enterotoxigenic *E. coli* are inhibited (8, 9, 58). Consequently, gastric conditions created by coarse feed are interpreted to create additional "barrier" against fecal/feed-oral pathogenic bacteria transmission. Moreover, lower pH in digesta matrix sustains a higher proportion of short chain fatty acids (SCFA) in undissociated form and therefore antimicrobial potency (57). It seems that weaned pigs can benefit tremendously from coarsely ground feed, however, there are apparently limited research investigating manipulation of weaned pig feed particle size to characterize impact on gut health and subsequent performance.

Feed particle size not only impacts gastric ecology but also other parts of the GIT particularly the large intestine. Studies have demonstrated that coarse diets were strongly associated with higher propionic and butyric acid levels in the cecum and colon contents (42, 44). It is possible that coarse feed particle size may promote an increase of bacteria populations producing SCFA and, thus, contribute to gastrointestinal health by preventing the proliferation and/or virulence of harmful bacteria such as *Salmonella* spp. and *E. coli*. Studies have demonstrated that change in feed presentation could be associated with microbiota modification (different composition and/or metabolic activities) in the GIT of pigs (28). As alluded to studies have indicated that larger particle size increases flow of starch in the large intestines and this has been shown to increase SCFA production limiting growth of coliforms and *Salmonella* (28, 44, 45). Phenomenon of retrograde movement has been demonstrated in pigs and poultry where anti-peristaltic low amplitude waves in the hindgut (cloaca and colon) result in movement of digesta back to the ceca and distal ileum (46). The risk of this phenomenon is potential contamination of small intestines with hindgut pathogens. Cappai et al. (60) hypothesized that diet form could prevent retrograded contamination of small intestine by stimulating efficient functioning of the ileocecal valve. Pigs were fed diets differing in grinding intensity (roller vs. hammer) and sieve sizes (1 vs. 6 mm). Coarse meal significantly increased thickness of ileal cecal valve which was interpreted to have potential of preventing of digesta backflow into foregut. According to literature data, decreasing the quantity of fine particles in pig feed is strongly recommended (7). Generally, based on existing literature the quantity of finer particles (<0.4 mm) should be as low as possible due to the negative effect on GIT health and the quantity of the coarsest fractions (>1.6 mm) should also be low due to decreased nutrient utilization whereas the share of medium-sized particles (>0.5 to <1.6 mm) considered optimal for pig's digestive system should be as high as possible (38, 39, 43).

Poultry

As in pigs, functional gizzard in poultry has been regarded as an important barrier in preventing pathogenic bacteria from entering the distal intestinal tract (22, 46). As alluded to, a well-developed gizzard enhances the grinding action, generates

stronger reverse peristalsis contractions, increases proteolysis, and stimulates secretion of HCl which reduces the pH. The feed pH is close to neutral, high feed intake orchestrated by HTP treatments such as pelleting (discussed later) results in elevated gizzard pH unless gastric juice secretion can increase in accordance with intake (46). Thus, the gizzard pH is reported to be higher in birds fed pelleted diets compared to birds fed mash diets linked to smaller particle size in pelleted feeds. Many experiments have demonstrated that when broilers are fed structural components in form of whole or coarsely ground cereals, or fiber materials, such as hulls or wood shavings, the pH of the gizzard content decreases by a magnitude of 0.2–1.2 units (27, 46). This has been associated with increased gizzard volume and longer retention time leading to higher HCl secretion (61). Harmful bacteria entering the intestinal tract via the feed have a greater chance of being suppressed in a highly acidic environment. Huang et al. (35) used the *S. enterica serovar Typhimurium* DT12 model developed by Mikkelsen et al. (44) to evaluate whether physical properties of feed influenced *Salmonella* colonization in broiler alimentary tract. Birds given fine particle size (0.3 mm) diet had a lower *S. enterica serovar Typhimurium* DT12 death rate compared with those receiving coarse particle size (0.9 mm) diet. A lower *S. enterica serovar Typhimurium* DT12 death rate in gizzard contents was associated with a relatively higher pH in the gizzard of birds fed fine particle diet.

There is dearth of data to support implications of changes in gizzard ecology on small intestine and ceca function and health. However, GIT ecology that favors growth of *Clostridium Perfringens* has been recognized as one of the key risk factors for the development of necrotic enteritis (NE); the most threatening disease in the broiler industry worldwide (62–64). The hallmark of this disease is the presence of typical necrotic lesions particularly in the mid-region of the GIT with detrimental effects on the digestive and absorptive capacity (64). An important factor worth considering with respect to NE is the role of *Eimeria* spp. the causative agent for coccidiosis. Coccidial infection damages the intestinal epithelium, allowing leakage of plasma proteins into the intestinal lumen—a rich nutrient substrate that *C. perfringens* can exploit for proliferation and toxin production (62). Therefore, GIT ecological conditions that prevent proliferation of *Clostridium Perfringens* and *Eimeria* are seen critical in controlling NE (62). Feed particle size may affect the physiological and morphological characteristics of the GIT and thus microbial status. Finely ground feed stimulated fast growth of *C. perfringens* than coarsely ground feed (65). Branton et al. (66) observed that birds fed coarsely ground wheat diet had 18.1% mortality due to NE, whereas birds fed finely ground wheat diet had 28.9% mortality. This was linked to course feed stimulation of gastric function, including secretion of HCl and better utilization of nutrients in the small intestines (65, 67). The peculiarity is that large flow of undigested protein and amino acids in the ceca results in production of unfavorable metabolites such as phenols, thiols, amines, ammonia, indoles that are toxic but most importantly increases the pH of the ceca content creating perfect conditions for proliferation of pathogenic bacteria such as *Clostridium* spp. (62, 63). Therefore, increased protein and amino digestion due to well-developed gizzard as result of coarse feed particle size can reduce pathogens in the lower GIT. However, studies examining interaction between experimental infection with *Eimeria* and whole wheat feeding in broilers have not been conclusive. Based on studies reviewed by Yegani and Korver (67), feeding whole wheat vs. finely ground wheat improved digestive tract function in healthy birds however responses in the context of *Eimeria* challenge were variable and ranged from no effects to exacerbation of infection. Because it is being increasingly recognized that poultry have a requirement for a certain degree of physical structure in their feed to meet their innate feeding behavior development, the inclusion of dietary structural components, such as coarse particles, insoluble fiber sources, and whole grains should be given consideration in the context of gut health in antibiotic and anti-coccidial free feeding programs.

HYDROTHERMAL PROCESSING

Common hydrothermal processes (HTP) in feed manufacturing includes pelleting, extrusion and expansion. The principle behind these processes are agglomeration of small particles into larger ones by means of mechanical compression in combination with application of moisture, heat, shear forces, and steam pressure (21, 24). Pelleting is the most prevalent HTP method for manufacturing pigs and poultry diets. Currently, most of pigs and poultry feed are fed as pellets or crumbles. Offering feed in pellet or crumble form improves the economics of production by bettering feed efficiency and growth performance (22, 28). These improvements are attributed to decreased feed wastage, higher nutrient density, reduced selective feeding, increased starch gelatinization, improved palatability, decreased time and energy spent for eating, and more importantly increased feed consumption (22, 23, 28). The ingestion of optimal level of dietary nutrients is very much dependent on the level of feed intake. In the case of pigs and poultry in most commercial situations, *ad libitum* provision of feed is practiced, in which the animal is permitted to give expression to its appetite (or voluntary feed intake). However, the level of consumption observed in practical commercial situations is often lower than the potential feed intake due to physical or physiological constraints and/or negative interaction with environmental situations (68, 69). Therefore, feed processing regimen such as pelleting that stimulate feed intake is well-received by the industry. The pelleting process can also increase nutritive value of the diet. Increased energy utilization has been reported in pelleted compared with mash feed (28, 70, 71). It has been shown that broilers fed pellets have lower heat increment and utilize more of the feed energy for productive purposes than those fed mash (72). A primary reason for the increase in productivity has been linked to behavior, more specifically, reduced energy wastage due to less time eating and more time resting (70). Heat and moisture applied in HTP have also been shown to positively affect nutrient (starch, protein) digestibility depending on the ingredients (23). Volumetric density is also reduced in mash feed and this can impact the ability to consume sufficient nutrients for

maximum production, particularly when diets are low in nutrient density (68, 69). Particle size in mash diets can further impact diet palatability and this effect is modified by pelleting (10). Regardless of the mechanism, pelleting diets affects the effective caloric value of feed. As energy is the most expensive component in monogastric diet, gaining extra calories by simply pelleting the diet is quite attractive to the industry. Indeed it has been suggested that the extra productive energy provided by pelleting can be favorably used as a non-nutritional factor by the feed industry to reduce dietary energy content (10).

There have been some informative reviews on aspects of HTP technologies for achieving end-product quality particularly the pellet quality (22, 24–26, 28). A major concern in the feed industry is that of ensuring food safety. There is a direct link between animal-feed quality and hygiene issues and the safety of human food of animal origin. It follows, therefore, that feed production and manufacture should be considered as an integral part of the food production chain (73), subject to quality assurance and food safety systems (74). Therefore, with evolving consumer demands and regulatory regimens, the quality of feed is no longer appreciated only in terms of supplying nutrients, but also in terms of hygienic status, direct effects on animal health and food safety. Understanding how feed manufacturing strategies affect bacteria inactivation in feedstuffs and/or gut microbial activity may become an important aspect of efficient animal production without antibiotics (22, 28, 75).

HYDROTHERMAL PROCESSING AND MICROBIAL STATUS IN THE FEED AND GASTROINTESTINAL ECOLOGY

Impact on Feed Microbial Load

Currently, there are no regulations dictating techniques related to microbial control in feed processing. Consequently, feed manufacturing techniques differ based on throughput demands, geographical and climate restrictions, ambient conditions, diet formulation, ingredient availability, and various feed processing equipment (75). There are numerous studies indicating HTP significantly reduces microbial load in feed (22). Most *salmonella* and coliforms can be eliminated by pelleting at temperatures above 80°C, while spore-forming bacteria are resistant to pelleting process as high as 90°C (75–77). Heat resistance also varies among non-spore-forming bacteria. For example, *Salmonella typhimurium* was more resistant to pelleting at 82.2°C with 15% moisture than *Salmonella enteritidis* whereas *S. enteritidis* was more resistant to pelleting at 87.8°C with 15% moisture compared with *Salmonella haardt* (78). The main factors determining the efficacy of HTP on feed decontamination are temperature, processing time, pressure and moisture (22). It is important to note these data are specific to feed microbial levels during and immediately after manufacture and do not predict microbial levels post processing. It is well-known that hydrothermally processed feed is at risk of recontamination during the cooling process, transportation, delivery, storage in feed bins, and feedlines (79). The most crucial stage for recontamination of the processed feed is the cooling process since high volume of air traverses through coolers and dust collected from coolers might have a greater likelihood of contamination compared with the dust obtained from other areas (76). However, there are studies indicating that HTP reduces prevalence of *salmonella* in chickens (22). However, it is yet to be determined whether this can be maintained in commercial poultry and pig operations.

Impact on Gut Physiology

It is well-known that dietary components *per se* (ingredients, nutrients and additives) can modulate development and functionality of the gastrointestinal tract including histomorphology, immune and endocrine systems as recently reviewed (80, 81). By modifying feed ingredients and feed presentation, feed processing will further impact these aspects as discussed below.

HISTOMORPHOLOGY

Hydrothermal processing further reduces feed particle size as exemplified by minimization of the differences in the particle size distribution of coarse and medium grindings (82). During pelleting process, the feed is passed through steam, which softens the feed particles before they are pressed through the die by the rolls in the pellet press, causing an additional grinding effect. Generally, there are limited studies on the impact of HTP on gut microstructure and morphology. The limited studies have concluded that HTP induced changes in the gut morphology and function cannot be separated from the effects on the microstructure and particle size of feed (34, 37, 61). As a consequence there are numerous studies that reported decreased gizzard and pancreas weights in birds fed HTP feed (**Table 2**) compared with mash feed linked to particle size reduction (22). The use of structural components, therefore, becomes even more critical in diets subjected to HTP. Numerous studies have shown that birds fed a pelleted diet had significantly decreased relative gizzard weight linked to the lack of stronger mechanical gizzard stimulation (33–37, 50). Comparative feeding of pelleted and mash feed in pigs showed that pelleting increased stomach ulceration linked to diminution of feed particle size during pelleting (39, 42, 43). It is thought that weaker mechanical stimulation by the feed might explain the higher pH found in the gizzards of pellet-fed birds due to a decrease in HCl secretion than in mash fed chicks (35). While HTP has a hypotrophic impact on the gizzard and sometimes in proventriculus, HTP can also affect intestinal morphology in poultry but published data does not give a clear picture regarding the trends and patterns (**Table 2**). However, regardless of diet form, the morphological changes observed in the distal part of the poultry gut could not just be because of particle size reduction by HTP. Such effects could be linked to changes in chemical characteristics of feed, nutrient bioavailability, digesta viscosity, microbial growth, and activity (22). For example, mash-fed hens had a higher glucose transport rate than hens fed expanded diets attributed to higher villus surface and increased expression of mucosal glucose transporters (37). Increased ceca weight in broilers fed pelleted feed relative to broilers fed mash-fed was linked to increased

TABLE 2 | Impact of hydrothermal processing on gastrointestinal physiology in pigs and poultry.

Species	Processing, main cereal	Effects, processed vs. mash	References
BROILER CHICKENS, AGE IN DAYS			
21–42	Pelleting, corn	No effect on gizzard but increased duodenal villi height and crypt depth	(33)
1–21	Pelleting, corn	Reduced gizzard weight, but increased ceca weight	(35)
1–21	Pelleting, wheat	No effect on gizzard but increased duodenum and jejunum villi height and crypt depth	(34)
1–42	Pelleting, corn	Reduced gizzard weight but no effects on small intestine weight	(36)
1–21	Pelleting, wheat and sorghum	Reduced gizzard and small intestine weight	(50)
LAYERS, AGE IN WEEKS			
20	Expansion, corn and wheat	Reduced gizzard weight. Reduced duodenal villi height but increased ileal villi height	(37)
PIGS, BODY WEIGHT RANGE, KG			
50–100	Pelleting, corn	Increased stomach keratosis	(39, 43)
5–100	Pelleting, corn	Increased stomach ulcers	(42)

flow of undigested starch in the ceca leading to increased fermentation capacity (35). In a more recent study addition of oat hulls in pelleted wheat diet increased gizzard weight and holding capacity (83). Further studies are needed to determine the mechanism behind the stimulation effects of HTP on gut physiology, morphology and immunology.

APPETITE CONTROL AND NEUROMODULATION

Among the nutrients in pig and poultry diets, starch is quantitatively the most important. Diets may contain up to 50% starch on a DM basis, and starch is the most important source of energy. In monogastric farm animals, enzymatically digestible vs. fermentable starch increases net portal glucose uptake and as a consequence increases energetic efficiency of starch use for protein and fat tissues accretion (84, 85). Therefore, large part feed processing focuses on optimizing starch gelatinization for increased glucose absorption in the small intestine (86). However, heat processing of feed ingredients may result in formation of resistant starch through retrogradation. Resistant starch is considered a functional component as it positively influences the functioning of the digestive tract, microbial flora, the blood cholesterol level, glycemic index and assists in the control of diabetes (87, 88). Heat processing of feedstuffs has been shown to influence kinetics of starch degradation in the digestive tract of pigs by shifting site and extent of digestion with implications on voluntary food intake and adiposity (89, 90). Studies in rodents have provided evidence that fermentation of resistant starch is an important mechanism for increased endogenous secretion of the gut hormones glucagon-like peptide 1 (GLP-1) and peptide YY; satiety-stimulating hormones that are released mainly in the ileum and colon (91, 92). This in turn influences insulin release when GLP-1 binds to receptors on pancreatic β cells. The general concept is that the resistant starch escapes to the large intestine impacting luminal microbiota composition, luminal SCFA concentrations, and the expression of host genes involved in SCFA uptake, SCFA signaling, and satiety regulation (92). The mechanisms relating to starch chemistry upon processing, SCFA production and endocrine responses requires a better understanding to optimize glucose homeostasis. Moreover, understanding the mechanisms involved in the complex interactions between the diet, intestinal microbiota, and intestinal tissue can assist in supporting GIT function and health via targeted modifications of the diet. Recent data in pigs indicated that molecular and morpho-function of mandibular gland of pigs may be influenced by physical form of diet. A coarser diet was shown to increase the expression of leptin and its receptor in the epithelial cells of striated ducts in growing pigs (93). Further studies in piglets demonstrated differential expression and localization of cannabinoid receptors type 1 (CB1) and cannabinoid receptors type 2 (CB2) in the mandibular glands in response to variable chewing activity due to different diets form (94). The authors opined that these findings suggested a link between the diet form and the functional molecules involved in appetite regulation.

Impact on Microbial Activity

The diversity of the microbiota in a gut section reflects in part the types of nutrient substrates in that section. Gastrointestinal microbiota derives most of their carbon and energy from luminal compounds (dietary and/or endogenous) which are either resistant to attack by digestive fluids or absorbed so slowly by the host that bacteria can successfully compete for them (8). Since bacterial species differ in their substrate preferences and growth requirements, the chemical composition and structure of the digesta largely determines the species distribution of the bacterial community in the GIT. Consequently, bacterial community structure and metabolic function is very much dependent on digesta biochemical conditions, because of feed composition and attendant host physiological responses such as endogenous secretions. It is inevitable that the use of any feed processing technology that influences the digestibility of the diet will change the selection pressures on the resident microbiota which in turn will moderate the efficiency with which the host utilizes its feed (8). As alluded to HTP improves

digestibility of nutrients and thus likely alter gut ecology. Generally, a large part of starch in feed ingredients is digested in the small intestine of pigs and poultry. However, especially in heat-processed ingredients, a fraction of the starch may be retrograded and designated as resistant starch. The latter fraction cannot, by definition, be enzymatically degraded in the small intestine by host enzymes and passes to the large intestine where it can be fermented by residing microbiota. There are numerous studies demonstrating that resistant starch modulated intestinal microbiota and increased the expression of genes responsible for gut development through the production of SCFA creating acidic and hostile environment for pathogen overgrowth (95). Moreover, there are numerous reports indicating that HTP changes physical chemical property of dietary fiber through increased solubility and particle size reduction (96). Acid extrusion (incubation in acids followed by extrusion) fiber rich corn distiller's grains with solubles facilitated more rapid degradation of non-starch polysaccharides and shifted fermentation to more proximal gastrointestinal segments (97). However, there are limited studies on the effect of HTP of feed on the bacterial composition and activity in the gastrointestinal tract of poultry and pigs.

Pigs

Heat treatment of cereals for piglets (corn and barley) and steam pelleting increased post-weaning growth performance and changed fermentation profiles in the hindgut indicating that the microbiota composition or their fermentation capacity had changed (98). Investigations on the impact of mash and pelleted diets on adhesion of *Salmonella enterica serovar Typhimurium* DT12 to pig ileum showed that mash diets were better in protecting than pelleted diets (45). The authors explained that pelleted diets stimulated secretion of mucins that facilitated *Salmonella* colonization. Total *E. coli* load was markedly lowered in both the caecal and colon contents of mash-fed pigs relative to pigs fed pelleted diets (42). Interestingly, cecal contents of pigs fed pelleted diet had higher content of genes for fimbriae F4 compared with cecal contents of pigs fed mash diet (42). These fimbriae are important virulence factor that facilitate enterotoxigenic *E. coli* binding to the specific receptors on intestinal epithelial cells resulting in colonization and subsequently in the secretion of enterotoxins such as STa, STb, and LT leading to diarrhea in piglets (8, 55). Enterotoxigenic *E. coli* (ETEC) strains causing diarrhea are more often detected in neonatal and newly weaned pigs (55). Thus, reducing the prevalence and the persistence of ETEC in pig herds may contribute to protecting pigs from contamination between production cycles and to reducing the risk of cross-contamination of piglets in the production system. It would be interesting to test feed texture in animals experimentally infected with ETEC to better understand the mechanism involved and record degree of diarrhea mitigation.

Poultry

In vitro simulation studies of gizzard contents of birds fed pelleted diets showed lower *Salmonella enterica serovar Typhimurium* DT12 death rate compared to gizzard content of birds fed mash diet (35). However, *in vivo* experiment showed that birds fed pelleted diets had significantly higher concentrations of *Salmonella enterica serovar Typhimurium* DT12 in the GIT than did mash-fed birds (35). Bjerrum et al. (99) reported that birds fed pelleted feed had higher numbers of *Salmonella* in gizzards compared with those given whole wheat. Interestingly, pelleted diets have been shown to increase concentrations of SCFA in the gizzard compared with mash feeds. However, the increased SCFA in gizzard was not accompanied with lower pH in gizzard of birds fed pelleted diet (35). Feeding pelleted diets increased ceca concentration of SCFA which was accompanied with decreased pH (35, 65). This was explained to be related to the fact that pelleting induced substantial reduction in particle size such that nutrients that entered the cecum were easily available for microbial fermentation. In poultry, the ceca is the reservoir for *Salmonella* (100). It is therefore of interest that birds fed pelleted feed had higher concentration of *Salmonella* than did mash-fed birds (35). It appears that the reduction of ceca pH orchestrated by increased concentration of SCFA in broilers fed pelleted diets was not effective in reducing *Salmonella* colonization (35). Markedly increased concentrations of *Salmonella* in the ceca of pellet-fed birds demonstrated that the gizzard pH orchestrated by increased HCl production in relation to feed structure might be a better strategy of reducing the ceca concentration of *Salmonella*. Indeed, studies have demonstrated that pelleting of feed increased the incidence of *Salmonella* in the contents of gizzards and ceca of growing broilers providing evidence that the gizzard may be an important critical control point for reducing *Salmonella* contamination in growing broilers (22). Increasing processing temperature led to an increase of *lactobacilli* in the crop and ileum, whereas *clostridia* and *enterobacteria* seemed unaffected by HTP (101). The impact of different HTP treatments in the crop and small intestine were mostly confined to *lactobacilli* and lactic acid concentration (101). This study concluded that typical HTP applied in feed does not significantly influence GIT microbial dynamics in poultry. Although the number of studies investigating the effects of HTP GIT microbiology of poultry are limited, a better understanding of the effects of steam conditioning time and temperature manipulations could help producers maintain hygienic, physical, and nutritional quality of feed in antibiotic free feeding programs.

FEED PROCESSING AND EFFICACY OF EXOGENOUS FEED ENZYMES

Although pigs and poultry are highly efficient in converting feed to food products, they still excrete significant amounts of undigested nutrients. For example, broilers lose almost 25–30% of ingested dry matter, 20–25% of gross energy, 30–50% of nitrogen, and 45–55% of phosphorus intake in the manure (10). Pigs of different breeds and ages were observed to digest 78% of gross energy in typical corn and soybean meal diet (102). Addition of 30% corn dried distiller's grains with solubles to this diet resulted in further reduction of digestible gross energy. The undigested nutrients are excreted in the manure

with negative implications on production efficiency, profitability and sustainability of farm operations. The peculiarity is that feedstuffs contains anti-nutritional factors (ANF) such as phytic acid or fractions that are not degraded sufficiently or indeed at all by the conditions and the array of digestive enzymes in the gastrointestinal tract (8, 9). This inherent digestive inefficiency in monogastric animals is seen as the reason of commercial development and application of exogenous feed enzymes technology. Indeed, amongst biotechnological feed additives, feed enzymes have made the most progress and impact in the feed industry over the last three decades (8, 9). As such the utility of feed enzymes in terms of nutrition and gut health and function are widely researched (8, 103–105). However, exogenous enzymes added to the diet must exert their effect during the short time from when the feed is moistened in the anterior digestive tract to the point that feed residues have passed the small intestine (46). Furthermore, the enzyme must be able to withstand the rigors of feed processing and digestive processes such as pH and endogenous proteases. This complicated matrix of conditions has been partly associated with the variation in the efficacy of exogenous feed enzymes (9, 11).

Moderate HTP temperature (65–85°C) improves availability of nutrients due to gelatinization of starch, rupture of the cell wall matrix and deactivation of enzyme inhibitors present in cereals (106). However, there is a wide range of temperature and time combinations used in the commercial feed manufacturing. As protein, exogenous feed enzymes are susceptible to hydrothermal denaturation, early studies indicated that the magnitude of enzyme inactivation increased with conditioning temperature and time (107). The advances in technology over the last two decades have addressed the challenge of feed enzymes thermostability through strategies such as post-pelleting spraying, granulation with hydrophobic materials and molecular engineering approaches to bolster intrinsic thermostability (11). However, the susceptibility of exogenous enzymes to HTP, regardless of the production and applied protection technologies is different. For example, effects of different pelleting temperatures 60, 70, 80, 90, and 100°C on the activity of fungal amylase and bacterial amylase added in barley, wheat and soybean diet suggested that fungal amylase can be pelleted at temperatures of up to at least 80°C and bacterial amylase up to 90°C without a considerable loss in analyzed activity (108). More than 65% of activity of a blend of cellulase, β-glucanase, and xylanase was lost in a barley, corn, dried grass, wheat bran, peanut meal and soybean meal diet subjected to extrusion (109). However, and surprisingly, the enzyme treated diet still improved the energy and fat utilization in laying hens compared with the control. In a second experiment in the same study, 52% of the blend activity was lost after pelleting, however, enzyme improved nutrient utilization in unprocessed and pelleted diet to the same extent (109). Such observations might suggest the residual activity was still efficacious post-processing or initial enzyme dosing was excessive of available substrates. Moreover, it has been demonstrated that effects of exogenous enzyme was more pronounced in diets subjected to HTP (110). Peculiarity is that HTP changes the structure of non-starch polysaccharides (NSP) by increasing the ratio of soluble to insoluble fractions (106). Under such circumstances the magnitude of enzyme response will be greater to counteract deleterious effects of solubilized NSP. It has also been speculated that high conditioning temperature may destroy cell walls, releasing substrates that would otherwise not be accessible to the exogenous enzyme (111).

Most exogenous feed enzymes have an optimum pH of between 4 and 6, but great variation may exist between different sources of enzymes, which results in a spectrum of catalytic activity between lower and higher pH (112). Therefore, it is essential to understand these digestive conditions and how they may vary to predict efficacy of exogenous enzymes. It is obvious that functionality of the stomach may have a large effect on responses to enzyme supplementation. Intermittent feeding will increase retention time and decrease pH of the crop, and structural components will increase retention time and decrease pH in the gizzard, as discussed previously. Supplemental phytase was able to degrade 50% of the phytic acid during 100 min of retention in the crop of broiler chickens (113). Despite this, an experiment designed to increase retention time in the crop and gizzard failed to demonstrate any improved efficacy of phytase (114). Functionality of the posterior digestive tract may also be affected by functionality of the gizzard due to structural components. A dysfunctional gizzard may allow too much and poorly degraded nutrients to be passed to lower gut. The implication of such eventuality may be morphological and microbiological changes in the lower gut and possibly affect efficacy of feed enzymes. Taken together, it appears that there are several fundamental mechanisms that may underlie a wide range of situations in which interactions between feed processing *per se* and feed enzymes application may occur. An understanding of these mechanisms may provide an opportunity to develop strategies for application of feed enzymes and other heat sensitive feed additives when added in feeds subjected to diverse processing regimens.

FUTURE PERSPECTIVES; TOWARD OPTIMAL FEED PROCESSING

The benefits of feed processing in terms of animal performance and economics are not questionable. Concerns pertaining to aspects such as pellet quality, nutrient digestibility, protein denaturation and milling efficiency will continue to stimulate innovations in feed manufacturing. However, advances in feed processing optimization will be challenged by emerging consumer and regulatory trends for restriction or cessation of production practices such as use of antimicrobial growth promoters. For example, feed processing should take consideration of increasing focus on dietary approaches (ingredients and physical characteristics) for maintaining healthy and functional gastrointestinal tract. Clearly coarse particle size stimulates stomach development and functionality. A dysfunctional stomach may allow too much and poorly degraded nutrients to be passed through, and thus an increased level of undigested nutrients may enter the ileum and ceca.

This may lead to morphological and microbiological changes, however, there is dearth of data to support implications of such changes on gut function and health. Optimal particle size could be designed in the grinding process using roller or hammer mill. However, since most pigs and poultry are fed diets subjected to hydrothermal processing, additional reduction of feed particle size is inevitable. Because fine grinding is generally favored for high pellet quality, and because it is difficult to avoid further reduction in feed particle size during the pelleting process, fine particle size is almost inevitable in pelleted feeds. The possibilities to decrease the intensity of grinding of particles during pelleting, by variation of parameters of pelleting process, are very limited. Modified extrusion process (i.e., processing using expander) followed by shaping element as applied in pet industry could be alternative for pelleting to preserve particle size, however there is dearth of data to application of this approach in pigs and poultry feed manufacturing. Strategies such as addition of concentrated fibrous material may be more applicable in pelleted feed, but data is largely lacking as to applicability in practical diets. The need to achieve high physical quality and to reduce potential levels of feed-borne pathogens such as *Salmonella* has led to the application of relatively high conditioning temperatures during conventional pelleting processes, a practice that does not favor high nutrient utilization. However, the true impact of high conditioning temperatures application on nutrient utilization of pelleted diets has been neglected due to focus on physical pellet quality and feed safety. Further research is warranted to identify and evaluate other possible approaches to manufacture high-quality pellets at low conditioning temperatures. Advances in enzyme technology will continue and one can expect that better forms of enzymes will be developed in the future. The "next-generation" enzymes will be close to being "perfect," with rapid and high specific catalytic activity (per unit of protein), good thermostability, high activity under a wide range of gut pH, resistance to proteolysis and good stability under ambient temperatures. Therefore, with evolving pig and poultry production practices, the regimens for feed processing will no longer be appreciated only in terms of optimizing nutrients utilization, but also in terms of impact on feed hygienic status, efficacy of feed additives, animal health and food safety.

AUTHOR CONTRIBUTIONS

AM is a graduate student of EK, she gathered some of the research articles used in the review and write-up related to feed structure in poultry. EK searched literature and wrote significant portion of the review and had overall conceptual and editorial responsibility.

FUNDING

Natural Sciences and Engineering Research Council of Canada.

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

frontiers in Veterinary Science

REVIEW
published: 21 February 2019
doi: 10.3389/fvets.2019.00046

Nutritional Intervention for the Intestinal Development and Health of Weaned Pigs

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Weaning imposes simultaneous stress, resulting in reduced feed intake, and growth rate, and increased morbidity and mortality of weaned pigs. Weaning impairs the intestinal integrity, disturbs digestive and absorptive capacity, and increases the intestinal oxidative stress, and susceptibility of diseases in piglets. The improvement of intestinal development and health is critically important for enhancing nutrient digestibility capacity and disease resistance of weaned pigs, therefore, increasing their survival rate at this most vulnerable stage, and overall productive performance during later stages. A healthy gut may include but not limited several important features: a healthy proliferation of intestinal epithelial cells, an integrated gut barrier function, a preferable or balanced gut microbiota, and a well-developed intestinal mucosa immunity. Burgeoning evidence suggested nutritional intervention are one of promising measures to enhance intestinal health of weaned pigs, although the exact protective mechanisms may vary and are still not completely understood. Previous research indicated that functional amino acids, such as arginine, cysteine, glutamine, or glutamate, may enhance intestinal mucosa immunity (i.e., increased sIgA secretion), reduce oxidative damage, stimulate proliferation of enterocytes, and enhance gut barrier function (i.e., enhanced expression of tight junction protein) of weaned pigs. A number of feed additives are marketed to assist in boosting intestinal immunity and regulating gut microbiota, therefore, reducing the negative impacts of weaning, and other environmental challenges on piglets. The promising results have been demonstrated in antimicrobial peptides, clays, direct-fed microbials, micro-minerals, milk components, oligosaccharides, organic acids, phytochemicals, and many other feed additives. This review summarizes our current understanding of nutritional intervention on intestinal health and development of weaned pigs and the importance of mechanistic studies focusing on this research area.

Keywords: amino acids, feed additives, intestinal development, intestinal health, weaned piglets

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *25 September 2018*
Accepted: *04 February 2019*
Published: *21 February 2019*

Citation:
Xiong X, Tan B, Song M, Ji P, Kim K, Yin Y and Liu Y (2019) Nutritional Intervention for the Intestinal Development and Health of Weaned Pigs. Front. Vet. Sci. 6:46. doi: 10.3389/fvets.2019.00046

INTRODUCTION

Weaning is the most challenging stage that has significant bearings on pig welfare and growth performance in swine industry. During weaning period, piglets are immediately imposed to a number of environmental and psychosocial stressors that predispose them to diarrhea and gut damage, which can adversely impact their survival at a very early and most vulnerable stage. The post-weaning mortality ratio is 6–10%, but sometime may rise up to 20%. Thus, in the last decade, animal nutritionists have made great effort to optimize feed formulation to meet requirement of newly weaned pigs, and to explore different nutritional factors or management that focus on promoting the overall health of weaned pigs. In addition, antibiotics used to be a powerful component in the herd health programs for protecting weaned pig health. It has been reported that global consumption of antibiotics in livestock production was estimated at 63,151 tons in 2010 and is projected to increase by 67% by 2030 (1). In the U.S., antibiotics use in livestock industry is estimated to account for 71% of the nation's annual antibiotic consumption (2). However, these practices also contribute to the spread of antibiotic-resistant pathogens in both livestock and humans, rising a significant public health threat. Use of in-feed antibiotics for production purpose in livestock industry is completely banned in the U.S. (3) starting in January 2017, which is remarkably increasing the challenge of keeping pigs healthy, especially in post-weaning period. Therefore, another urgent need in animal science society is to develop strategies to replace antibiotics for food-producing animals without hampering animal production. Although the manipulation of genetics, management, and health also plays substantially important role in protecting animal health and promoting their production performance, in the current review, we only focus on nutritional interventions on intestinal health of weaned pigs.

WEANING STRESS ON INTESTINAL DEVELOPMENT AND HEALTH

Many factors contribute to post-weaning stress, including hierarchy stress, new housing environment, transferring to solid feed, and others (4). Weaning stress is generally companying with reduced feed intake, poor growth performance, as well as increased disease susceptibility (5, 6). Weaning stress also negatively impacts intestinal development, physiology, microflora, and immunity as thoroughly discussed by other review articles (7–9). The focus of this review is to briefly highlight weaning stress on intestinal development and health by adding more recently published research.

Weaning Stress on Pig Intestinal Physiology

Intestinal epithelium is characterized by rapidly proliferating cells in crypts, which then invaginating into the underlying mesenchyme and villi (10). The intestinal epithelial cells continuously and rapidly turn over in 4 to 5 days (11). The stem cells in crypts produce proliferating transit-amplifying cells that undergo a series of transitions, and ultimately differentiate into four differentiated cell types comprising one type of absorptive (enterocytes) and three types of secretory cell lineages (enteroendocrine cells, goblet cells, and paneth cells) (12). Absorptive enterocytes constitute up to 90% of epithelial cells in the crypt-villus axis (13). Paneth cells migrate to the base of crypts, whereas enteroendocrine cells and goblet cells migrate to villi (14). The proliferation, differentiation, and apoptosis of intestinal epithelial cells play important roles in intestinal development, maintenance, and recovery from tissue damage (7).

Several recently published research articles revealed the impacts of weaning stress on the expression of proteins and metabolites in enterocytes of piglets (15–20). Weaning significantly down-regulated the expression of proteins involved in the tricarboxylic acid cycle, β-oxidation, and the glycolysis pathway in the upper villus and middle villus of the jejunum in early-weaned pigs, but up-regulated proteins involved in glycolysis in crypt cells (15). During the post-weaning period, the expression of proteins related to various cellular metabolic or biological processes, such as energy metabolism, protein amino acid glycosylation, ion transport, mTOR signaling pathway, and differentiation and apoptosis, were reduced in jejunal differentiated epithelial cells (villus upper cells) of piglets (17). Proteins involved in the respiratory electron transport chain, Golgi vesicle transport, protein glycosylation, as well as the metabolism of nutrient such as lipids, monosaccharides, and nucleotides were also down-regulated in the jejunal differentiating epithelial cells (the middle villi cells) of piglets during the post-weaning period (20). These results indicated that weaning influenced energy metabolism, cellular macromolecule organization and localization, and protein metabolism, thereby further impacted the proliferation of intestinal epithelial cells in weaned piglets (18). In addition, polyamine metabolism and ornithine decarboxylase expression were also altered by weaning and may be used as a marker of intestinal growth and restitution in pigs (21).

Weaning stress could also induce tremendous morphological/physiological changes, such as villous atrophy and crypt hyperplasia (22, 23), which further disturb the digestive and absorptive capacity and performance of weaned pigs (4, 24). Brush border enzyme activities and electrolytes secretion in the small intestine have been used as important indicators of maturation and digestive capacity in weaned pigs (25, 26). Due to the change of diet, the activities of enzymes at brush border, such as lactase, sucrase, and maltase, are dramatically reduced between 3 and 5 days after weaning (27, 28). The malabsorption of nutrients in the small intestine is exacerbated by the reduced electrolytes absorption and secretion in newly weaned pigs (29).

The epithelial cells and the mucin layer in the small intestine provide the first line of defense to protect weaning pigs from various harmful microorganisms, toxins, or antigens in the intestinal tract (30). Gut permeability is straightly regulated by tight junction proteins, such as zona occludens 1, claudin, and occludin that are expressed by the epithelial cells (31). It has been reported that weaning stress reduced goblet cells number and mucin production, disrupted epithelial barrier function,

increased intestinal permeability, lowered tight junction protein expression, and increased disease susceptibility in weaned pigs (32–34). It was observed that the intestinal barrier damage caused by weaning stress was not restored and returned to pre-weaning levels on d 7 post-weaning (35).

Host-Microbial Nutrition Interactions in Post-weaning Gut Microflora Dysbiosis and Diarrhea

Porcine gut microbiome exhibits dynamic composition and diversity that shifts overtime (36, 37). The primary pig gut microbiota at birth was shaped by the sows' milk and featured with more abundance of lactic acid bacteria (38). However, weaning transition reduced the relative abundance of *Lactobacillus* group, increase *Clostridium* spp., *Prevotella* spp., *Proteobacteriaceae,* and *E. coli*, resulting in a loss of microbial diversity (39–41).

The composition and diversity of gut microbiota of weaned piglets is also highly impacted by the levels and sources of dietary proteins or fibers that are offered to post-weaning pigs (42). Nutritional interactions between intestinal cells and gut microflora are remarkably important for the recycling and maintenance of gastrointestinal tract nutrient pool (**Figure 1**) (43–46). In contrast, a balanced nutrient pool is also critical for the renewal and proliferation of intestinal cells, as well as maintaining a balanced microbial community (12, 47). During the post-weaning period, piglets often have sharply reduced feed intake due to weaning stress. Hence, the nutrients for bacterial survival and proliferation is also limited. Pathogenic bacteria are able to utilize special nutrients (i.e., ethanolamine) that cannot be catabolized by commensal bacteria, thereby, enhance the expression of their virulence factors (48, 49). For instance, both *Salmonella* and enterohemorrhagic *E. coli* could use ethanolamine as carbon or nitrogen source to gain nutritional advantages in competing with other microflora (12, 48, 50). Enterohemorrhagic *E. coli* can also utilize fucose to activate type III secretion system, which facilitates the adhesion of those pathogenic bacteria to host enterocytes (46, 51). As a result, weaned piglets are more susceptible to intestinal inflammation and post-weaning diarrhea due to rapid proliferation of pathogenic bacteria and the loss of microbial diversity (52).

Weaning Stress on Intestinal Mucosal Immunity

The barrier-related mucosal homeostasis is very important for the recognition of exogenous dangerous stimuli, but the same time it has to make sure our body is not hypersensitive to innocuous antigens (53). For example, in the intestine, epithelial cells are primarily responsible for fluid secretions and nutrients absorption, as well as providing a selective barrier against noxious antigens in the lumen. The cross-talk between intestinal epithelial cells and underlying lamina propria cells transfers immune-related signals to the local adaptive immunity, which subsequently help to maintain gut immune homeostasis (54).

The neonates are born with few lymphocytes and relatively low expression of co-stimulatory molecules (55, 56). In addition, the neonates also have a biased intestinal adaptive immunity due to a comparatively higher T helper 2 immune response rather than T helper 1 (57). To develop a stable number of lymphocytes in un-weaned pigs, it may take about 6 weeks (58). Therefore, newly weaned pigs at age of 2 to 4 weeks do not have mature intestinal immunity, which increase their disease susceptibility.

The impacts of weaning stress on intestinal immunity has been thoroughly revealed by McCracken et al. (59) and Pié et al. (60). Briefly, there are several major changes in intestinal immunity of weaned pigs compared with pre-weaning pigs. First, weaning sharply increases both intestinal CD4+ and CD8+ T lymphocytes in pigs on d 2 post-weaning (59) and enhances mRNA expression of inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, and IL-8) in the middle of jejunum during the first 2 day post-weaning (60). Those observations indicate that weaning induced a transient gut inflammation in pigs. Second, weaning stress up-regulates matrix metalloproteinase (i.e., stromelysin) by activating immune cells in the lamina propria, which may contribute to villus atrophy (59). Third, weaning stress may down-regulate the MHC I expression in jejunal mucosa of pigs, which is possibly due to the increased plasma cortisol concentration (59, 61). Fourth, the concentration of fecal IgA is continuously decreased from day 5 after birth and remained very low until at least 50 days of age, which may enhance the vulnerability of pre- and post-weaning piglets (62).

Weaning Stress on Intestinal Oxidative Status

Weaning stress is also associated with increased oxidation processes, which leads to a high release of free radicals, also called reactive oxygen species [ROS; (63)]. The excessive production of ROS could modify certain cellular proteins and activate the up-regulation of pro-inflammatory cytokines, which may further negatively affect the expression of tight junction proteins and cause increased gut permeability (64, 65). Animal cells generally have complex and protective mechanisms to against the formation of oxidative stress, including prevention of ROS formation, ROS scavenging antioxidant systems, and elimination and/or reparation of damaged molecules (66). Therefore, the balance between oxidation and anti-oxidation is very important to cell integrity and health.

A series of antioxidant enzymes play critical roles to protect organisms against harmful pro-oxidants (67). For example, superoxide dismutase provides an efficient dismutation of O_2^- into H_2O_2, which is scavenged by glutathione peroxidase and catalase (68). A study from Yin et al. (69) thoroughly investigated the impacts of weaning on the development of antioxidant system of pigs. They observed that plasma superoxide dismutase activity was decreased 1 day post-weaning and then gradually recovered at 3, 5, and 7 day post-weaning. They also observed that weaning down-regulated the expression of genes encoded superoxide dismutases (i.e., *CuZnSOD* and *MnSOD*) and glutathione peroxidases (i.e., *GPx1* and *GPx4*) in jejunum of piglets (69). A likely reason is that excessive ROS inhibits

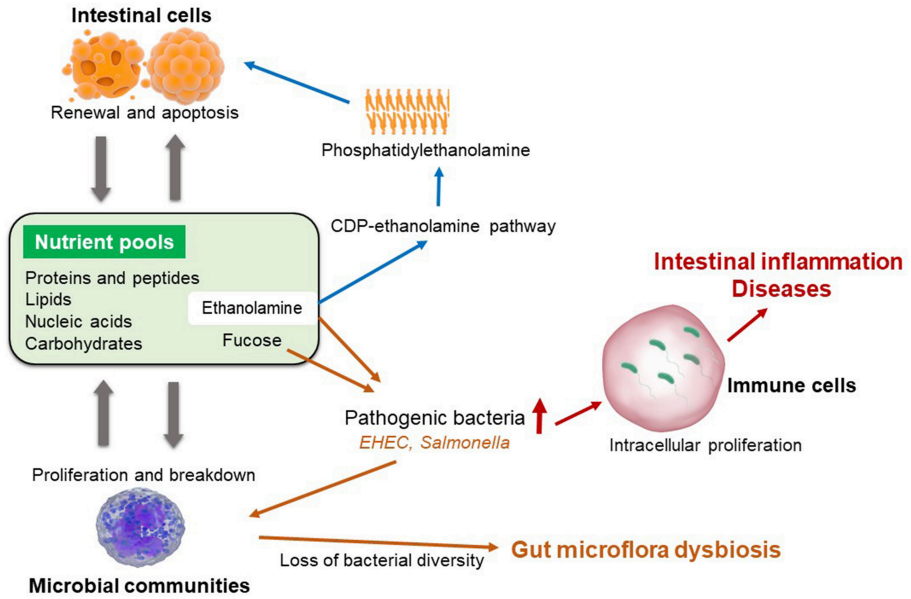

FIGURE 1 | Maintenance of intestinal nutrient pool and the pathogenic baceterial specific nutrition metabolism.

the phosphorylation and degradation of IκBs and Keap1, which, therefore, stimulates proteasomal degradation of Nrf2 and p65 and suppresses Nrf2 and p65 signals (69, 70).

HOW TO DEFINE A HEALTHY GUT

A healthy gut is critically important to the overall metabolism, physiology, disease defense, and growth performance of weaned pigs. Recently, the item "gut health" has attracted much attention in the newly weaned pigs due to the negative effects of weaning stress. However, it still lacks a precise and unifying definition of "gut health." Several review articles have comprehensively summarized timely information for this particular topic in newly weaned piglets (71–74) and provided slightly different definitions on "gut health." Based on Kogut and Arsenault (71), a healthy gut was defined 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." Celi et al. (72) emphasized the importance of effective digestion and absorption of feed, effective structure and function of gut barrier, host interaction with gut microbiota, and effective immune status. The latest publication from Pluske et al. (74) stated that gut health should be more general and described as a generalized condition of homeostasis in the gastrointestinal tract. They remarked that the generalized criteria to assess gut health of weaned pigs could include effective nutrient digestion and absorption, effective waste excretion, a functional and protective gut barrier, a stable and appropriate microbial community, a functional and protective gut immunity, a minimal activation of stress/neural pathways, and the absence of diseases (74). It is not our intention to reiterate all details included in these publications and compare their definitions. In this regard, we completely agree that a healthy gut should enhance the overall capacity/ability of the host to respond and adapt to challenges/stress and should be concomitant with optimal performance as described by Pluske et al. (74).

NUTRITIONAL INTERVENTION ON INTESTINAL DEVELOPMENT AND HEALTH OF WEANED PIGS

Many nutritional strategies have been applied to improve health and maximize the production of weaned pigs (75–78). Those strategies include but not limited to: optimization of feed formulation, utilization of low protein diet in post-weaning period, enhancement of feed processing and manufacturing, and supplementation of different feed additives. They are targeting different aims: (1) improvement of nutrient digestion and absorption, (2) regulation gut microbiota to more favorable bacterial species, and (3) immune modulation to enhance disease resistance of weaned pigs. In this review article, we will only focus on the impacts of several selected feed ingredients or additives (functional amino acids, phytochemicals, antimicrobial peptides, and short-chain fatty acids) on intestinal health of weaned pigs. Those feed additives may or may not have nutritional contribution to human or animal, but they play very important roles in health maintenance or regulation. Many other ingredients or additives are also shown promising results in weaned pig health, but will not be covered in the current article.

Functional Amino Acids

A growing body of literature indicates that some of traditionally classified dispensable amino acids, such as, arginine, glutamine, glutamate, and proline play important roles in the regulation

of gene expression, intracellular signaling pathways, nutrient metabolism, and oxidative defense (79–81). This group of amino acids is defined as functional amino acids (82). It has been known that the deficiency of a functional amino acid may impair the whole-body homeostasis. For example, dietary deficiency of arginine could result in metabolic, neurological, or reproductive dysfunction (83). The importance of functional amino acids has been thoroughly reviewed by Wu et al. (81) and Wu (84, 85). The major objective of this review section is to highlight recent published research articles focusing on the effects of functional amino acids on intestinal health and development of weaned pigs.

Arginine is remarkably deficient in sow milk (86, 87), but the concentration of arginine in tissue proteins in piglets are relatively higher compared with other amino acids (88). This observation has remarkably increased the research attention in the nutritional significance of arginine. It has been reported that supplementation of L-arginine (0.2 to 1%) enhanced growth performance and alleviated the negative effects of different insults or challenges in young pigs (81, 89–91). Supplementation of 0.4 to 0.8% L-arginine in pre-weaning diet enhanced intestinal growth and development in early post-weaning period (92). In addition, supplementation of 0.6% L-arginine enhanced small intestinal growth, goblet cell number in intestinal mucosa, intestinal heat shock protein-70 expression in weaned pigs (81). Increasing evidence confirmed the positive impacts of arginine on preventing intestinal dysfunction as a substrate for the synthesis of nitric oxide, polyamines, creatine, and protein (93). It was also reported that arginine could improve DNA synthesis and mitochondrial bioenergetics of intestinal epithelial cells, therefore improve the regeneration and repair of the small intestinal mucosa in animals (94). The underlying biochemical mechanisms may be closely related to the activation of PI3k-Akt pathway, mTOR and TLR4 signaling pathways, and/or the enhanced intracellular protein turnover (94, 95). Moreover, the increased nitric oxide from arginine metabolism could also regulate intestinal blood flow, integrity, secretion, and epithelial cell migration (96).

Besides arginine, other functional amino acids in the arginine family, have been also well investigated in the last decades, including glutamine, glutamate, aspartate, proline, etc. For example, it was reported that the administration of proline improved mucosal proliferation, intestinal morphology, as well as tight junction and potassium channel protein expression in early-weaned piglets (97). Dietary supplementation of glutamine was also shown to prevent intestinal atrophy, increase enzyme activities, and promote growth performance of weaned pigs (98). One dipeptide that is composed of glutamine (glycyl-glutamine), appear a great substitute for glutamine to increase intestinal integrity and enzyme activities and growth performance of weaned pigs (99–101). Another dipeptide, alanyl-glutamine, also has the biological effects similar to free glutamine, as regarding their effects on proliferation, mitochondrial respiration, and protein turnover in the porcine intestinal cells (102). Alanyl-glutamine may be another effective substitute for glutamine as energy and protein sources in the intestinal tract, which has to be further investigated with *in vivo* animal model. Several mechanisms are highly involved in the benefits of glutamine or glutamine dipeptides on intestinal health. First, glutamine, glutamate, and aspartate could provide major fuel for small intestinal epithelial cell proliferation and provide energy required for intestinal ATP-dependent metabolic processes (103). Second, catabolism of glutamine provides precursors for polyamine synthesis, which is important for proliferation, differentiation, and repair of intestinal epithelial cells (104). Third, glutamine is also a major precursor for the synthesis of glutathione, an important antioxidant in cells regulating the homeostasis of free radicals (105, 106). Fourth, glutamine supplementation may enhance intestinal secretory IgA production via regulating the intestinal microbiota and/or T cell-dependent and T cell-independent pathways (107).

Although it is beyond the scope of functional amino acids, several indispensable amino acids, such as tryptophan and sulfur amino acids, have also attracted large attention recently (108–110). A growing evidence has revealed that supplementation of these amino acids beyond the current NRC requirement brought positive effects on intestinal health of weaned pigs by regulating host physiology, metabolism, oxidative status, and immunity (108–110). The modification of gut microbiota and their metabolites by these amino acids was also highly correlated to the enhanced gut barrier functions of weaned pigs (109).

Phytochemicals

Phytochemicals, naturally occurring plant chemicals/metabolites, are one of most powerful candidates as potential alternatives to in-feed antibiotics because of various biological functions. First, most of phytochemicals exhibit a wide spectrum of antibacterial activities against both gram-negative and gram-positive bacteria, including *E. coli*, *Salmonella*, Clostridium, Mycobacterium, etc. (111, 112). Second, certain phytochemicals have been recognized as potential anti-viral agents (113, 114), which is probably beyond provision of antibiotics. Third, the immune-regulatory activities of certain phytochemicals have been identified in both human and animal models (114–118). Last but not the least, phytochemicals could act as antioxidants to remove free radicals from the body and protect animals from oxidative damage (119). Several commonly used phytochemicals and their main components are summarized in **Table 1**.

The protective effects of phytochemicals on poultry and livestock have been thoroughly reviewed in Lillehoj et al. (121). Previous research revealed that dietary supplementation of phytochemicals enhanced disease resistance (i.e., reduced frequency of diarrhea) and growth performance (114, 122, 123). These benefits were likely driven by improved gut health, such as, improved intestinal barrier integrity (122, 123). For example, supplementation with phytochemicals extracted from different seasonings improved intestinal villi height and upregulated mRNA expression of the *MUC2* gene in ileum (118). Feeding capsicum oleoresin from pepper, turmeric oleoresin or curcumin extracted from ginger up-regulated the expression of genes related to tight junction (e.g., genes encode claudins and occludin) and cell-cell junctions in the ileum of *E. coli* challenged pigs (118, 124). A recent publication from Yuan et al. (125) also reported that the flavones extracted from the leaves of *Eucommia*

TABLE 1 | Several commonly used phytochemicals and their main components exhibiting different biological activities, modified from Liu (120).

Scientific name	Common name	Main components	Biological activities
Allium saticum	Garlic	Allicin	Antimicrobial Anti-inflammatory
Capsicum	Pepper	Capsaicin	Antimicrobial Anti-inflammatory
Cinnamomum verum J. Presl *Cinnamomum osmophloeum*	Cinnamon	Cinnamaldehyde	Antimicrobial Anti-inflammatory Antioxidant
Eugenia caryophyllus Spreng. *Eugenia caryophylata* Thunb	Clove	Eugenol	Antioxidant
Foeniculum vulgare	Fennel	Anethol	Antioxidant
Funicular vulgare	Fennel	Anethol Eugenol	Antimicrobial
Origanum vulgare spp. *Origanum onites* *Origanum minutiflorum*	Oregano Thyme	Carvacrol	Antimicrobial Anti-inflammatory Antioxidant
Punica granatum	Pomegranate	Ellagic acid	Anti-inflammatory
Syzygium aromaticum (L.) *Eugenia caryophyllata*	Cloves Fennel	Anethol Eugenol	Antimicrobial Anti-inflammatory
Thymus vulgaris L. *Thymbra spicata*	Thyme Fennel	Thymol Carvacrol Terpinene	Antimicrobial Anti-inflammatory Antioxidant
Zanthoxylum schinifolium	Rutaceae	Citronellal β-Phellandrene	Anti-inflammatory
Zingiber officinale	Ginger	Curcumin Gingerol	Antimicrobial Anti-inflammatory Antioxidant

ulmoides enhanced intestinal morphology and integrity of diquat challenged pigs by improved intestinal barrier function.

The immuno-regulatory and antioxidant properties of phytochemicals are also responsible for their positive effects on animal health. Lang et al. (126) reported that garlic extract could inhibit the secretion of chemokines from intestinal epithelial cells, thus suppress the recruitment of various circulating leukocytes into the inflamed tissue. Dietary supplementation of phytochemicals (10 mg/kg of capsicum oleoresin, garlic, or turmeric oleoresin) downregulated the expression of genes related to antigen processing and presentation and other immune response-related pathways, indicating that these phytochemicals may attenuate the immune responses caused by *E. coli* infection (118). Supplementation of flavones extracted from the leaves of *Eucommia ulmoides* also alleviated the inflammatory responses of weaned pigs induced by diquat (125). Several commonly used phytochemicals (extracts from oregano, thyme, ginger, fennel, pepper, clove, basil, cinnamon, garlic, mint etc.) are also showing strong antioxidant activities in both *in vitro* cell culture and *in vivo* animal models (127–130). The antioxidant property of phytochemicals is mainly associated with the phenolic compounds that have high reactivity with peroxyl radicals, which are free radical species for the oxidation of proteins and lipids (131, 132). Otherwise, surfur-containing volatiles in garlic extracts express strong antioxidant activity due to the formation of unstable degradation products as radicals-trapping agents (129). However, limited research have been reported the effects of phytochemicals on intestinal oxidative status/responses of weaned pigs.

Antimicrobial Peptides

Antimicrobial peptides, also known as host defense peptides, have been considered as potential alternatives to antibiotics in livestock and poultry (133–135). Antimicrobial peptides are polypeptides, naturally produced by different organisms from prokaryotes to mammals. Therefore, antimicrobial peptides could be directly isolated from bacteria, insects, plants, and vertebrates, or could be synthesized as recombinant molecules (136). They are small and positively charged, and contain both hydrophobic and hydrophilic regions. The majority of antimicrobial peptides are belonged to either defensins or cathelicidin family, whereas defensins are further divided into α-, β-, θ-defensins on the basis of the spacing patterns of their cysteine residues (134). Compared with cathelicidins that are highly expressed in mammalian neutrophils, defensins are more abundant in epithelial and phagocytic cells in different tissues, including intestinal mucosa (137).

Antimicrobial peptides possess a strong and large-spectrum activity against gram-negative and gram-positive bacteria, fungi, parasites, and viruses (138). Compared with traditional antibiotics, one obvious advantage of antimicrobial peptides is they could kill pathogenic bacteria (e.g., *P. aeruginosa* and *Staphylococcus aureus*) that are resistant to specific antibiotics

(134, 139). As mentioned above, most antimicrobial peptides are small, positively charged, and amphipathic molecules that allow them to actively interact with bacterial membranes through different models (barrel-stave model, carpet model, or toroidal-pore model) (140, 141). As a consequence, antimicrobial peptides could disturb the structure of cell membrane, penetrate into cells, regulate intracellular pathways, and/or cause cell death. Other mechanisms may be also involved in the antibacterial properties of antimicrobial peptides, such as inhibiting cell wall synthesis, suppressing protein and nucleic acid synthesis, and inhibiting enzymatic activities in bacteria (142).

The protective effects of antimicrobial peptides on intestinal health have been reported in weaned pigs. Supplementation of recombinant lactoferrin increased gut morphology (e.g., greater villi height) and growth performance of piglets (143). Xiao et al. (144) reported that feeding 0.4% of a mixture of antimicrobial peptides (including bovine lactoferrin and plant defensins) and active yeast alleviated the negative effects of mycotoxin by increasing intestinal integrity and reducing intestinal permeability of weaned pigs. Several defensins were shown to enhance mucosa barrier function by up-regulating the expression of mucin and tight junction proteins (145). The potential benefits of antimicrobial peptides are also related to other modes of action, such as regulating immune responses and gut microbiota (146). Supplementation of recombinant lactoferrin or lactoferramoin-lactoferricin increased *Lactobacillus* and *Bifidobacterium* counts but reduced total *E. coli* and *Salmonella* in the small intestine of weaned pigs (146, 147). Addition of cecropin A/D reduced incidence of diarrhea and enhanced intestinal *Lactobacilli* counts in *E. coli* challenged piglets (148). As reviewed in Zasloff (136), fully processed active peptides probably act as epithelial "preservatives" to protect host against intestinal infectious agents. They may also work as effector molecules of innate and adaptive immunity by regulating inflammatory responses and chemotactic activity in pigs (149, 150).

There are two ways to incorporate the benefits of antimicrobial peptides into animal health and nutrition. One is direct supplementation of exogenous antimicrobial peptides to animal feed, while the other one is to use dietary supplements/ingredients to stimulate the secretion of endogenous antimicrobial peptides by the host (135). Although exogenous or recombinant antimicrobial peptides have shown a great potential to be used as alternatives to replace antibiotics, the effectiveness of those candidates should be carefully verified because the majority of exogenous antimicrobial peptides would be digested in the upper gastrointestinal tract without reaching to the lower part where most pathogens reside. Therefore, the stimulation of endogenous antimicrobial peptides secretion by nutritional manipulation may be a better approach. For instance, Robinson et al. (135) have completely reviewed the regulation of antimicrobial peptides synthesis by butyrate and vitamin D in livestock and poultry and pointed out the importance of antimicrobial peptides-inducing compounds in antibiotic-free animal production.

Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are fatty acids with a chain of <6 carbon atoms, which are primarily produced by hindgut fermentation of dietary fiber. The SCFAs are a major fuel source for colonocytes, and are essential for maintaining the normal metabolism of colon mucosa, including colonocyte growth and proliferation (151, 152). In particular, as much as 90% of butyric acid is metabolized by colonocytes (153). However, the benefits of SCFAs is probably not limited to the colon: (1) SCFAs may function as a direct energy source for enterocytes, thus, increase proliferation and reduce apoptosis of enterocytes (154, 155), (2) SCFAs may modulate the expression of genes involved in gut motility, host defense, and inflammatory responses (154, 156), (3) SCFA could stimulate the formation of intestinal barrier and protect intestinal barrier disruption (157), and (4) SCFA may affect the composition of gut microbiota (158–160). The most abundant SCFAs in the gastrointestinal tract are acetate, propionate, and butyric acid. Despite being the least abundant of the 3 primary SCFAs, butyric acid has attracted significant research attention due to its' importance of maintaining gut health in both human and animals.

Butyric acid, also known as butanoic acid, is one of the SCFAs that are produced by microbial fermentation in the gastrointestinal tract of pigs (161). Especially, the propionic and butyric acids produced in the gastrointestinal tract are considered important metabolites that have antibacterial effects on pathogenic bacteria (162). In particular, butyrate has received particular attention and has been widely investigated as an attractive potential alternative to replace in-feed antibiotics. Addition of butyric acid directly to a swine diet may be limited because of its highly volatile and corrosive characteristics (163). Therefore, some products of butyric acid have been used in combined forms with calcium or sodium.

It has been reported that dietary supplementation of 0.1% sodium butyrate reduced diarrhea, enhanced gut integrity, increased serum IgG, but decreased serum pro-inflammatory cytokines in weaned pigs under normal conditions (158, 159, 164, 165). Machinsky et al. (166) also observed a positive effect of sodium butyrate on the protein digestibility of pigs. Another alternative form of butyrate is glyceryl tributyrate, also called tributyrin. Tributyrin is a naturally present triglyceride in butter at the minute amounts. The major advantage of tributyrin vs. sodium butyrate is that tributyrin is a delayed release source of butyrate. Tributyrin stays intact in the stomach and is slowly released as butyrate and/or monobutyrin in the small intestine where pancreatic lipase appears. Feeding 0.1% tributyrin reduced intestinal injury caused by intrarectal administration of acetic acid, as indicated by improved tight-junction formation and activated epidermal growth factor receptor signaling (167). Supplementation of tributyrin also improved the growth and intestinal barrier functions in intrauterine growth-restricted piglets (168).

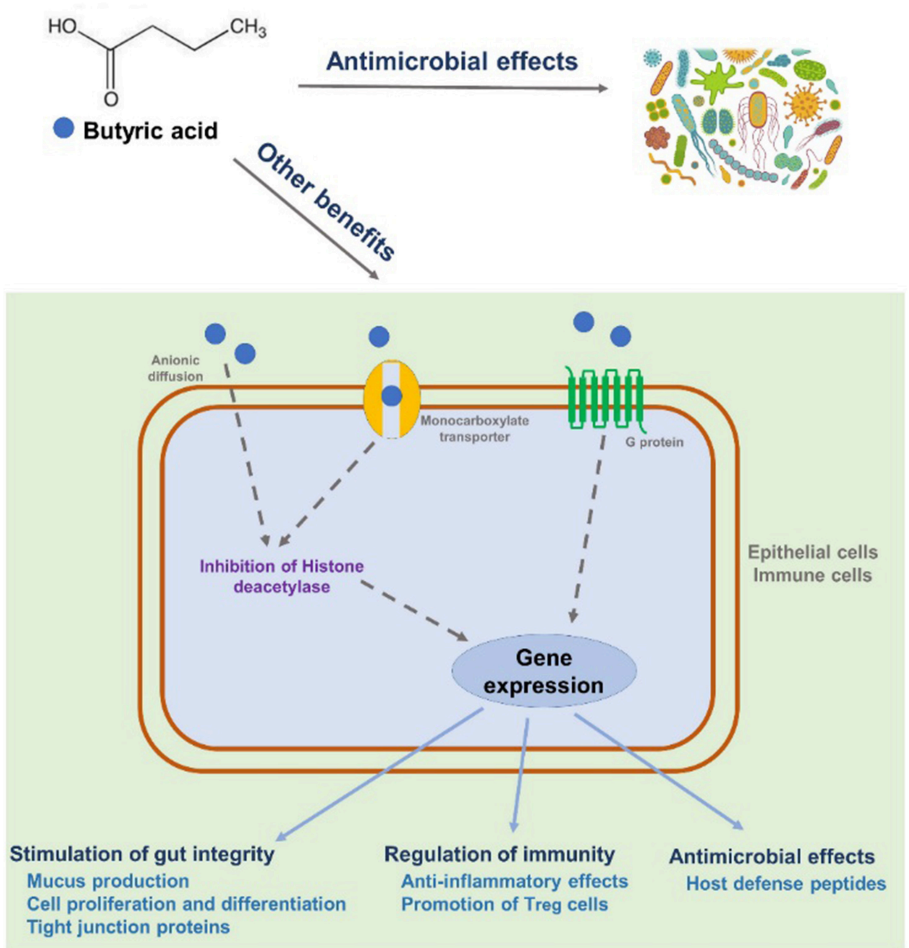

FIGURE 2 | Proposed mechanisms of action on the beneficial effects of butyric acid.

Despite many years of research, the exact mechanism of action of dietary butyrate supplements has not been fully elucidated, but the following mechanisms have been proposed (**Figure 2**). Butyric acid penetrates into epithelial cells either by simple diffusion or monocarboxylate transoporter (169). Butyric acid could also bind to G-protein-coupled receptor expressed in epithelial cells or immune cells. The binding will mediate a cascade of immune regulation (170). A brief summary for the anti-microbial and/or immuno-regualtory effects of butyric acid is shown below. First, butyric acid regulates a large amount of gene expression as one of histone deacetylase inhibitors by removing acetyl groups from the N-terminal tail of the histones (171, 172). Recent studies also revealed that the inhibition of histone deacetylase is highly correlated to the regulation of inflammatory responses and immunity by butyric acid in both human and rodents (157, 173, 174). Second, butyric acid and its derivatives have been shown to possess strong antimicrobial activity against both gram-positive and gram-negative pathogenic bacteria both *in vivo* and *in vitro* (175, 176). The antimicrobial activity of butyric acid is likely due to the ability of this acid to penetrate the bacterial cell wall and acidify the cell cytoplasm, thereby causing bacterial death (177). Third, butyric acid could enhance the expression of host defense peptides in different types of porcine cells, which is remarkably important in modulating host immune system and against a range of pathogens including antibiotic-resistant strains (178, 179). Last but not least, butyric acid may be able to alleviate intestinal injury by promoting tight-junction formation (167, 180).

CONCLUSIONS

A healthy gut is extremely important, as the gut is a nutrient digestion and absorption organ, a chemo-/nutritional sensing organ, as well as the largest immune organ in the body. The young pigs in post-weaning period have limited luminal nutrition supply and are immediately imposed to tremendous challenges, which cause changes in the structure and function of the intestinal tract. These changes may include but not limited to disrupted intestinal structure, reduced digestive and absorptive capacity, damaged intestinal barrier, loss of microbial diversity, and unbalanced intestinal immune homeostasis. A

large amount of research have been conducted to increase our understanding of the importance of gut health on animal production and performance, although the definition of a healthy gut is still not unified. Currently, the most summarized and generalized one is that a healthy gut may contain several key criteria, such as, effective nutrient digestion and absorption, effective waste excretion, a functional and protective gut barrier, a stable and appropriate microbial community, a functional and protective gut immunity, a minimal activation of stress/neural pathways, and the absence of diseases. To promote gut health of weaned pigs, particularly under the restriction of the use of antibiotics in feed, a wide arrange of nutritional interventions have been proposed and investigated. Increasing evidences show that supplementation of extra functional amino acids or specific phytochemicals could provide very positive impacts on intestinal integrity and immunity of weaned pigs. Antimicrobial peptides and their inducing compounds such as butyrate derivatives have also emerged as a potentially viable alternative to replace antibiotics and to maintain intestinal health. There are much more candidates of feed additives/nutritional interventions than the four listed in this review, which may be effective in regulating intestinal environments and enhancing weaned pig performance. It is very important to keep in mind that the efficiencies of each candidate may differ on the basis of their modes of action, the basal diet formulation, and the health status of pigs. Moreover, the importance of omics approaches (i.e., metagenomics, transcriptomics, proteomics, metabolomics, etc.) should be highly recognized as well, although it is not discussed in the current review. These novel approaches have been widely adopted to explore the mechanisms of nutritional interventions on animal health and production by investigating the impacts of nutrition on intestinal microbiota and their metabolites, and the interactions of nutrition, genes and their encoded products (proteins and peptides, etc.).

AUTHOR CONTRIBUTIONS

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

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

frontiers in Veterinary Science

MINI REVIEW
published: 04 March 2019
doi: 10.3389/fvets.2019.00060

Oxidative Stress in the Poultry Gut: Potential Challenges and Interventions

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The gastrointestinal tract (GIT) provides the biological environment for nutrient digestion and absorption, and protection from pathogens and toxins. Broilers are fast growing because of the great potential of intestinal epithelia for nutrient absorption, and efficient conversion of nutrient to muscle. Physiologically, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by GIT epithelial cells either from oxygen metabolism or by enteric commensal bacteria and regulate gut health. However, increased production of ROS elevates free radical production and antioxidant insults resulting in oxidative stress. Oxidative stress in poultry GIT is derived from nutritional, environmental heat stress, and pathological factors, which alters overall performance as well as meat and egg quality. Supplementation of exogenous vitamins, antioxidants, and plant extract having antioxidant properties scavenge ROS and are beneficial in mitigating oxidative stress in the GIT. This review highlights the involvement of oxidative stress in the gastrointestinal functionality of poultry and potential intervention strategies to maintain redox balance in the GIT.

Keywords: oxidative stress, gastrointestinal tract, antioxidant, poultry production, heat stress

OPEN ACCESS

Edited by:
Pietro Celi,
DSM, United States

Reviewed by:
Maria Walsh,
DSM, Switzerland
Vincenzo Tufarelli,
Università degli Studi di Bari, Italy

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *17 October 2018*
Accepted: *11 February 2019*
Published: *04 March 2019*

Citation:
Mishra B and Jha R (2019) Oxidative Stress in the Poultry Gut: Potential Challenges and Interventions. Front. Vet. Sci. 6:60. doi: 10.3389/fvets.2019.00060

INTRODUCTION

Poultry is one of the fastest growing animal industry and has a substantial contribution to food security and nutrition. Poultry meat and eggs are among the most common animal source of food consumed at the global level. The poultry and egg industries are among the largest agricultural commodities globally. Over the period, immense improvements have been made in genetics, feed conversion ratio, fat reduction, and breast size of broiler chickens and significant improvement in the hen-day egg production and egg quality in laying hen (1, 2). In the poultry industry, the feed is the major component of the total cost for meat and egg production. Also, feed exposes the birds to a wide variety of factors through the gastrointestinal tract (GIT), and affect poultry health and production.

The GIT is a highly complex and dynamic organ, which plays a critical role in nutrient absorption and immune response (3). Intestinal mucosa, a site for nutrient absorption, is composed of heterogeneous cell populations, epithelial cells, and connective tissues. The intestinal epithelia are exposed continuously to a wide variety of potentially harmful substances and act as a selective barrier between the tissues and luminal environment of the GIT. There are several stressors such as feed toxin; infectious agents induce the cellular free radicals' generation results in redox imbalance. This stress can negatively affect the delicate balance among the components of the chicken GIT, which in turn, affect the health status and productivity of poultry. The purpose of this review is to

provide updated information on different oxidative stressors, to elucidate the impact of oxidative stress on the pathophysiology of poultry GIT and potential interventions to mitigate the effects.

OXIDATIVE STRESS

Stresses in commercial poultry result from environmental, nutritional, microbiological, and management factors which negatively impact poultry health and production. Oxidative stress is downstream of all these stresses. Oxidative stress in the cells/tissues results from an imbalance between free radical production and endogenous antioxidant defense and leads to lipid peroxidation, protein nitration, DNA damage, and apoptosis. Cells are exposed continuously with the free radicals generated during the physiological oxygen metabolism (4). Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) at certain levels are signaling molecules involved in homeostasis. However, excessive production of ROS and RNS or their inefficient scavenging leads to oxidative stress. ROS, including superoxide, hydrogen peroxide, and the hydroxyl radical radicals, are generated by oxygen metabolism and further balanced by the rate of oxidant formation and the rate of oxidant elimination. The intracellular reduction of ROS is physiologically scavenged by superoxide dismutase, catalase, and glutathione peroxidase (GPX) (5). Superoxide dismutase (SOD1 and SOD2) catalyze the dismutation of the superoxide anion (O_2^-) to H_2O_2 (6), which in turn, is decomposed into H_2O and O_2 by catalase, while GPX reduces lipid hydroperoxides by incorporating glutathione (6). The RNS that are by-products of nitric oxide synthases (NOS) are expressed in selected cells of the intestinal mucosa and submucosal regions. The NOS metabolizes arginine to citrulline and forms the nitric oxide radical (NO•) which is crucial for cellular function including neurotransmission and immunomodulation. However, overproduction of nitric oxide radicals' damages intestinal mucous membrane and impaired nutrient utilization (7). Both ROS and RNS can contribute to lipid peroxidation especially cell membrane lipids and lipoproteins since they are rich in polyunsaturated fatty acids. The end product of lipid peroxidation is 4-hydroxynonenal, which increases oxidative damage to the cell membrane and impair the cell signaling and mitochondrial dysfunctions. Inflammation in GIT is mediated through several stressors/infections which in turn generate ROS and disrupt redox balance.

OXIDATIVE STRESS ASSOCIATED WITH ENVIRONMENTAL HEAT STRESS

The high temperature is one of the most challenging environmental stressors associated with poultry production (8). Heat stress is a major source of systemic oxidative stress since it causes a redox imbalance between the pro- and anti-oxidants in favor of prooxidants. Heat stress has been shown to alter the feed intake, poor growth performance, immunosuppression, hypoxia, and high mortality (9, 10). Heat stress also deteriorates the meat quality of chicken (11). Birds under cyclic heat stress display less crypt depth, mucous area, and villus height of small intestine (12), leading to negative impact on nutrient absorption. Also, heat stress causes intestinal epithelial cell injury, and apoptosis contributes to intestinal hyperpermeability which causes the influx of bacterial products from the intestinal lumen into the circulatory system and affects organ systems. The ROS components such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are produced in the mitochondria and act as signaling intermediates (5). Under physiological conditions, generated antioxidant enzymes rapidly eliminate ROS. Several studies link oxidative stress with heat stress or lipopolysaccharide (LPS), and suggest synergistic augmenting of cell death and increased ROS generation in specific cells (13). Heat stress also activates the chicken hypothalamus, pituitary adrenal axis, resulting in elevated serum corticosterone, which in turn decreases food intake, body weight gain, relative immune organ weight, and innate immunity. This neuroimmune dysfunction further alters intestinal-immune barrier, allowing pathogenic bacteria to migrate through the intestinal mucosa and generating an inflammatory infiltrate. Inflammation of the intestine also decreases nutrition absorption and consequently decrease in weight gain (14).

OXIDATIVE STRESS ASSOCIATED WITH FEED TOXINS

Poultry feeds/feed ingredients are often contaminated with a wide range of environmental toxicants, bacterial and fungal toxins, and known to affect the gut health. The intestinal luminal epithelial cells and the tight junction proteins between two adjacent epithelial cells from the barrier and thus preventing paracellular absorption of toxins. Oxidative stress alters not only the cellular processes but also the intestinal barrier function. Mycotoxins are metabolites produced in a strain-specific way by a wide range of fungi, particularly molds. The common mycotoxins are aflatoxin, zearalenone, deoxynivalenol, trichothecenes, fumonisin, T-2 toxin, and ochratoxin. Once these toxins come in contact with the epithelial cells or during the absorption, the GIT is greatly impacted by the induction of oxidative stress. It has been shown that the chronic, long-term exposure to even low levels of mycotoxins may impact the immune system and intestinal integrity and compromise the blood phagocytic activity in chickens (15). Trichothecenes are a group of mycotoxins (deoxynivalenol, T-2 toxin, and fumonisin B1) which are mainly produced by fungi of the genus Fusarium. These mycotoxins generate ROS which induces lipid peroxidation, alters the cellular redox signaling, antioxidant status, and membrane integrity of the cells. Trichothecenes also increase the intestinal epithelial permeability. Together, mycotoxins increase cellular apoptosis and affect poultry health and production.

Arsenic is widely distributed in water, food, and the environment. It is highly toxic and causes adverse effects

on digestion and absorption of nutrients, resulting in potential losses to poultry growth. Heavy or chronic arsenic exposure induces lipid peroxidation, decreases antioxidants, and eventually trigger apoptosis in several body tissues of poultry (14). Copper, arsenic, and their combination induce the inflammation and the destruction of the intestinal mucosa (16).

Ammonia is one of the primary sources of the air contaminant in the poorly ventilated poultry house. High levels of ammonia decrease growth rate, body weight, and alter feed efficiency (17). Longer exposure to ammonia also causes several health issues and compromise the welfare of broilers (18). The absorption capacity of the intestine depends on the number and size of villi. Chicken exposed to high concentration of ammonia has much lower villus height and crypt depth among different segments of the small intestine. Ammonia also exerts negative impacts on immune organ development of chickens, which may cause enormous damages to nutrient absorption and immune system (18). It has also been reported that ammonia exposure increases the activity of creatine kinase and decreased activity of serum T-SOD producing oxidative stress and apoptosis of mucosal structure (19).

OXIDATIVE STRESS ASSOCIATED WITH MICROORGANISMS

In poultry production, intestinal health and function play a critical role in efficient feed utilization and growth, and the overall profitability of the farm. The GIT microbiota mainly consists of bacteria, fungi, and protozoa. Microbiota population varies across the compartment with maximal at the distal segments of the GIT (20). Intestinal epithelial in response to commensal bacteria generate ROS, which serves as a second messenger and participates in cellular signaling. Tight junctions between intestinal epithelial cells from the barrier and prevent the invasion of the microorganism (21, 22). Studies have suggested that interaction of mucosa with microbes or their toxin triggers oxidative stress. Coccidiosis is among the most common parasitic diseases of poultry. Eimeria primarily produces oxidative stress, thereby destroy the intestinal epithelial barrier and tight junctions, lipid peroxidation, antioxidants insult, as a result, infected birds display reduced feed intake, absorption of nutrients and decreases weight gains (23). Environmental heat stress affects the intestinal epithelial cells and further stimulate intestinal bacteria and bacterial LPS. The LPS is known to induce apoptosis and injury in various cell types (24).

ANTI-OXIDATIVE SYSTEMS IN GIT

The intestinal mucosa is responsible for the absorption of nutrient, and the antioxidant system maintains diverse microbiota in the luminal epithelia. The intestinal mucosa is directly exposed to both feed and non-feed substances. Above physiological level, production of ROS/RNS results in intestinal inflammation and impair the absorption capacity. It has been reported that the broilers fed with the oxidized oils/fats imbalance antioxidants and immune response within the intestinal mucosa (25). As the first line of defense against oxidative stress, the intestinal mucosa contains an extensive antioxidants defense system including enzymes (CAT, SOD, or GPX) and non-enzymatic endo- and exo-genous scavengers like glutathione, transient ions (e.g., $Fe2^{+}$, $Cu2^{+}$) or flavonoids (26). Glutathione and SOD are intracellular antioxidants, widely distributed in the small intestine, and their abundances are at a higher level during intestinal development (27).

MITIGATION OF OXIDATIVE STRESS

The feeds intake, digestion, and subsequent absorption of nutrients in the intestine produce free radicals and imbalance antioxidant system in the intestinal mucosa resulting oxidative stress (28). Also, oxidative stress damages to the intestinal mucosa impede the efficient digestion and absorption of nutrients and adversely influences normal animal growth (29). Dietary inclusion of antioxidant compounds reduces intestinal free radicals, and also help in maintaining the intestinal mucosa. Several studies have suggested that oxidative stress predisposes the birds to various pathological and welfare situation. Therefore, it is essential to formulate a cost-effective strategy to mitigate oxidative stress. Supplementations of vitamin C and E, improve antioxidant ability and immune performance (30). Alpha lipoic acid, possesses both fat and water soluble, is a potent antioxidant and is protective against oxidative damages in the poultry intestine (31). The inclusion of polyphenol compounds also exhibits potent antioxidant activity (32). Equol which is derived from the isoflavonoid daidzein, a major isoflavone of soybean, can hinder oxidative modification induced by ROS (33). Equol protect protects intestinal epithelial cells from oxidative damage by promoting the expression of antioxidant genes, increasing the activities of antioxidant enzymes, and by enhancing antioxidant capacity (34). Dietary galacto-oligosaccharides, as a prebiotic, stabilizes intestinal integrity and prevent against oxidative damages (35). The supplementation of antioxidant-containing plants extracts such as Tulbaghia violacea is shown to have a beneficial effect on the rate of Eimeria oocyst shedding (23). Dietary supplementation of L-glutamine also prevents Necrotic enteritis in antibiotic-free diets (36). Dietary glutamate and N-acetylcysteine induce several antioxidant genes and inflammatory biomarkers in the intestinal mucosa which alleviate LPS-induced intestinal inflammation (37) and can be potentially be used in the chicken diet. Therefore, based on the stress, an individual ingredient or in combination can be used to mitigate oxidative stress in the GIT.

CONCLUSION

Oxidative stress in the poultry GIT is produced by the nutritional factors, environmental factors like heat stress, and pathological factors. These stresses have a negative impact on broiler growth and production as well as the quality of

meat and egg produced. Currently, different antioxidants like exogenous vitamins, antioxidants, and plant extract are used individually or in combination to prevent the oxidative stress in poultry. These anti-oxidants scavenge the reactive oxygen species and reactive nitrogen species to mitigating oxidative stress in the GIT at varying level. Further studies are required to investigate the effects of antioxidants in different combination to mitigate oxidative stress in broiler chicken.

AUTHOR CONTRIBUTIONS

BM reviewed the literature and drafted the manuscript. RJ reviewed the manuscript and provided critical review and suggestion and comments.

FUNDING

CTAHR, USDA/NIFA-Multistate Fund.

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

frontiers in Veterinary Science

REVIEW
published: 04 March 2019
doi: 10.3389/fvets.2019.00048

Dietary Fiber and Intestinal Health of Monogastric Animals

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Animal performance, feed efficiency, and overall health are heavily dependent on gut health. Changes in animal production systems and feed regulations away from the use of antibiotic growth promoters (AGP) have necessitated the identification of strategies to optimize gut health in novel and effective ways. Among alternatives to AGP, the inclusion of dietary fibers (DF) in monogastric diets has been attempted with some success. Alternative feedstuffs and coproducts are typically rich in fiber and can be used in the diets to reduce feed costs and optimize gut health. DF are naturally occurring compounds with a diverse composition and are present in all plant-based feedstuffs. DF stimulate the growth of health-promoting gut bacteria, are fermented in the distal small intestine and large intestine to short-chain fatty acids and have beneficial effects on the immune system. Maternal DF supplementation is one novel strategy suggested to have a beneficial programming effect on the microbial and immune development of their offspring. One mechanism by which DF improves gut health is through maintenance of an anaerobic intestinal environment that subsequently prevents facultative anaerobic pathogens from flourishing. Studies with pigs and poultry have shown that fermentation characteristics and their beneficial effects on gut health vary widely based on type, form, and the physico-chemical properties of the DF. Therefore, it is important to have information on the different types of DF and their role in optimizing gut health. This review will provide information and updates on different types of DF used in monogastric nutrition and its contribution to gut health including microbiology, fermentation characteristics, and innate and adaptive immune responses.

Keywords: dietary fiber, gut health, gut microbiota, immunity, nutritional strategy, pig, poultry, gene expression

OPEN ACCESS

Edited by:
Minoru Tanaka,
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Reviewed by:
Takeshi Ohkubo,
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *17 December 2018*
Accepted: *06 February 2019*
Published: *04 March 2019*

Citation:
Jha R, Fouhse JM, Tiwari UP, Li L and Willing BP (2019) Dietary Fiber and Intestinal Health of Monogastric Animals. Front. Vet. Sci. 6:48. doi: 10.3389/fvets.2019.00048

INTRODUCTION

Although dietary fiber (DF) is abundantly present in common feedstuffs, its concentration in monogastric animal diets has increased proportionally with the increased incorporation of coproducts. It is well-known that DF can contribute nutritional value to animals, directly by providing energy (1, 2) and indirectly by improving gut health and immune function (3–6). Yet, DF has historically been considered as an antinutritional factor due to its negative impacts on nutrient utilization (4, 7). However, DF has recently gained special attention due to its functional value in improving gut health of monogastric animals (8). Maintaining or improving gut health is essential to enhance feed efficiency, promote growth performance, and maintain the overall health of monogastric animals. Antibiotic growth promoters (AGP) have been used in feeding programs for over 60 years to maintain or promote gut health and improve growth performance of

production animals. However, due to potential public health risks, use of AGP have been banned or tightly regulated in several countries. To overcome the negative impacts of AGP regulation and ban on health and productivity of animals, several alternatives have been proposed and tested; with DF being considered to be one of the effective alternatives to AGP (8).

DF are naturally occurring compounds with a diverse composition and are present in all plant-based feedstuffs including cereals, tubers, and agro-industrial byproducts (8–10). Despite some adverse effects on nutrient and energy digestibility, there is growing interest for including DF in monogastric animal diets due to its potential beneficial effects on the gut health, welfare, and the environment (11). DF escapes digestion by host endogenous enzymes in the proximal small intestine and is utilized by the residing microbial population as a fermentative substrate in the distal small intestine and large intestine. Microbial fermentation of DF produces metabolites including short-chain fatty acids (SCFA), which in turn, promotes the growth of beneficial gut bacteria, supports intestinal integrity, and proper immune function. Studies with pigs and poultry have shown that fermentation characteristics and their beneficial effects on gut health vary widely based on type, form, and the physico-chemical properties of the DF (8) as well as the matrix in which it lies (12). Therefore, it is important to have information on the different types of DF and their specific roles in optimizing gut health of monogastric animals.

This paper has reviewed different types of DF used in monogastric animals (primarily pigs and poultry) and their role in modulating intestinal health. To gain a better understanding of this topic, we have discussed the effects of DF on pigs and poultry nutrient utilization and its fermentation characteristics. For further comprehension, we have highlighted the influence of DF on intestinal mucosa and histomorphology, microbial profiles of both host animals and progeny, and innate and adaptive immune response. Finally, we have emphasized the effect of DF on intestinal disorders and diseases.

DIETARY FIBER

Dietary fiber can be defined in many ways; most commonly being based on the chemical composition and the physiological functions. Based on chemical composition, DF is the sum of non-starch polysaccharides (NSP) and lignin. From a nutritionist's point of view, it can be simply defined as carbohydrates that are indigestible by endogenous enzymes. Common feed ingredients rich in fiber are cereals like barley, wheat, oats, and other coproducts like distillers dried grains with solubles, canola meal, and wheat millrun. Generally, DF includes cell wall components cellulose, hemicellulose, and other structural and non-structural compounds resistant starch (RS), inulin, chitin, pectin, β-glucan, and oligosaccharides. The utilization of DF in pig and poultry diets depends on the fiber content, the degree of microbial fermentation in the large intestine, the extent of absorption, and other factors (8, 13). Soluble fiber sources are rapidly fermented by resident microbes in the distal small intestine and large intestine, increase digesta viscosity, reduce digesta passage rate through the intestine, and can decrease feed intake due to increased satiety. On the other hand, insoluble fiber passes through the intestine undigested, increases passage rate and fecal bulking; however, monogastric species have a limited capacity to ferment insoluble fiber as they lack specific microbial species (4, 14). Therefore, it is essential to understand the components of DF and its nutritional and physiological effects in animals before incorporating it into monogastric diets. For details on the composition of DF, its sources and utilization in different parts of the gastro-intestinal tract (GIT), readers are referred to Jha and Berrocoso (8), which provides an extensive updated review on these topics.

THE CONCEPT OF INTESTINAL HEALTH

The GIT is the largest group of organs in the body. It is not only the site of digestion and absorption of dietary nutrients but provides protection against pathogens and toxins. Moreover, it hosts a large population of microbiota and immune cells. Thus, a healthy intestinal tract is of utmost importance for overall sound health and improved productivity of animals. However, the definition of "intestinal health" or "gut health" is not yet clearly defined, despite it having been a focus of major research efforts in the last few decades. Conway (15) proposed that gut health is the function of three major components: the diet, the mucosa, and the commensal microbiota. Later, Montagne et al. (16) elaborated that it includes a diet that would provide sufficient nutrients, mucosa that maintains the gut integrity, and a microbial community that maintains a balanced, healthy environment. Since the GIT of pigs and poultry contains about 70% of total body immune cells, it should be included in the definition of "intestinal health." Thus, we suggest that intestinal health should be considered in a holistic way including the diet, mucosa, microbiome, and immune system (**Figure 1**). The GIT of pigs and poultry consists of hemopoietic cells (macrophages, dendritic cells, and T-cells), non-hemopoietic cells (epithelia, Paneth cells, and goblet cells), and the microbiome (bacteria, archaea, protists, fungi, and viruses) all of which contribute to gut health. The innate and adaptive immune systems constantly communicate with the microbiome to maintain homeostasis. Any imbalance in the immune system or the microbiome can lead to dysbiosis, resulting in increased susceptibility to various diseases (17). The intestinal mucosa is composed of the epithelium, the gut-associated lymphoid tissue (GALT), and the mucus overlying the epithelium. The intestinal mucus, host epithelial cells, GALT, and microbiome interact with each other forming a fragile and dynamic equilibrium, which is critically important for efficient functioning and absorption capacity of the digestive system. The physical (epithelial cells, intercellular tight junction, and mucus) and chemical (acidity, proteolytic enzymes, lysozymes, and antibacterial proteins) barriers play an important role in maintaining gut barrier function and preventing the microbial population from translocating and causing systemic immune activation. Besides acting as a physical barrier, the epithelial cells also secrete cytokines and chemokines that regulate chemotaxis of immune cells. Paneth cells located at

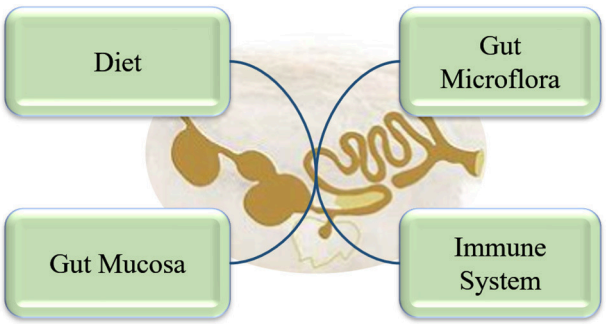

FIGURE 1 | Components of gut health in a holistic approach.

the base of crypts of many vertebrate species, including poultry. It contains defensin rich granules that are released in response to bacterial-induced inflammation (not during protozoal or fungal infection) via exocytosis (18). Three mucosal barrier factors help to maintain and restore the mucosal integrity of intestine; diamine oxidase, trefoil factor, and transforming growth factor-α. Occludin, claudin, and zona occludens-1 are the three tight junction proteins that maintain the paracellular barrier (19). Goblet cells in the GIT produce mucin, which also plays an important role in maintaining gut barrier function. Mucin production can be increased several bacteria, including *Lactobacillus* (20), which can help to improve the gut barrier as pathogenic microbes are impeded by the dense mucous layer. However, optimal gut health is not characterized by complete absence of pathogenic microbiota, rather an intestinal microbiome with a high microbial and functional diversity.

DF AND NUTRIENT UTILIZATION

The significant fraction of NSP in any cereals fed to pigs or poultry consists of arabinoxylan, followed by cellulose, and mixed linked β-glucan (8, 21). Cellulose is a polysaccharide consisting of chains of glucose molecules. It differs from starch in the orientation of the glycosidic bonds. While starch has α-glycosidic bonds, those in cellulose are in a β-orientation. Lignins are cross-linked phenol polymers and are present in a more significant proportion in rye than in wheat and oat, with a concentration in bran higher than in whole grain (21). Among the commonly used cereals in the diets of pigs and poultry, the concentration of β-glucans is the highest in oat (4%), intermediate in wheat and rye (0.7–1.7%), and lowest in corn (0.1%) (21). The structure of the cell wall of cereal grains is complex, and their composition and properties vary depending upon the location of tissues. The kernel of the cell wall consists of xylans, cellulose, and a significant amount of lignin. This layer is thick and hydrophobic. On the other hand, endosperm (aleurone layer) is thin and hydrophilic and consists of mainly two polysaccharides, arabinoxylans, and β-glucan (22). NSP present in cell walls, along with lignin, are not digested by endogenous enzymes but can influence digestion and absorption by encapsulating nutrients and by increasing digesta viscosity (23). The concentration of DF in brans are generally far greater than in whole grains. Most brans contain a higher amount of insoluble fiber than cereal grains with the exception of oat bran which is more soluble as it contains a larger aleurone and sub-aleurone layer and higher amounts of β-glucan (24). The aleurone layer in wheat contains a large amount of arbinoxylan as well as phenolic phytochemicals. The aleurone layer is a part of the endosperm and contains higher amounts of insoluble polysaccharides than the remaining endosperm layers (21). The aleurone and pericarp also contain increased amounts of ferulic acids than in the starchy endosperm layer (25). Ferulic acid is the most abundant phenolic acid present in most cereals and wheat and rye brans, which are esterified to arabinoxylans. The physicochemical properties of DF are affected by the crosslinking of diferulates with lignin, with insoluble DF possessing 100 times higher diferulates than soluble DF (26).

Amount of DF and nutrient utilization are inversely proportional to each other. Increases in the amount of DF reduce growth performance of monogastric animals. However, the inclusion of NSPase or the fiber degrading enzymes has been found to be one of the best methods of eliminating the negative effects of DF on growth depending on the type and structure of fiber present in the ingredients used (23, 27, 28). Structural component, orientation, substitution, presence of functional group; all has a role to play in determining the effect of DF in gut immunity. The immunomodulating effect of DF has been reported to have overall health benefits to host animals (23) describing its potential to be used as an alternative to AGP (27). Increased regulations and the banning of sub-therapeutic antibiotics in monogastric diets have led nutritionists to look for alternative strategies to maintain animal growth performance. Therefore, dietary inclusion of oligosaccharides and soluble fiber is one potential alternative strategy to help support gut health and animal performance.

DF FERMENTATION AND EFFECTS

The diet of pigs consists of a considerable amount of carbohydrates, which partially escapes small intestinal digestion, and passes through to the large intestine where it is fermented by microbes. Microbial fermentation of DF results in the production of SCFA, branched chain fatty acids (BCFA), lactate, amines, indoles, phenols, and various gasses like hydrogen, carbon dioxide, and methane (11). The substrate that is being provided to microbes to ferment directs the end metabolites. In the absence of adequate DF, proteolytic fermentation can take place in the colon producing BCFA and potentially harmful metabolites like ammonia indoles, and phenols. Ammonia is produced from the deamination of amino acids and hydrolysis of urea whereas phenols are produced due to carboxylation of amino acids. Hence, the composition of SCFA produced in the gut can be manipulated by changing the substrate that reaches the colon (4, 5, 29).

Starch digestion in pigs is more desirable than its fermentation to SCFA because starch digestion products are more efficient sources of energy (30, 31). The SCFA are thought to provide

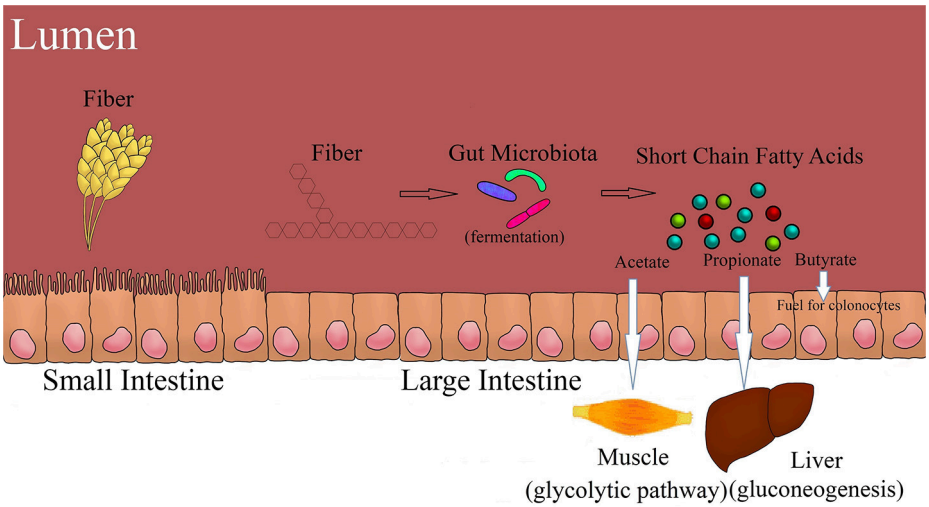

FIGURE 2 | Fiber fermentation and its primary utilization pathways.

up to 15% of the maintenance energy requirement of growing pigs and 30% in gestating sows (1). However, an increase in the concentration of SCFA, more specifically of butyrate, can improve the gut mucosal health as well as the immune system of pigs. Energy provided by butyrate to the host is vital to maintaining the gut ecosystem as well as the health of pigs. In the absence of fermentable carbohydrates as an energy source, microbial fermentation shifts toward amino acids and utilize carbon skeleton from amino acids as energy source, and the resulting metabolite ammonia is absorbed and disposed of in the form of urea (11). On the other hand, in the presence of energy from fermentable carbohydrates, ammonia is removed as microbial biomass (32), i.e., the resident microbes in the large intestine retain more nitrogen for their growth.

The most abundant end product of fermentation in the proximal GIT is acetate, which contributes to more than 90% of total SCFA produced. However, conditions change in the distal GIT, where the concentration of lactate decreases and the concentration of SCFA increases with a ratio of approximately 60% acetate, 25% propionate, and 15% butyrate. Degradation of DF is highest in the proximal colon, and so is the production of lactic acid and SCFA. However, the progressive decrease in the flow of digesta toward the distal colon changes the fermentation metabolite and bacterial profile (4, 6). Modification in the structure of DF due to cross-linking, transglycosylation, or esterification prevents hydrolysis of starch both by the host and bacterial enzymes. Most of the SCFA (more than 90%) absorption occurs in the anionic dissociated form, as they are weak acids. The SCFA produced are absorbed from the apical membrane by three primary methods; passive diffusion in lipid soluble form, anion exchange between bicarbonate and SCFA (33), and by the help of active transporters like Monocarboxylate transporter 1 (MCT1) and Sodium coupled monocarboxylate transporter 1 (SMCT1). Fermentation starts only after the DF gets depolymerized by microbial hydrolytic enzymes. The faster the rate of depolymerization of a substrate, the faster the carbohydrates will be available for fermentation by the bacteria. The DF which are heavily branched provide a larger surface area for enzymes to act on and are more rapidly fermented (30). On the other hand, degradation of linear polymers or high amylose starch is slowly fermented as their degradation yields larger fragments (larger oligomers), which are further utilized by bacteria and produce metabolites like SCFA and gases. The major fermentation metabolites and its primary utilization pathway are summarized in the **Figure 2**.

The solubility of DF also affects SCFA production, as insoluble DF are less fermentable compared to soluble DF because insoluble DF contains ∼100-fold more ferulic acid (26). Besides SCFA production, soluble DF influences gut health by decreasing fecal bulk, delaying emptying of liquids by increasing viscosity of gastric chyme, lowering pH in the intestinal lumen as well as altering bile acid profiles (34). Soluble DF are responsible for changing viscosity of luminal digesta (23, 35). When soluble DF comes in contact with water, it absorbs it and swells, increasing the viscosity of digesta. Viscosity of DF is also affected by the molecular weight of individual DF. Structural variation, the degree of polymerization, branching, and chemical modification in the DF subsequently determine its fermentation characteristics. Solubility and viscosity of DF also affect the end product of fermentation.

DF AND INTESTINAL MUCOSA/HISTOMORPHOLOGY

Gut health is essential to maintain growth performance and overall health of monogastric animals. The primary role of intestinal mucosal tissue is digestion and absorption of nutrients. Feed ingredients are hydrolyzed and broken down by the host into smaller compounds; the mucosa obtains glucose from starch, amino acids, and peptides from proteins, and fatty acids and monoglycerol from lipids. The DF are fermented

resulting in SCFA, which promote proliferation of the mucosal epithelium and villus height (36). The epithelial layer of mucosa regulates the exchange of nutrients to the body (16). Besides the intestinal secretions and glycoproteins produced by the brush border membrane, mucosal epithelium also greatly influences the adherence capacity and the metabolic activity of intestinal microbes. Hence, the intestinal mucosa acts as a barrier to the pathogenic bacteria and toxic compounds. Both innate and adaptive immune systems participate in the building of intestinal mucosal barrier.

The inclusion of DF often increases the endogenous losses, resulting in a perceived decrease in the digestion of energy and nutrients in monogastric animals. Therefore, DF has been recognized as "anti-nutritive" for monogastric animals. Moreover, these negative effects are more prominent to chickens and piglets than in growing and finishing pigs (37). However, moderate levels of dietary fiber may increase gut size, length, volume, and morphological structure of pigs, poultry, and other non-ruminant animals. The addition of soluble fiber to the diet of piglets generally causes an increase in the viscosity of the intestinal content, which may increase the rate of villus cell losses leading to villus atrophy (38). The villus height to crypt depth ratio is a useful criterion for estimating the likely digestive capacity of the small intestine. In growing pigs, the inclusion of 10% high fiber source in diets over 14 days caused an increased width of villi and depth of the crypts in the jejunum and ileum. The inclusion of high fiber in diets also increased the rate of cell proliferation and crypt depth in the large intestine, when compared to the same diet containing no straw (39). However, the height of villus and the depth of crypt in the gut is not immutable; it changes with the location of the small intestine. Therefore, it is critical to understand the mechanisms of nutrient absorption, and the location of specific nutrient utilization in the gut to develop the optimal feeding system to obtain the best production performance.

DIETARY FIBER AND INTESTINAL MICROBIOTA

Direct Fiber Supplementation on Microbial Composition

The complex carbohydrates and plant polysaccharides indigestible by monogastric animals provide an essential fermentative substrate to the microbiome (including bacteria, fungi, protozoa, and archea) and are known to impact bacterial composition, diversity, and metabolic capabilities (40). It is likely the microbiome as whole that contributes to fiber breakdown; however, only the role bacteria play in this complex process has been well-defined. The GIT of poultry and swine are highly diverse containing over 1,000 bacterial species mainly belonging to predominant phyla Firmicutes, Bacteroidetes, and Proteobacteria (41–43). It must be taken into consideration that the nutritional and health benefits residing bacteria provide to their host is a result of the entire community and their metabolic capabilities, not the presence or absence of a single species. It is through glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases that gut- associated bacterial communities are able to breakdown and ferment complex carbohydrates into SCFAs (44).

The microbial process of fiber fermentation is considerably more variable than host macronutrient digestion due to the range in fiber sources and the physicochemical properties of that fiber (i.e., solubility, viscosity, and water-holding capacity) (31). Recently recognized in humans is the substantial effect colonic transit time has on microbial composition (45). Therefore, soluble fiber has the ability to increase the viscosity of intestinal digesta and the transit time, hence increased intestinal mass. Retained digesta in intestinal lumen for longer time provides opportunity for proliferation of selective microbiota. This might be the probable mechanism which cause fiber and its type alter microbial profiles. Resistant starches are also involved in increasing the viscosity of digesta. However, RS are easily degraded to small molecular weight residue whereas DF are more resistant to depolymerization. This might be the reason for RS to have better response than DF. In weaned and growing pigs, changing passage rate and site of digestion of starch from the proximal to distal intestine through the inclusion of purified resistant starch selectively promotes bifidobacteria (46, 47) and lactobacilli as reviewed in a recent meta-analysis (48). Fermentable fiber from barley high in β-glucans also shifts the site of nutrient digestion from the small to large intestine subsequently increasing relative abundance of Firmicutes genera; *Dialister*, *Sharpea,* and *Ruminococcus* (49). However, increasing digesta viscosity in poultry with soluble fiber (barley β-glucans or wheat arabinoxylans) can be detrimental to growth and has shown to favor expansion of potential pathogens, *E. coli* and *Clostridium perfringens* (50–52). Viscosity caused by certain fiber results in villus cell loss as it prevents the enterocytes from reaching to the nutrients. Long term impact of such fiber inclusion results in atrophy of villi. Supplemental enzyme has shown positive response in minimizing this impact (23). The villus height to crypt depth ratio is a useful criterion for estimating the likely digestive capacity of the small intestine. In pigs, arabinoxylans enrich butyrogenic species and others commensals including *Faecalibacterium prausnitzii, Rosburia intestinalis, Blautia coccoides, Eubacterium, rectale, Bifidoabcterium, and Lactobacillis* spp. (53). A more in depth review of how specific fiber types and feed ingredients promote beneficial bacteria can be found elsewhere (8).

In comparison to swine, the literature exploring the complex interactions between gut microbiota and fiber in poultry is scarce. However, recently over 200 different non-starch polysaccharide-degrading enzymes (mainly oligosaccharide degrading enzymes vs. cellulases and endohemicellulases) were found encoded within the metagenome of broiler microbiota, suggesting poultry microbiota are capable of utilizing soluble forms of dietary fiber (41). The importance of supplying dietary fiber to the microbiota is truly demonstrated in fiber deficient diets, where resident polysaccharide degrading bacteria begin to utilize the mucus layer of the intestine, which can reduce intestinal barrier function leaving the host increasingly vulnerable to pathogen invasion (54). Feeding highly digestible low fermentable wheat based diets to pigs increases abundance of *Akkermansia*, a microbe

known to utilize host-glycans, emphasizing the adaptability of the microbiota to utilize host substrates when dietary fiber is scarce (49).

Maternal Fiber Supplementation on Progeny Microbiota

In natural settings offspring of monogastrics derive their gut-associated microbiota through vertical transmission during the birthing or hatching process. The minimal distance between the digestive tract and birthing canal is likely no evolutionary coincidence. In commercial swine production piglets fecal microbiota first resembles that of the environment (floor, sow milk, and sow nipple); however, soon reflects that of the sow, emphasizing the importance of the sow microbial composition (55). Although hens externalize eggs through their vent, a common external opening for excretion of fecal matter, the practice of cleaning eggs pre-hatch removes many co-evolved avian microbes leaving newly hatched chicks to colonize with environmentally derived non-host-adapted microbiota.

Due to the fact piglets receive their colonizing microbiota from the sow (55), beneficial manipulation of sow microbiota with dietary fiber may directly influence the intestinal microbiota of her piglets. The concept of fetal programming through maternal nutrition is not new, and it has been shown that maternal seaweed extract supplementation can reduce both sow fecal *Enterobacteriaceae* populations at parturition and piglet *E. coli* populations at weaning (56). Both wheat bran and inulin supplementation of sows during gestation and lactation have shown to impact piglet microbiota and fermentation profiles (57) with inulin also able to reduce enterobacteria (58). Although fiber supplementation of sow diets has shown to impact piglet microbial profiles, the changes observed may be more related to altered colostrum and milk composition rather than maternal microbial changes. After parturition there is a 1–3 week period whereby piglets rely exclusively on the sow for nutrition and research in humans has demonstrated the importance of milk composition in shaping the neonatal intestinal microbiota (59). In particular, the composition of milk oligosaccharides is of great interest, as these heterogeneous mix of soluble glycans are indigestible by the host but provide a fermentative substrate for the colonizing intestinal microbiota (60, 61).

Sows also produce milk oligosaccharides that are fermentable by piglet microbiota (62), which suggest they play a key role in colonizing microbiota composition (63). Current literature suggests dietary supplementation of sows with short-chain fructo-oligosaccharides (scFOS) during nursing can increase microbial fermentative capacity in their suckling piglets, stimulating the development of intestinal immune defenses including increased ileal cytokine secretions, mucin secreting goblet cell numbers, and improved vaccine-specific IgA levels (64). Increased fermentative capacity in piglets suckling from scFOS supplemented sows may be from altered porcine milk oligosaccharide composition, as recent literature has suggested that supplementing nursing sows with chitooligosaccharides (COSs) significantly alters milk oligosaccharide composition (65). The effects of supplementing sows with soluble fiber (pregelatinized waxy maize starch and guar gum) can also be immediately recognized by the improved piglet growth rates and associated increase in plasma growth hormone, insulin-like growth factor-1, and reduced incidence of diarrhea (66). In the study by Cheng et al. (66), piglets suckling from soluble fiber supplemented sows also had remarkable changes in their microbial composition, with increased relative abundance of *Bacteroides*, *Lactobacillus*, *Roseburia*, *Fusobacterium*, and *Acinetobacter* that was accompanied by improvements in markers of intestinal integrity (plasma zonulin, endotoxin, and diamine oxidase). Maternal fiber supplementation can also affect other colostrum and milk components essential for piglet immune development. Sows supplemented with scFOS have shown to have increased colostral IgA and transforming growth factor beta-1 which subsequently supported piglet mucosal immune development by increasing secretory IgA production in Peyer's patches and activated T cells (67). This emphasizes the important and often overlooked concept of maternal nutritional programming on offspring microbial and immune development.

IMMUNE PROGRAMMING WITH SCFA

It is well-accepted that the gut-associated microbiota have co-evolved with their respective host and play a vital role in immune maturation and function and protection against pathogens (68, 69). The relationship between gut microbiota and immune development is exemplified in germ-free animal models, which have defective immune systems whereby colonization with live microbial communities recapitulates immune development and function (70). Uncovering the mechanisms of how microbial communities benefit host immune function is in its infancy; however, appear highly connected to microbial fermentation metabolites, SCFAs. The production of SCFAs, particularly butyrate, can enhance intestinal epithelial cell barrier function, the first line of defense against invading pathogens (71) and helps maintain this physical barrier by stimulating goblet cell differentiation and mucus production (72). Short chain fatty acids promote the differentiation and function of colonic regulatory T cells, which maintain gut homeostasis by inhibiting effector T-cell function and increasing IL-10 production, important in preventing excessive inflammation (73, 74). The presence of specific nonpathogenic bacteria, such as *Bacteroides thetaiotamicron*, can also inhibit host inflammatory responses by promoting the nuclear exportation of NF-κB, a transcription factor that triggers proinflammatory gene expression (75). Although intestinal inflammation may sometimes be necessary to clear intestinal pathogens, restoring intestinal homeostasis as quickly as possible is necessary to maintain animal health and performance.

MAINTAINING AN ANAEROBIC ENVIRONMENT WITH SCFA

The fermentation metabolite butyrate is used preferentially as an energy substrate by intestinal epithelial cells and plays a major role in maintaining homeostasis by keeping the intestine

anaerobic. During microbial colonization the GIT goes from being aerobic to anaerobic. In a homeostatic state the intestine remains anaerobic with anaerobic bacteria outcompeting aerobes and facultative anaerobes. During dysbiosis facultative anaerobic Proteobacteria, such as *E. coli* and *Salmonella,* characteristically expand at the expense of oxygen sensitive butyrate producers, disrupting the anaerobic intestinal environment (76). Referring to dysbiosis as "dysanaerobiosis" elegantly summaries the change in intestinal environment from hypoxic to micro-aerophilic and the subsequent shift from obligate anaerobes to facultative anaerobes (77). Inclusion of dietary fiber may help prevent or ameliorate the micro-aerophilic environment that occurs during dysbiosis by providing a fermentative substrate to anaerobic butyrate-producing bacteria (**Figure 3**). In a homeostatic environment host intestinal tissues use butyrate as an energy substrate via β-oxidation, a process that consumes considerable amounts of oxygen helping to maintain an anaerobic environment (76, 78). In the absence of butyrate, enterocytes use anaerobic glycolysis to obtain energy, a process that increases epithelial oxygen concentrations creating a favorable niche for facultative pathogens such as *Salmonella* to flourish (76, 79). To maintain and improve piglet and poultry gut health, nutritional strategies should aim at restoring the hypoxic intestinal environment through the expansion of butyrate producers to prevent facultative anaerobic expansion.

DIETARY FIBER ON INTESTINAL DISORDERS/DISEASES

Inclusion of dietary fiber can support colonization of beneficial commensal microbiota that competitively exclude pathogens, enhance maturation, and barrier function of the GIT through metabolite production, and directly block adhesion of pathogenic microbes to the intestinal epithelium by providing alternative adhesion sites (80). One of the most common causes of reduced animal performance and economic loss in swine production is the incidence of post-weaning diarrhea caused by opportunistic pathogens such as *E. coli* and *Salmonella*. Historically highly digestible low fiber diets have been used for newly weaned pigs in efforts to improve digestibility and animal performance. However, it has since been proposed that there is likely at least a minimum dietary fiber requirement for piglets to achieve optimal gut health (81). As such, inclusion of insoluble non-starch polysaccharides (iNSP) such as oat hulls have shown to reduce diarrhea incidence in piglets (81, 82). Although oat hulls are highly insoluble and lignified in nature, they are also able to reduce fecal biogenic amines, cadaverine, and β-phenylethylamine, from protein fermentation, signifying oat hulls can beneficially influence dietary fermentation patterns (82). Inclusion of 40 g/kg of wheat bran in piglet diets, another dietary source of iNSP, can also reduce intestinal enterobacteria populations and increase butyric acid concentrations in young piglets, further suggesting the ability of piglet gut microbes to utilize insoluble fiber and provide protection (83). Additionally, when challenged with *E. coli* K88, piglets supplemented with coarsely ground wheat bran had reduced diarrhea severity, increased SCFA concentrations (84), and reduced ileal *E. coli* K88 adhesion (85).

There is conflicting evidence as to whether or not inclusion of soluble fiber is detrimental or beneficial to disease resistance in piglets and has been reviewed previously (80, 86). An older literature has reported that increasing dietary soluble non-starch polysaccharides (sNSP) from 1 to 6% can increase haemolytic *E. coli* in the small intestine from 1.3×10^4 to 8.0×10^9 (87). Although increasing levels of dietary sNSP can increase SCFA concentrations, digesta viscosity is also linearly related with sNSP intake and is suggested be the cause of intestinal *E. coli* proliferation (88). However, more recently sNSP have shown to be protective against post-weaning diarrhea, likely through the promotion of commensal microbiota proliferation, SCFA production, and subsequent maintenance of an anaerobic environment. Inclusion of 50–150 g/kg of inulin was shown to increase the *Lactobacillus*:coliform ratio and SCFA concentrations (89) while reducing the occurrence of diarrhea when challenged with *E. coli* (89, 90). Enrichment of commensal microbiota such as *Lactobacillus* with sNSP (91) may induce growth inhibition or competitive exclusion to *E. coli* (92).

As discussed above another mechanism by which DF may reduce diarrhea incidence and pathogen colonization is by improving intestinal barrier function. It has been shown that inclusion of 10% wheat bran fiber or pea fiber into piglet diets can improve intestinal barrier function (increased villous height: crypt depth ratio, colonic goblet cells, and peptide trefoil factors) potentially mediated through changes in microbial composition, namely increases in *Lactobacillus* and *Bifidobacterium* populations (20). Furthermore, wheat bran fiber and pea fiber were observed to reduce diarrhea incidence in comparison to maize fiber and soybean fiber (20), suggesting source, compositional and functional characteristics of fiber are important factors to take into consideration. There is also evidence that fermentable fiber can benefit pre-weaned pigs, where piglets fed milk replacer supplemented with 7.5 g/L of either FOS or soy polysaccharides vs. methylcellulose can increase SCFAs in the colon, improve intestinal function (increased glutamine transport), and can inhibit *Salmonella* induced diarrhea (93).

Swine dysentery (SD) is another common contagious diarrheal disease observed in the grower-finisher phase of swine production caused by the intestinal spirochaete *Brachyspira hyodysenteriae*. Recent work has shown that diets high in fructans and galactans from chicory root and sweet lupins can protect pigs from infectious SD (94, 95), which may be due to increased abundance of commensal microbiota, *Bifidobacterium thermacidophilum* subsp. *porcinum* and *Megasphaera elsdenii*, lactate producers and lactate utilizing butyrate producers, respectively (95). More recent research also observed that although lupins can delay the onset of disease, 80 g/kg inclusion of inulin can reduce the risk of developing SD (96). A study by Hansen et al. (97) also confirmed that increasing dietary inulin from 0 to 80 g/kg reduces the risk of pigs developing SD when challenged directly with *Brachyspira hyodysenteriae* and the protective effect was accompanied by a linear increase in cecal SCFAs and reduction in

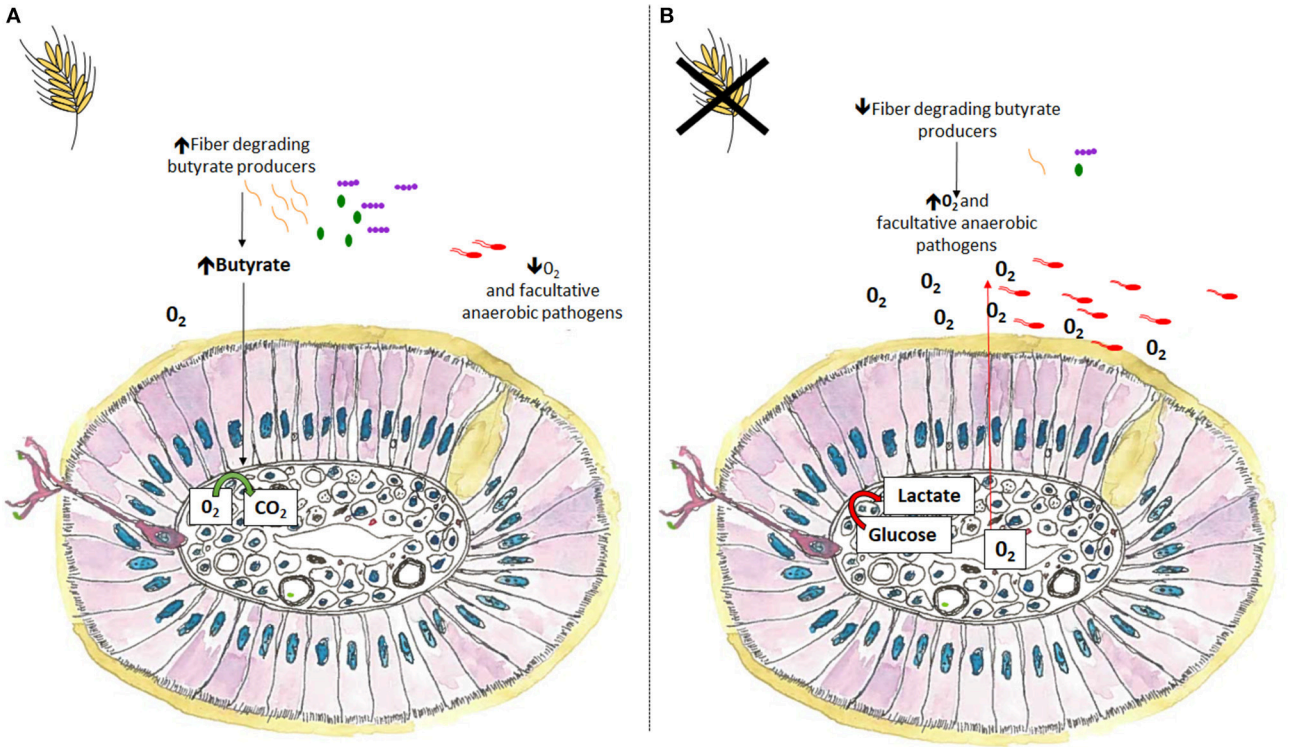

FIGURE 3 | A transverse cross section of colonic villi in the presence or absence of dietary fiber. **(A)** Inclusion of dietary fiber helps maintain intestinal homeostasis and improves disease resilience by maintaining a hypoxic environment. Dietary fiber facilitates the expansion of anaerobic butyrate producers, which subsequently increases butyrate concentrations, reducing luminal oxygen, and limiting the expansion of facultative anaerobic pathogens. **(B)** Alternatively, in the absence of dietary fiber facultative anaerobic pathogens, including certain *E. coli* and *Salmonella* species may expand at the expense of oxygen sensitive butyrate producers. In the absence of butyrate, enterocytes use anaerobic glycolysis to obtain energy, a process that increases epithelial oxygen concentrations creating a favorable niche for facultative pathogens such as *Salmonella* to flourish.

protein fermentation metabolites. It is hypothesized that inulin acts by modifying microbial fermentation patterns, potentially reducing the protein:carbohydrate ratio in the hindgut increasing carbohydrate fermentation while suppressing protein fermentation, thereby inhibiting SD colonization (97).

A severe intestinal disorder in poultry is necrotic enteritis and is caused by the pathogen *C. perfringens*. Feeding whole wheat has been shown reduce and *C. perfringens*, the causal pathogen of necrotic enteritis (98, 99). It is suggested by authors that whole wheat improves gut health of chickens by reducing gizzard pH, increasing retention time and viscosity creating an inhospitable environment for pathogen survival into the lower intestinal tract (98). Acetylated resistant starch has also been shown to improve gut health and reduce severity of a *C. perfringens* challenge through reducing luminal pH through specific SCFA delivery (100).

Controlling *Salmonella* colonization in poultry flocks is another global priority to reduce potential zoonotic contamination of meat products. A 1% inclusion of wheat bran with a reduced particle size (280 μm) into broiler diets was able to reduce levels of cecal *Salmonella* colonization (1.3 vs. 3.6 Log CFU/g in control) and *Salmonella* shedding post-challenge. *In vitro* fermentation of 280 μm wheat bran resulted in increased production of butyrate and propionate compared with larger particle sizes (101). Inclusion of whole wheat in broiler diets has also shown to increase gizzard fermentation reducing gizzard pH and subsequent *Salmonella* Typhimurium post-challenge; further suggesting feed structure and particle size can influence pathogen colonization (99). Incubating *Salmonella* with wheat bran (280 μm) fermentation products can reduce hilA expression, a transcriptional activator of *Salmonella* pathogenicity island I vital for *Salmonella's* entry into epithelial cells (102). A component of wheat bran, arabino-xylooligosaccharides, can also reduce *Salmonella* colonization of the cecum and subsequent *Salmonella* shedding (103). Other fiber types including FOS and mannan-ologisaccharides have shown to inhibit the growth and colonization of *Salmonella in vitro* (104) and *in vivo* (105). Although there is much evidence to suggest supplementing dietary fiber to pigs and poultry is beneficial to gut health and disease resistance, research needs to focus on defining the mechanisms of action to help develop optimal nutritional strategies to further improve animal health. It must be recognized that there are likely numerous nutritional strategies that utilize dietary fiber to improve gut health of pigs and poultry depending on environment, health status, life stage, and feeding objective (growth vs. longevity).

CONCLUSION

Although dietary fiber was recognized as an anti-nutritional factor in the past, there is increasing interest in its inclusion in monogastric animal's diets due to potential functional benefits to the host, primarily on the intestinal health. The benefits are primarily due to fermentation of DF in the distal GIT. The fermentation metabolites and interaction of DF with the intestinal environment affect the intestinal histomorphology, mucosa, microbial community, and immune system, altogether named as "intestinal health." Based on the available information, it can be concluded that inclusion of dietary fiber can be a strategy to improve gut health, thereby overall health and production of monogastric animals in the post-antibiotic era. However, type, form, physico-chemical properties as well as the amount of DF inclusion in diets need to be considered strategically as there is wide variation in their composition and subsequently their effects on intestinal health of monogastric animals.

AUTHOR CONTRIBUTIONS

JF, UT, and LL wrote this review manuscript. BW reviewed the manuscript and provided critical suggestions and comments. RJ decided on the review topic, reviewed the literature and manuscript, and provided critical suggestions and comments.

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

frontiers in Veterinary Science

REVIEW
published: 13 March 2019
doi: 10.3389/fvets.2019.00073

Trace Mineral Supplementation for the Intestinal Health of Young Monogastric Animals

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Growth performance and feed efficiency are essential parameters when evaluating profitability of livestock. However, animal performance does not always reflect optimal gut health. Decades of research have supported the theory that improved animal performance such as average daily gain and feed efficiency can be impacted by intestinal health or the ability of the intestinal mucosa to absorb nutrients, but dysfunction may be found when the animal is stressed. Most of the early research focused on enteric infections causing diarrhea and nutritional alternatives to antibiotics which has led to findings related to pharmacological supplementation of trace minerals above the nutrient requirements for non-ruminants. While pharmacological concentrations of copper (Cu) have been shown to enhance growth, the mechanism in the gut is elusive. High concentrations of zinc (Zn) fed to newly weaned nursery pigs reduced the incidence of diarrhea from the proliferation of enterotoxigenic *Escherichia coli* (*E. coli*) and *Clostridium* and improve gut morphology. There are numerous publications where pharmacological supplementation of Zn as zinc oxide (ZnO) were fed to newly weaned pigs. Pharmacological Zn has been reported to shape the intestinal microflora as well as the diversity of the microflora during the first 2 weeks post-weaning. Both Fe deficiency and fortification impact bacterial growth in the intestine. Therefore, this paper will focus on the role of trace minerals that potentially impact optimal gut health of young monogastric animals.

Keywords: monogastric animal, intestinal health, nutrition, zinc, microorganisms

OPEN ACCESS

Edited by:
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Reviewed by:
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University of Arkansas, United States

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *01 October 2018*
Accepted: *19 February 2019*
Published: *13 March 2019*

Citation:
Shannon MC and Hill GM (2019) Trace Mineral Supplementation for the Intestinal Health of Young Monogastric Animals. Front. Vet. Sci. 6:73. doi: 10.3389/fvets.2019.00073

INTRODUCTION

Monogastric animal production and profitability have relied on genetic improvements, meeting nutritional requirements, and animal health in order to maximize growth performance and ensure improved efficiency. For both pig and broiler production, producers have been working for decades to increase efficiency and reduce the cost of production. Siegel (1) reported that broiler feed conversion ratio from 1985 to 2010 has improved by almost 35% from 2.3 to 1.5, respectively. Similarly, data (2) from the swine industry support an improvement in feed conversion from 3.6 to 2.7 between 1986 and 2016. However, it is believed that there are still improvements to be made based on genetic potential and nutritional interventions associated with intestinal microflora of monogastric animals. The diversity of microbiome is important in digestion of animal feeds in the gastrointestinal tract. This was demonstrated by Frese et al. (3) and Bian et al. (4), who characterized the fecal microbiome of nursing or weanling pigs and determined that gut microbial populations are clearly established based on dietary content consumed. De Rodas et al. (5) recently reported

the bacterial microbiome throughout the gastrointestinal tract from birth to market weight to have increased Clostridia and a decreased Gammas proteobacteria with age. Hence, reminding us that the microbiome is not just influenced by feedstuffs but age.

INDUSTRY MANAGEMENT ISSUES

The swine industry is continuing to evaluate herd efficiency and health as related to standard operating procedures. The weaning period on a swine farm is probably the most stressful phase for the young pig which often results in disruptive intestinal barrier integrity due to enterotoxigenic infectious such as *Escherichia coli*, and ultimately a reduction in growth performance for the first few days post-weaning often referred to a post-weaning lag. Moeser et al. (6) reported that weaned pigs at an older age (22–24 d of age) were less likely to have an exacerbated immune response from an *E. coli* challenge when compared to pigs weaned at a younger age (16–18 d of age). Therefore, early-weaned pigs that are stressed during the weaning process may have an altered intestinal microflora and permeability. Additionally, Moeser (7) has reported that the sex of the animal, as well as, weaning age affects gut health; therefore, possibly influencing the permeability of the intestinal lining. He notes that it will "limit or tightly regulate the exposure of environmental antigens (e.g., feed antigens), toxins, and microorganisms to the gut mucosal immune system" (7). At 24 h post-weaning, De Rodas et al. (5) found that relative abundance of Lactobacillaceae was reduced. As important, the introduction of feed from 21 to 33 days of age had more effect on the microbiome than age, changes in type of feed or change in location for the entire study.

Therefore, in order for the swine industry to overcome the weaning stress issues, much research has evaluated the impact of weaning age and weaning weights on growth performance of nursery pigs. Feed additive antibiotics have historically been used to control pathogenic infections in swine production. However, culturally, antibiotic usage for general control of pathogens have been more closely regulated in order to curtail the antibiotic resistance in the human populations (8). Therefore, finding nutraceutical feedstuffs that can alter gut health and also improve animal performance is essential. Since the challenge of today's modern hog operations who maintain an antibiotic free system is the prevention of gastrointestinal disease especially when the production is located in high swine concentration environment.

The intestinal microbiota of young piglets can adapt quite quickly to dietary changes (liquid diet to a solid diet) or other environmental stresses. During this critical time, intestinal pathogens often thrive resulting in observations of diarrhea in newly weaned pigs. So, any nutritional interventions through trace mineral supplementation to alleviate post-weaning diarrhea of newly weaned pigs will ultimately improve gut health and overall animal well-being.

Unlike pig production, broiler production does not have a post-weaning lag period associated with weaning the piglet from the sow, but post-hatch chicks often experience a delay in access to feed and water due to the time between hatching and delivery to the farm. The yolk sac functions as an internal nutritional reserve, but often newly hatched chicks will have decreased growth performance, gastrointestinal development, and immunosuppression due to water and feed deprivation (9–11). Zinc is an essential trace mineral for poultry and required for normal growth, reproduction and development of the enzymatic systems (12). The supplementation of dietary Zn has subsequently been introduced into broiler chickens and has been shown to alleviate the loss of intestinal mucosal barrier function (13). Hu et al. (14) concluded that Zn supplemented at 60 ppm improved growth, intestinal micro-flora, gut morphology, and barrier function in broilers.

COPPER AND ZINC

The biological necessity of trace minerals for growing animals was determined many years ago (15). However, researchers continue to determine the mechanism and impact of their interactions on performance as well as intestinal microbial population. Emphasis in this area of research has increased in recent years due to the increased concern of antibiotic use in livestock production and the need to find alternatives to antibiotics.

The Cu requirement for young nursery pigs weighing 5–20 kg has been established as 5 to 6 mg/kg/d (16). Copper has been reported to provide antimicrobial effects when fed above the requirement in pharmacological concentrations (100–250 ppm). Currently, there is no agreement relative to the mechanism involved, but Shurson et al. (17) reported an acceleration in intestinal cell turnover while Zhao et al. (18) found reduced villus height. Interestingly early work showed that copper sulfate stimulates average daily gain in nursery pigs (19–21) and today many forms of Cu are effective in stimulating gain Spears laboratory (22) reported that the villus height was greater in the duodenum and reduced in jejunum in pigs fed 225 mg Cu as sulfate compared to control pigs (6.7 ppm Cu). Additionally, duodenal malondialdehyde concentrations followed this same pattern. However, hepatic cytochrome c oxidase assembly protein 17 mRNA was less and expression of antioxidant l mRNA greater in the sulfate supplemented pigs compared to the controls. Pigs fed 225 Cu as sulfate or tribasic Cu chloride (TBCC) had greater Cu concentrations in the mucosa of the small intestine than pigs fed 5 ppm Cu., and sulfate resulted in higher concentrations in the mucosal duodenum than pigs fed TBCC. The mRNA of duodenal antioxidant 1 was downregulated in sulfate pigs compared to the control pigs while hepatic Cu transporting β-polypeptide ATPase was upregulated in pigs fed 225 ppm Cu vs. controls (23).

These new laboratory techniques may yield information relative to the mechanism(s) involved in performance enhancement via trace mineral supplementation. However, there is not a consistent response to pharmacological Cu suggesting that various mechanisms and interactions on gut health may be involved.

Pharmacological Cu is frequently fed during the early part of the grow/finish phase at 150–200 ppm. It is interesting to note that in the first dietary phase of grow/finish (44 days post weaning; 63 days of age) the dominant operational taxonomic unit classified as Campylobacter were negatively correlated with body weight in the De Rodas et al. (5) study while being fed 150 ppm Cu that followed 200 ppm Cu in all nursery diets. Campylobacter is a commensal bacterium that is present in pigs at most ages.

Zinc is an essential nutrient for normal development, growth, DNA synthesis and many cellular functions, and the zinc requirement for young nursery pigs weighing 5–20 kg has been established as 80–100 mg/kg/d (16). However, many researchers further evaluating the 3,000 ppm Zn supplementation work by Poulsen (24) observed that higher concentrations of Zn as ZnO may improve young pig growth performance (25) as well as reduce scouring (26, 27). Carlson et al. (28) and Case and Carlson (29) determined that the feeding of pharmacological concentrations of Zn in the form of ZnO at a rate of 3,000 mg/kg/d improves growth performance of newly weaned nursery pigs. The observed improvement in growth performance has mainly been attributed to the decrease in the presence of *Escherichia coli* bacteria count (30). Mechanistically, Carlson et al. (28) observed alternations in the duodenum such as deeper crypts and greater total thickness, as well as increased intestinal metallothionein (MT) concentrations in nursery pigs fed diets supplemented with 3,000 ppm Zn as ZnO. Thus, her work indicates that high concentrations of Zn have an impact on intestinal health. Based on knowledge from human medical research, animal research looked closer as the intestinal pre-infection microorganisms and a decrease in diversity of the gut microbiome after weaning (31). High concentrations of dietary ZnO have been shown to be beneficial for maintaining the stability of the intestinal microflora, to support a large diversity of coliforms in weaned pigs (32), and to reduce the susceptibility of the pigs to *Escherichia coli* infections (33). Li et al. (34) confirmed these findings when feeding 3,000 ppm Zn as ZnO to 21 d-old weaned pigs resulting in increased mucosal thickness and villous width of the small intestine.

Vahjen et al. (35) determined that 40–42 d old pigs showed no changes in the order level of ileal microbiome when fed 3,000 ppm Zn as ZnO, but genus level changes were observed. For example, *Streptococcus* increased while *Sarcina* decreased. In addition, Li et al. (34) reported no effect of pharmacological concentrations of Zn as ZnO supplementation for nursery pigs on the number of *Enterobacteriaceae*, *Clostridia*, and *Lactobacilli* in ileal digesta and feces. In contrast, Broom et al. (36) and Jensen-Waern et al. (37) found that pharmacological concentrations of Zn as ZnO reduce fecal counts of *Lactobacilli* and *enterococci* during the post-weaning period of pigs, but only temporarily. In agreement, Hojberg et al. (38) reported that feeding weaned piglets 2,500 ppm of Zn as ZnO reduced the MRS counts (lactic acid bacteria) and Rogosa counts (*lactobacilli*) for all segments of the gastrointestinal tract. Impact of ZnO fed at pharmacological concentrations on the microbiome cannot be determined in the De Rodas et al. (5) research since 3,500 was fed the first 8 days after weaning and 2,000 was fed from days 8 to 22 post weaning to all pigs.

Studies have shown that *Lactobacilli* are considered to have beneficial effects on human and animal health (39, 40) due to its antimicrobial activity against microbial pathogens (41). *Lactobacilli* are among the earliest bacteria to colonize the gut (41). The populations of *Lactobacillus* are thought to be found in high populations in weaned pigs. However, De Rodas et al. (5) reported that *Lactobacillaceae* were reduced 24 h after weaning. Studies *in vitro* and in animals have shown that *lactobacilli* may prevent *Escherichia coli* from colonizing in the jejunum and produce substances directed against the enterotoxins resulting in an inhibition of *Escherichia coli*-induced enterotoxin reactions (42–44). In agreement, Conway (45) and Chan et al. (46) studying the concept of competitive exclusion of pathogenic *Escherichia coli* by lactobacilli in the intestine and urinary tract *in vitro*, respectively, found that the colonization of the *lactobacilli* sterically hindered the adhesion of *Escherichia coli* to the surface. Importantly, Sawai (47) reported ZnO inhibits *Staphylococcus aureus* and *Escherichia coli* growth in the intestine. Thus, providing another mode of action of ZnO fed in pharmacological concentrations.

Roselli et al. (48) using cell culture techniques reported that ZnO may protect intestinal cell from *Escherichia coli* infections by inhibiting the adhesion and internalization of bacteria, preventing the disruption of barrier integrity, and modulating cytokine gene expression, but not by a direct antibacterial effect. Feed grade sources of ZnO vary in color, texture, content, and processing method. Also, ZnO sources tested by chick assays ranged in bioavailability from 37 to 93% based on weight gain and tibia Zn (49). Mavromichalis et al. (50) observed that nursery pigs fed ZnO sources with either high (95%) or low (35%) bioavailability did not affect the growth performance and gut morphology during the entire 21-d assay.

Olukosi et al. (51) reported in broilers that form and amount of Cu and Zn affected performance, percent of breast meat, and concentration of hepatic Cu. As seen by Carlson et al. (28) in pigs, villus height and the villus height to crypt depth ratio were higher in the duodenum when broilers were fed a ZnO source. This data suggests less intestinal permeability when utilizing *ex-vivo* Using chambers; Hu et al. (14) observed reduced colonic permeability to mannitol and inulin.

In the pre-ruminant calf, Jenkins and Hidiroglou (52) showed that 700 and 1,000 ppm Zn reduced weight gain, feed intake and efficiency compared to intakes from 40 (NRC recommendations) to 500 ppm. Dosing calves with 40 g Zn/d resulted in neonatal calves recovering from diarrhea 1 day earlier than controls (53). Perhaps these findings are a result of the observations of Rodriguez et al. (54) in guinea pigs and shigellosis infected children (55) that the increased intestinal paracellular permeability observed during fasting and malnutrition is prevented by pharmacological Zn. Additionally, Zn has been reported to promote epithelialization and anti-infective in wound healing (56).

The phytate component in feed ingredients is reported to affect Zn solubility and gut pH (57). Additionally, when pharmacological Zn and Cu are fed, the Zn: phytate and

Cu: phytate ratios are altered. Soluble complexes are present at intestinal pH values when the ratio exceeds 10:1, but insoluble complexes are formed when more than one divalent cation per phytate is formed. Hence, the observed effect of pharmacological Zn and or Cu on phytase activity (58) may explain the observed variation in performance outcomes when pharmacological Zn or Cu is fed to pigs. In broilers, Morgan et al. (59) reported that phytase activity impacted Zn concentration in the gizzard and ileum but not the duodenum indicating the importance of not making assumptions on Zn metabolism between species.

Hill's laboratory (60–62) has explored many of the Cu and Zn enzyme's activity and associated gene expression, but few swine and poultry researchers have determined Zn and Cu biological signals (63) such as transporters even though it has been shown in humans that dietary Zn intake influences Zn transporters in the plasma membrane of the intestine (64).

OTHER IMPACTS ON INTESTINAL HEALTH

The physical process of weaning pigs from the sow regardless of piglet age has been characterized as the decrease in intestinal barrier function of the gut in the newly weaned pig resulting in decrease pig performance caused by increased intestinal permeability creating alternations in intestinal microbial populations as well as inflammation (65). There are several layers of protection in the intestinal lining against pathogens and toxins. The protection is very important during weaning in a baby pig's life due to the physiological stress of separation from the sow and converting to a dry grain based diet. During weaning, villus height decreases and the intestinal lining becomes more susceptible to pathogens causing reduction in growth performance through lower nutrient absorption. A practical solution for the swine industry for years has been using feed-grade antibiotics; however, more recently other dietary nutritional interventions have been researched such as feeding higher concentrations of trace minerals zinc and/or copper.

As noted earlier, Zn has been known to be essential in many biological functions of mammals, such as anti-inflammation, anti-diarrhea, and maintaining epithelial barrier integrity (48, 66). Feeding pigs' dietary concentrations of Zn as ZnO greater than the requirement has shown to impact intestinal morphology of growing pigs (28). Subsequent research supports similar findings with feeding Zn to growing pigs improves intestinal microflora and barrier function (67). Intestinal counts of *Clostridium* and *Escherichia coli* in the intestinal segment of the jejunum decreased linearly when nursery pigs were fed dietary concentrations of zinc (67).

Heat, crowding (68), and weaning are stressors (69) known to negatively impact intestinal health, feed consumption and weight gain. Sanz Fernandez et al. (70) reported that pigs exposed to 36°C with ∼50% humidity demonstrated increased ileal and colonic permeability that was decreased by feeding a diet containing 220 ppm Zn. It is not clear if the effect on the gut health is totally due to reduced feed consumption, change in diet form, or decreased energy because husbandry practices often confound the research findings (71, 72).

IRON AND MANGANESE

The Fe requirement for young nursery pigs weighing 5–20 kg has been established as 100 mg/kg/d (16). Iron is an essential nutrient needed for hemoglobin in red blood cells where most of the body's Fe is found. However, newborn pigs are unique because of their rapid growth and low concentration of Fe in milk and hence need supplementation of 100–200 mg of injectable Fe in the first 3 days of life (19). Before gut closure, these newborn pigs can very efficiently absorb Fe from the intestinal mucosa (73). Oral Fe within the first few hours of life can be absorbed, but must be administered before gut closure to large molecules. Interesting, research with humans suggests that there is a lack of Fe homeostasis in young infants (74). In rats before weaning, pups cannot regulate Fe homeostasis regardless of Fe status, but 10 days later Fe transporters (DMT1 and ferroportin) in small intestine were affected by Fe status. There is no data to suggest if this occurs in pigs and poultry.

Lactoferrin is absorbed across the intestinal cell via the lactoferrin receptor and is found in colostrum, milk, saliva, tears, and nasal secretion as part of the immune system. Its roles include antimicrobial, immunomodulatory, and Fe binding. The number of eosinophils is higher in the intestine of healthy pigs. The migration of eosinophils, which increase during inflammation, is inhibited by lactoferrin. The receptors of lactoferrin in the pig are found on the duodenal brush border of villi, crypt, and within the lamina propria (75) Hence, this important Fe binding protein not only is important in Fe absorption in the duodenum but in controlling bacteria in the gut. The human small intestinal lactoferrin receptor has been cloned in the pig (76).

Iron toxicity has been shown to occur at 600 mg/kg of Fe in 3–10 d old pigs. In addition, growing-finishing pigs only require 40–50 mg/kg of Fe, but typically growing pig diets will contain four to five times more Fe than required. The excessive concentrations of Fe in commercial swine diets, that may be unavailable to the animal, is due to differing Fe bioavailability of Fe in dietary ingredients such as blood meal, dicalcium phosphate, and limestone (16, 77).

There is an interdependency of the transport mechanisms and regulation of Mn and Fe, two transition elements. Both utilize transporters especially divalent metal transporter-1 (DMT-1). Mammals will have abnormal accumulation of Mn when Fe is low in the diet/body and if Mn is excessive or low, Fe homeostasis is altered. For example, Hansen et al. (78) reported that pigs fed high Fe diets had lower gene expression of Fe encoded proteins in the liver (Hepcidin) and duodenum (DMT1) as well as Mn was lower in liver and greater in duodenum. These results indicate that dietary Fe supplementation may impair absorption of Mn, but not Cu and Zn.

The acidic environment of the stomach and effect of diet usually result in the Fe that reaches the stomach to be in the

ferrous form, but as the pH increases and ferric Fe solubility decreases. Most Fe is absorbed in the duodenum, but the remainder goes to the colon where it is utilized by bacteria. There is the potential for limited Fe absorption from the colon. Rats treated with antibiotics had decreased absorption of Fe (79).

Iron is required for most bacteria to flourish since it serves as a co-factor in re-dox reactions, metabolic pathways and of course the electron transport chain reactions. A few bacteria such as *Borrelia burgdorferi* that causes Lyme disease use Mn instead in proteins requiring Fe. Most bacteria have increased viability in the presence of Fe, and an excess is believed to exacerbate most gut infections.

The research data associated with iron supplementation and the impact on gut health is inconclusive at best. Iron is in abundance and often not considered deficient or limiting in today's animal production. There is research that supports Fe supplementation and increases the presence of beneficial microbiota that may improve the overall gut health of the animal (80, 81). However, in a review, Lönnderdal (82) reminds us that excessive Fe has a negative effect on growth and microbial health of the gut if animals or humans had adequate Fe before supplementation. There was an increase in the proportion of anaerobes except when Fe supplementation was high. It is thought to occur because high Fe results in an increase in free reactive Fe that induces free radical damage in the gastrointestinal track by release of oxygen by Haber-Weiss reaction. This increase in oxidative stress will decrease the strict anaerobes.

The interaction of Fe and Mn may be the most important information that is known about Mn on gut health. In humans and experimental animals, it has been shown that Mn is not well-absorbed, and it appears from experimental animal studies that fiber, phytic acid, oxalic acid, Ca and P reduce its availability for absorption (83). Perhaps more importantly, Mn and Fe compete for binding sites that effects absorption and ultimately body stores (84).

It is believed that Mn absorption in poultry is less than pigs, but there is no definitive research with today's genetics in either species. Using polarographic analysis, solubility in buffers, and deionized water, Li et al. (85) reported that bioavailability of five different organic Mn sources was closely related to chelation strength. While organic Mn has been promoted to be of value in preventing leg abnormalities, this incidence of swelling of the tibia-tarsal joint abnormality is not prevented by Mn dietary supplementation. This broiler problem is more severe if poly-unsaturated fatty acids are added to the diet for growth promotion.

When inorganic and chelated minerals (Cu, Zn, Fe, Mn) fed at reduced dietary concentrations were compared to control fed pigs from weaning to finishing, reduced mineral concentrations regardless of source did not affect performance but resulted in reduced fecal excretion (86). Besides the role of Mn in metabolic functions of enzymes, supplementation of Mn has been reported to result in greater lean color scores and more vivid red color in pork chops when provided as a sulfate but not an organic form (87). This might indicate that absorption differed perhaps due to valence changes.

CONCLUSION

Health of the gut is reflected in the performance of the animal from hatch/birth to death. Additionally, changes in the gut's morphology may not provide a means of evaluation of gut health. However, it is currently being used with the *ex vivo* studies with Using Chambers that determine the capacity of the intestine to transport nutrients. Hence, the techniques and technologies currently being used in research today will give additional information lacking in today's literature. Clearly, the published National Research Council's nutrient requirements for specific species should only be used a guidelines. However, researchers have determined that any disruption of the gastrointestinal tract will impact animal performance, and supporting the antioxidant system by the usage of trace mineral supplementation will ensure intestinal microbiota health and repair. In conclusion, much of the results of positive and negative attributes of trace mineral supplementation on gut health are influenced by genetics, production goals, and the environment.

AUTHOR CONTRIBUTIONS

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

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

frontiers in Veterinary Science

REVIEW
published: 21 March 2019
doi: 10.3389/fvets.2019.00082

Early Nutrition Programming (*in ovo* and Post-hatch Feeding) as a Strategy to Modulate Gut Health of Poultry

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Healthy gastrointestinal tract (GIT) is crucial for optimum performance, better feed efficiency, and overall health of poultry. In the past, antibiotic growth promoters (AGP) were commonly used to modulate the gut health of animals. However, considering the public health concern, the use of AGP in animal feeding is banned or regulated in several jurisdictions around the world. This necessitates the need for alternative nutritional strategies to produce healthy poultry. For that, several alternatives to AGP have been attempted with some success. However, effective modulation of the gut health parameters depends on the methods and timing of the compound being available to host animals. Routinely, the alternatives to AGP and other nutrients are provided in feed or water to poultry. However, the GIT of the newly hatched poultry is functionally immature, despite going through significant morphological, cellular, and molecular changes toward the end of incubation. Thus, early growth and development of GIT are of critical importance to enhance nutrients utilization and optimize the growth of poultry. Early nutrition programming using both *in ovo* and post-hatch feeding has been used as a means to modulate the early growth and development of GIT and found to be an effective strategy but with inconsistent results. This review summarizes the information on *in ovo* and post-hatch-feeding of different nutrients and feeds additives and their effects on gut development, histomorphology, microbiology, and immunology. Furthermore, this review will provide insight on the future of early nutrition programming as a strategy to enhance gut health, thereby improving overall health and production so that the poultry industry can benefit from this technique.

Keywords: broilers, gut health, histomorphology, immune system, *in ovo* feeding, post-hatch, nutritional strategy, poultry

OPEN ACCESS

Edited by:
Pietro Celi,
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Reviewed by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *28 January 2019*
Accepted: *27 February 2019*
Published: *21 March 2019*

Citation:
Jha R, Singh AK, Yadav S, Berrocoso JFD and Mishra B (2019) Early Nutrition Programming (in ovo and Post-hatch Feeding) as a Strategy to Modulate Gut Health of Poultry. Front. Vet. Sci. 6:82. doi: 10.3389/fvets.2019.00082

INTRODUCTION

Poultry production has increased at a faster rate than any other livestock animal globally. Among others, the nutritionally balanced-feeding program along with antibiotic growth promoters (AGP) in poultry diets played a significant role in achieving this success. However, the poultry industry is under pressure to redefine its nutrition program to grow safe and quality meat in the light of

public health concern due to the use of AGP in poultry diets. Maintenance or improvement of gut health is essential for optimum growth, better feed efficiency, and overall health of poultry (1). Also, a healthy gut is critically important for the efficient conversion of feed into absorbable form for optimal nutrient utilization, thereby better growth performance of poultry.

Gut health covers the efficient nutrient utilization, macro- and micro-structural integrity of the gut, the stability of the microbiota, and the status of the immune system (2, 3). Moreover, gut health is a complex field combining nutrition, microbiology, immunology, and physiology of animals. When gut health is compromised, digestion, and nutrient absorption are affected (3), which in turn, may have a detrimental effect on feed efficiency and greater susceptibility to diseases leading to economic loss. In addition, recent regulatory changes on the use of antibiotics, different feed requirements, and more feed-efficient animals highlight the need for a better understanding of the gut function and overall gut health. Therefore, understanding and improving gut health by different nutritional strategies are becoming a reality in the monogastric animal industries, especially when antibiotics are not allowed in food-animals feeding program (3–5).

Chicks have been shown to benefit from early access to feed and water. A healthy 1-day-old chick is a crucial link between the hatchery and the broiler farm. The delayed intake of water and nutrients to chicks could lead to a diminishing of their overall growth performance with adverse effects on breast meat. The most extreme consequence of delayed feeding is increased mortality (6). Early feeding strategies have been suggested and developed to diminish or possibly reverse the negative effects of delayed feeding. These strategies range from *in ovo* feeding to specially designed post-hatch diets (7–9). The importance of early nutrition and its effect on growth performance and different components of gut health (histomorphology, microbiota, and immune system) have already been extensively studied in the last two decades (10–15). Some studies have gone in detail about specific nutrient supplementation and its effect on the host. For example, probiotics supplementation in early life prevent pathogenic infections, amino acids (L-arginine, L-lysine, L-histidine, threonine) are beneficial in growth performance, vitamin C and E boost immunity, carbohydrates increase glycogen stores, and creatine supplement promotes muscle growth (16). Also, the *in ovo* injection of sulfur-containing amino acids (methionine plus cysteine) in the embryonated eggs exposed to heat stress have positive effects on gene expression and antioxidant indices as well as reduce the lipid profile of newly hatched broiler chicks (17). This paper has reviewed the current state of knowledge on *in ovo* and post-hatch-feeding as a strategy to enhance the gut health of poultry. It emphasizes on the effects of different nutrients on intestinal histomorphology, microbiota, and immune system using *in ovo* or post-hatch feeding system. The paper further provides the potential application of the current knowledge for the advancement of the poultry industry. Also, it has highlighted current limitations and future potential and research needs for effective use of early feeding in birds.

IMPORTANCE OF EARLY FEEDING

The perinatal period spanning from late-term embryo to few days post-hatch is an important period for the development of the gastrointestinal tract (GIT) and the immune system of poultry. Unlike mammals that can influence the development of fetus even after parturition, avian species can leverage expression only through the composition of the egg. Due to this restriction, all necessary nutrients, growth factors, and the machinery needed for the development are required to be present in the fertilized egg. Also, because of a rapidly growing embryo and the fast-metabolic turnover of modern chicks, some of the essential nutrients can become depleted or insufficient during adverse environment and disease challenges. This constraint of nutrient reserves may limit the maximal development and growth of newly hatched chicks. The availability of essential nutrients can be improved, and the existing challenges can be overcome to some extent by providing early nutrition to the embryos and chicks. Early nutrition or feeding in poultry production is a concept of providing the required nutrients to the birds either during the period when the embryo is developing or immediately after hatch until they attain a fully matured digestive system (8).

A prominent early nutrition technique that could provide further opportunity to influence the development of a chick inside the egg and overcome the constraints of nutrient limitation during late incubation phase is *in ovo* feeding (IOF). This technique provides the opportunity to supply essential nutrients, neutraceuticals, and functional foods to uplift the status of growth and development of the embryo. Several routes including amniotic (8, 18), yolk sac (19, 20), and air sac (13, 21) have been used. Amniotic fluid in the amnion surrounds the developing embryo and is believed to provide mechanical protection, prevent desiccation, and adhesion of the embryo (22). The amniotic fluid also contains protein, minerals, hormones, water, and other nutrients needed for growth and development of the developing chicks which begin to imbibe it around d 13 of incubation until internal pipping (23). This natural phenomenon of consumption of amniotic fluid by the late-term embryo toward hatch provides opportunities to add various forms of essential nutrients that would ultimately reach the GIT of chicks. Nutritional substances injected into the amniotic cavity are ingested and get deposited in the lungs and intestine due to the rhythmic respiratory movements of the late-term embryo (18, 24). *In ovo* feeding can also be used to provide adequate nutrients to the late-term embryo to protect it from the negative effect of starvation during the extended window of hatching (25). Afsarian et al. (26) found that *in ovo* injection of thyroxine along with manipulation of the eggshell temperature decreased the mortality rate occurring due to cold-induced ascites and improved chick quality and post-hatch performance. Recently, Yang et al. (27) observed that IOF of creatine pyruvate increased glucose concentration in thigh muscles of neonatal broilers, which suggests that the energy metabolism can also be altered in embryo and chicks by *in ovo* injection of different bioactive compounds. Moreover, *in ovo* application of prebiotics can be advantageous as it has

been reported to increase the numbers of beneficial bacteria and promote their early colonization in the intestine of neonatal chicks (28). Likewise, investigators are interested in inoculating eggs with probiotics and synbiotics as they can be used in small amount and provide improved immunity and a better post-hatch resistance against pathogens (29, 30).

Perinatal period is also very critical as the chicks must adjust to the nature of the changing nutrition mostly from yolk-based lipid diet to carbohydrate-based solid feed. It has been observed that chicks are more efficient in utilizing lipid following hatch and gradually gain the capacity to absorb more hexoses and amino acids (31, 32). The transition of a chick from nutrient utilization from embryonic reserves to feed forces for adjustment of the newly developed digestive system. Early post-hatch feeding is essential not only for normal growth and development but also for maintaining homeostasis. Early feeding of chicks can provide readily available energy to assist in restoring hepatic glycogen stores and maintain high body temperature during initial post-embryonic days (33). In contrast, higher inclusion of anti-nutrient like non-starch polysaccharides during the early growth period can deteriorate the feed efficiency and overall productivity of growing chickens (34). Due to the practical perspective of feeding program and due to limited information on the early nutritional requirement, poultry hatchlings (e.g., chicks, ducklings, and turkey poults) are fed starter diet after hatching till 2–4weeks (34–36). Several investigators have reported that chicken weight at 6–7 weeks had the linear relationship with their weight in the first week (37) and it was not due to the breeder age and day-old chicken weight (38). The pre-starter feed could be more expensive than starter feed, but such feeding last only for a shorter period of 3–4 days and has more favorable effect on the performance of birds (39). Broiler feeding in the first few days of life is one of the priorities that could affect growth, feed efficiency, uniformity, and finally the profit of farmers. Nutrient utilization in chicks at an early stage is dependent on digestion and absorption of nutrients in the GIT (40). Improved performance has been observed in broilers by feeding pre-starter containing carbohydrate and fat during the first hours of chicken life (39, 41). Some pre-starter diets are prepared with more focus on digestible nutrients than the total requirements, and it can precondition the chick to later digest complex substrates once they acquire matured enzyme production in the GIT (7, 42). Since highly digestible alternative substrates tend to be expensive, the use of different enzymes combination or the higher activity of enzymes than those applied in later phases of diet could improve the productive performance of birds. However, limited work has been conducted to date to estimate the nutrients requirement of first week chicks that could outperform in terms of market weight and disease resistance compared with those fed starter diet (43, 44).

The GIT, especially the small intestine of poultry has the highest post-hatch relative growth during the first week growing period (45, 46). Therefore, an early feed deprivation can lead to a decreased intestinal enterocyte length and villus surface area which negatively affects nutrient utilization and growth (47). Early feeding is expected to influence immune development either by providing nutrients for cell proliferation and differentiation or by providing substrates for antigenic and immunomodulator activity leading to the production of several immunoglobulins (48–50). It is understood that early access to nutrients is essential for a sound immunity and improved health of chicks and poults (51). By managing a proper nutritional strategy, a specific stimulus can be generated to guide this immune system toward a more appropriate and desired direction. The requirement of enhanced immunocompetence becomes exceptionally important in the view of reducing the dependence on antibiotic growth promoters (AGPs). One of the suitable alternatives to feeding AGPs can be a supplemental chicken cytokine (52). Also, early access to feed supplemented with mannanoligosaccharides and acidifier have been reported to improve the development of intestinal morphology and immune response of chickens to *C. perfringens* challenge compared to early feed restricted chicks (10). Recently, in a study on nursery pigs, Tiwari et al. (53) reported that the use of NSP degrading enzymes modulated the production of tight junction proteins that maintain the intestinal barrier function and hence can prevent the permeability of the gut to invading pathogens. However, Mateos et al. (54) reviewed literatures on the effect of early feed access or restriction and dietary changes in the prestarter diet and concluded that the difference in the productivity tends to disappear with increasing age of birds. Still, there is a scarcity of information on early nutritional modification and most of the studies until now have focused on early access of chicks to feed. Further research is warranted to determine if early prestarter diet would be worthy of optimizing metabolic homeostasis in poultry for prospective maximal growth and feed efficiency.

NUTRITION AND GUT HEALTH IN POULTRY

The term "gut health" is a very comprehensive topic that requires a holistic tactic involving nutrition, gut physiology, microbiology, and immunology. Nutrition and health are interdependent, and the interface between the two occurs largely in the gut. One of the most effective ways to influence poultry gut health is through nutrition programming. In fact, the early nutrition programing by the introduction of feed and water to the chick provides a good means to feed the gut. By doing this gut, development is most favorable. This will ensure the birds are better equipped to cope with the gut challenge. There are several nutritional strategies that can be adopted to influence gut health. In this section of the review, some prominent factors affecting intestinal health are briefly presented.

Diet Formulation

A balanced diet formulation to match nutrient content of feed with the nutrient requirements of the birds is considered as one of the most important aspects of the animal feeding program. In this respect, poultry diets should be formulated based on digestible

values instead of "total" values in order to maximize protein digestibility. The 'Balanced Protein' (or ideal amino acid profile) concept for all the essential amino acids should be applied. The emphasis is not on minimum crude protein but rather on the proper balance or ratio of each amino acid to lysine. The use of synthetic amino acids will be beneficial and can help to prevent excess crude protein. An excess of indigestible protein in the hindgut can predispose to several intestinal challenges (e.g., wet litter).

Starch Properties

Among the nutrients in poultry diets, starch is the most important nutrients and main source of energy in the broiler's diets (may contain up to 50% starch on a DM basis). Different cereal grains (wheat, corn, sorghum, rice) have different starch characteristics and physico-chemical properties. For example, wheat contains high levels of non-starch polysaccharides (NSP) such as β-glucans and arabinoxylans (55). The physico-chemical properties of NSP are responsible for their antinutritive activities in the broiler chicken, especially soluble viscous NSPs, which decreased the digestibility of protein, starch, and fat. In addition, high viscosity of the digesta has been shown to cause digestive and health problems, decreasing digeta passage rate, digestive enzymatic activities and nutrient digestibility, depressed feed efficiency, and growth rate of the birds (56) in diets based on barley, wheat, rye, or oats (high levels of NSP). On the other hand, insoluble and non-viscous NSPs may have a beneficial effect on gut health (57). Others NSP properties, such as resistance to the animal's digestive enzymes also promote to create a viscous environment within the intestinal lumen, resulting in excretion of sticky droppings (58). In addition, the rate of starch digestion can favor the growth of *Clostridium perfringens*. Toxins produced by *Cl. perfringens* are responsible for necrotic enteritis (10). Therefore, the application of appropriate xylanase enzymes is necessary to enhance digestion of starch and gut health because they have the ability to break down NSP and reduce digesta viscosity, increase digesta passage rate, and improve bird performance (59–61)

Physical Texture and Form of Feed

The physical form of the raw materials used in broilers diets may affect the morphological and physiological characteristics of the entire intestinal tract (62, 63), although available published works in this area of research are inconsistent. Small grain particles give a larger surface area, and this may give rise to rapid feed passage in the gut. However, many studies (64–66) have demonstrated that larger particles promote gizzard development and activity, and, consequently, feed is retained longer in the gizzard, and its particles are more uniform when pass to the small intestine, favoring their digestion. In addition, larger particle size of feed lead to longer feed retention time in the gut; this helps to release the starch entrapped in cereal grain cells and encourage more beneficial bacterial fermentation in the caeca. Therefore, the target for particle size should be 800–1000 microns. In this respect, feeding broiler chicken with whole wheat could reduce the numbers of *Salmonella typhimurium* and *Cl. perfringens* in the GIT of the birds (63, 67). In addition, the inclusion of whole wheat into the broiler diets increased growth rate and feed efficiency of the birds (68). In contrast, Svihus et al. (69) did not find significant effects of diets containing whole wheat on body weight gain and feed efficiency. However, the authors reported that the birds fed with whole-wheat diets were more efficient and the nutrients were better digested and absorbed than the birds fed with ground wheat diets. The authors suggested that the improvements in the digestion might result from the increased pancreas and liver secretions. Svihus et al. (70) compared the inclusion of ground or whole wheat on on passage rate through the anterior GIT, and reported that although the gizzard has high capacity for processing diets with whole wheat, the average passage rate for a diet through the gizzard does not seem to be affected by the form of the wheat. Based on these results, it can be concluded that when the GIT is healthy, the inclusion of whole wheat into the diet may help to improve gut development and utilization of feed nutrients and consequently broilers performance, but when the integrity of the GIT is impaired, the inclusion of whole wheat into the diet may decrease the performance of the birds. Also, the inclusion of whole wheat in diet will reduce the energy consumption for grinding and the final feed cost of the diets.

On the other hand, use of a hammer mill to grind grains is still acceptable for the production of broiler feeds, but it may be best used with a 6-mm sieve size and a rotation speed of 750 rpm (71). A roller mill will give a more even distribution of particle size and hence better overall uniformity. In addition, a roller mill tends to produce a sharp-edged particle with less dust while a hammer mill produces a more rounded particle, which results in considerably more dust and more durable pellets. The sharper edged particles produced by roller mills may provide physical stimulation of the gut lining and therefore lead to better gut health (72).

EFFECTS OF EARLY NUTRITION ON HISTOMORPHOLOGY

The GIT tissue acts as a physical and immunological barrier to the harmful chemicals and infectious agents that enter the host. It also provides a path to the nutrients for proper digestion and absorption (5). Yegani and Korver (5) also found that healthy gut morphology directly affects the metabolism of nutrients, disease resistance, and immune response by the host. Diet intake exposes the GIT to the external environment whose quality, quantity and timing largely affect the delicate balance between the host, diet and gut ecology. So, feed ingredient used in the diet should be favorable to host gut structure and commensal microbiota (1). In the adverse gut environment, birds are at high risk of developing necrotic enteritis, coccidiosis, and other toxin-producing pathogens. Gut histomorphology is one of the most commonly used parameters to diagnose the status of gut health.

The effects of early nutrition on histomorphology have been studied in the last decade (13, 15, 73). It is well-documented that the first days after hatch is a critical period for the development of the mucosa because of major change occurring in the source of nutrients as the yolk is replaced by an exogenous diet. The growth of the chickens is directly linked to the digestion and absorption of nutrients, which is a result of the morphological and functional development of the small intestine. Villus height, crypt depth, and villus height/crypt depth ratio, crypt proliferation, rate of enterocyte migration, mucosal enzyme activity and goblet cell development are good indicators for the functional capacity of the intestine (74). It is well-accepted that a deeper crypt is indicative of faster tissue turnover and, perhaps, higher demand for new tissue (53, 75). Furthermore, it has been reported that a high intestinal villus is associated with a well-differentiated intestinal mucosa with high digestive and absorptive capabilities (75). A meta-analysis study done for the effect of post-hatch feed and water deprivation (PHFWD) shows significant subnormality in the small intestine segment with reduced length and relative weight of duodenum, jejunum, and ileum, and shorter villus height and crypt depth during the first week of age (74). Therefore, *in ovo* and post-hatch feeding strategies should be taken into account as a strategy to modulate the gut health of poultry.

Early nutrition is a stimulus to the early development of GIT, early absorption of the yolk sac, improved performance and the health status of the birds later in life. In the case of PHFWD, chicks have significantly lower yolk sac weight at 3 days post-hatch than on the day of hatch (74, 76). reported that *in ovo* feeding on 17–18 day of incubation is functionally equivalent to 2-day-old bird intestine with the increased size of villi and increased capacity to digest and absorb disaccharides leading to greater body weight compared to control birds fed in conventionally. However, in the commercial setup newly hatched chicks do not get access to feed and water for at least 12–36 h due to the inevitable operation of the hatchery, sex determination, vaccination, and transportation to the production farms. There are many reports on the negative effects of post-hatch fasting period on the intestine function and growth performance in the long run (77, 78). Fasting could further increase the susceptibility to infection and compromise immunity leading to an increase in the production cost of chickens (33). In the post-hatch period, there is the rapid development of intestinal length, weight, and its enzymatical activities, where delay in feeding causes a reduction in development and expression of nutrient transporters affecting absorption of nutrients (5). Apajalahti et al. (79) relate dietary factors and their interaction with a microbial profile to find out the effects on the intestinal development, mucosal architecture, and the mucus composition of the GIT. Pre- or post-hatch intestine morphological development is also very critical to digest, assimilate and absorb nutrients from the gut. Researches by Biloni et al. (80) and Adeleye et al. (77) found that fasting (feed and water) birds for 24 h negatively affects the morphology of the gut with a decrease in duodenal and jejunal villus height compared to birds fed 4-h post-hatch.

Feeding a blend of perinatal supplement and probiotic supplement shows increased villus height, villus width, villus to crypt ratio, and villus surface area along with an increase in overall body weight supported by competitive exclusion of *Salmonella* (80). *In ovo* injection of prebiotic inulin has shown to increase the villus height on the first day and it is also known to increase mucin production to protect epithelial cells in the gut (81). Another research by Madej and Bednarczyk (82) found that *in ovo* inulin injection helps proliferate lymphoid tissue by T cells but no any effect on gut-associated lymphoid tissue. Berrocoso et al. (13) found some interesting results showing *in ovo* injection of 4.5 mg raffinose significantly affected the CD3 and ChB6 genes which are associated with the activity of T cell and B cell. The authors also found a linear increase in villus height and villus to crypt ratio of post-hatch birds with the increase in dose of raffinose. *In ovo* injection prebiotic galacto-oligosaccharides increased the overall body weight of broilers on 5th week due to its activity on sodium-dependent glucose co-transporters in the gut helps in monosaccharides absorption (83). The authors also found an increase in pancreatic secretion of trypsin and amylase on embryonic day 21 and day 7 post-hatch. *In ovo* injection of synbiotic preparation increases the number of goblet cells in the jejunum and ileum (84). These goblet cells produce mucus and protect the gut epithelium. The author also found an increase in width of the duodenal villi and crypt depth of 21-day old chickens fed *Laminaria spp.* as *in ovo* preparation. In conclusion, we can claim that early dietary intervention impact on all aspect of gut health such as gut microbial ecology, gut epithelium and immune system leading to benefit the overall growth performance of the chickens.

EFFECTS OF EARLY NUTRITION ON GUT MICROBIAL ECOLOGY

The confounding performance of modern chicken to gain body weight by 25% in a day by newly hatched chick and 5,000% by 5 weeks to 2 kg makes it more demanding to meet the nutrient requirement along with the enhancement of the microbial function to improve immunity, digestive and absorptive function to overall improvement of the bird growth (2). The established protective intestinal microbiota is very stable, but it can be influenced by different dietary, disease, and environmental factors. For example, feed additives (antibiotics, coccidiostats, buffers, or acidifiers that influence gut pH), disease and hygiene conditions (clean vs. dirty environment, pathogen load in the feed ingredients, humidity of the shed, litter type, and usage), and stress (change of feed, sudden disturbances, heat, or water stress) could also affect gut microflora. However, diet is perhaps the most critical factor influencing the gut microflora (1, 85). Young animals are more affected by dietary manipulations such as its composition, processing, digestibility, and feeding method may disturb the balance in the gut ecosystem, especially in young animals (79, 86–89). For example, corn and sorghum-based diet increased *Enterococcus*, barley-based diet increased *Lactobacillus*, oat increased *Escherichia* and *Lactococcus*, and

rye-based diet increased the *Streptococcus* (90). Researches have shown that the gut immunity changes with the changes in the gut microbial activity. Newly hatched chickens are very prone to colonize their gut by pathogenic bacteria as they provide near-sterile and suitable environment and continues to establish a relatively stable ecology with the animal age. Here interaction between microbiota and the host plays a deciding role to determine the future ecology and stability of such microbiota in the gut. Pourabedin and Zhao (91) explained this shift of Clostridiaeae and Enterobacteriaceae as dominant ileum microbiota families on day 7 to Lactobacillaceae and Clostridiacea on day 35. Apajalahti et al. (92) showed that bacterial densities one-day post-hatch in the ileum and cecum of the broiler reaches 10^8 and 10^{10} cells per gram of digesta, respectively, and attends optimum levels of 10^9 and 10^{11} per gram of ileal and cecal digesta, respectively, by 3 day post-hatch. This bacterial density remains stable for further 30 days or so in broiler chicken. Supplementing prebiotics in the diet at an early stage of life increases the abundance of *Lactobacilli* and *Bifidobacterial* and suppress *coliform* (93). Whereas, a study by Villaluenga et al. (94) found that *in ovo* administration of symbiotic on day 12 or 17 of incubation, increased the number of bifidobacteria in the post-hatch period. Another study by De Oliveira et al. (29) injecting *in ovo* probiotic preparation of *Enterococcus faecium* bacteria reduced the number of *Salmonella enteritidis* positive chicks post-hatch. This indicates that *in ovo* colonization of probiotics is potential enough to post hatch bacterial infection. In general, prebiotics stimulates the microbiome when administered *in ovo* at the early age of around 12 days and later probiotic at 17/18 days of incubation causes competitive exclusion. Thus, early beneficial microbiota colonization is very critical for the proper growth of chickens (80). Many hi-tech molecular techniques are used to study the gut microbiota; high-throughput sequencing is the most common one, along with targeted amplicon sequencing followed by shotgun sequencing, metaproteomics. These techniques have some limitations regarding characterizing the functional activity of gut microbiota, thus the holistic approach using multiomics might help to better understand these gut microbiota (85).

EFFECTS OF EARLY NUTRITION ON THE IMMUNE SYSTEM

Since there is a very short time for the chicks to grow to a marketable age, it becomes essential to adopt a sound management practice that would not only ensure a general well-being of birds but would also assist them to maintain a healthy gut microbiome, strong immunity and improved gut health. Several nutrients are important in the early development of the immune system. Vitamin A is necessary to maximize immuno-competence and for the optimum growth and feed efficiency of poultry (95, 96). Other nutrients which can affect early immune development are linoleic acid, iron, selenium, and some of the B vitamins (97). Much development of immune tissues in poultry occurs at late incubation and early post-hatch period. Thus, maternal nutritional status and deposition of nutrients as well as early nutrition play an important role in the modulation of the nutritional immune system. It has been known that vitamins A, D, and E have regulatory roles in the immune system (98). The complexity of the immune response requires various modes of communication of immune cells and immune-molecules. It has been found that the poultry is most susceptible to the invading pathogens during early hatch period as their immune system does not attain the full functionality by this age.

Nutritional modulation by providing micronutrients and required substrates through both *in ovo* and in feed during first-week post-hatch can support the proliferation of lymphoid organs and modify the population of the microbiome in GIT (97). This can ultimately result in improved immunity and enhanced integrity of the intestinal epithelium of growing birds.

The intestinal mucosa is exposed to a variety of non-self external materials including pathogenic microbes and its gut-associated lymphoid tissue (GALT) plays a significant role in the avian immune system (99). Hence, it is critical that delay in feeding is avoided as it can delay the onset of GALT function (11). *In ovo* injection of nutrients can play a vital role where delay in feeding is expected due to various limitation arising due to shipping and distribution of the day-old chicks.

In ovo manipulation of the embryo is not only a method of filling up the reserves with nutrients but it is also a tool that allows injecting other substances that can stimulate the immune system, modify the gut microbiome, and shift the level of production of metabolites. Dibner et al. (51) proposed the effect of early oral nutrition on the development of the immune system in hatchlings broiler chicks. According to the authors, early nutrition can provide the limiting substrates, affect endogenous levels of hormones or immunomodulators, and the presence of antigen in GIT can trigger the complete differentiation of immune cells like B-lymphocytes. Recently, the focus has been intensified on nutritional manipulation of growing embryo for improvement in immunity and health of birds during later growth periods. The improvement in the immune status of poultry by use of vitamins, amino acids, and prebiotics through *in ovo* feeding has been encouraging (13, 100, 101). Similar to the late *in ovo* feeding, Kadam et al. (102) injected amino acid threonine into the yolk sac of 14 d old embryo and found that it improved the humoral response of the broiler chicks. Bakyaraj et al. (103) reported that *in ovo* injection of amino acids, trace elements, and fatty acids and vitamins into the amniotic cavity of late-term embryo improved the cell-mediated immunity in chicks. A research conducted by Selvaraj and Cherian (104) using *in ovo* injection of fatty acids into amniotic cavity revealed that injection of linoleic acid (ω-6 fatty acid) increased cell-mediated immunity while the injection of ω-3 fatty acids of marine origin induced a humoral response. Likewise, glucose triggered humoral immunity while fructose and ribose modulated cellular immunity in broilers receiving *in ovo* injection in yolk sac/amnion on d 14 of incubation (105). It has also been found that *in ovo* feeding of amino acids can enhance the growth-related genes and modulate the expression of immune genes in broilers (106). Early improvement in the status of immunity in broilers is also dependent on the general

health status and nutritional state of the bird. Some substrates like mannanoligosaccharides have the potential to exert a direct effect on the maturation of enterocytes, enhancement of digestive capacity and improvement of epithelial barrier function when fed *in ovo* (107). Also, early feeding of lectin extract into the amniotic cavity of chicken in a study by Dalloul et al. (108), produced resistance against orally challenged coccidiosis. This protection of chicken against coccidiosis infection is a clear indication of immunopotentiating effect of nutritional programming. The nitric oxide (NO) is produced by cells involved directly or indirectly in immune response and has a key role in immune regulation and neurotransmission. Besides its role as a signal molecule, NO can also act as a non-specific component of the immune system by destroying the invading pathogens, tumor cells, and parasites. *In ovo* feeding of arginine to late-term embryo has been documented to increase secretory immunoglobulin A (sIgA) in the intestinal mucosa and activate Arg-NO signaling pathway in broilers (109). The increase in the level of NO and sIgA indicates that *in ovo* feeding of arginine can enhance the immunological barrier function of intestinal mucosa and improve the overall immunity and intestinal health of birds.

The application of some additives as a nutritional approach to tackle deficiencies during the early feeding period also have some prospect in reinforcing the immunity of growing poults. Feeding of β-glucan can strengthen the innate immunity by up-regulating the oxidative burst, phagocytosis and bactericidal killing capacity of heterophils and decrease the incidence of organ invasion by *Salmonella enteritica* in neonatal chickens (110). Early feeding of the amino acid is essential as their oxidation rate increases during inflammation, and a regular diet may not accommodate the requirement of growing and challenged birds. It is known that glutamine provides energy and nitrogen source for the proliferation of immune and intestinal mucosal cells and is required along with cysteine for the synthesis of antioxidants like glutathione (111, 112). However, it is suggested that breeding poultry for higher immunocompetence would have some negative impact on some production performances that is not desirable in modern poultry production due to the short time of feed to meat or egg turnover (113). Hence, further works are required to be conducted to ascertain the effect of early nutritional programming vs. delayed intervention for tackling challenges originating due to metabolic and infectious diseases in poultry production.

FUTURE POTENTIAL AND LIMITATIONS OF EARLY NUTRITION PROGRAMING

The continuous development and improvement of *in ovo* technology have established a new scope for perinatal nutrition, allowing and creating new challenges and opportunities for nutritionists to optimize poultry production. The *in ovo* injection of important nutrients or substances into the amnion is a novel way to feed critical dietary components to embryos. Indeed, *in ovo* feeding may "jump-start" development, improving the nutritional status of the perinatal chick. The *in ovo* feeding technique has several advantages, including improvements of total digestive tract capacity; increased body weight, growth rate, and feed efficiency; reduction of post-hatch mortality and morbidity; improvements in the immune system and the response to enteric antigens; reduction in incidence of developmental skeletal disorders; and increase in muscle development and breast meat yield. The next step in the early nutrition could be to imprint genes of a bird at a very early age and turn it into a more efficient animal later. In addition, the administration of digestible nutrients into the amnion of embryos can bring an improvement in bird quality, increased glycogen reserves, fast development of the total digestive tract superior skeletal health, better muscle growth rate, higher body weight gain, improved feed conversion, and enhanced immune function (16). Using nutrigenomic data, almost 30 percent of genes expressed different activity over time by *in ovo* feeding.

The main limitations still are associated with embryo development and nutrient metabolism. Another question is a limitation in the probiotic preparation that fits the specific needs of the individual bird. Future early nutrition would be feeding complex symbiotic that would replace feed additives and supplements in the post-hatch feed and is more beneficial to the overall poultry industry.

CONCLUSION

With the increase in productivity and highly feed efficient birds, the nutritional demand of embryos and early aged chicks has changed over decades. Early nutrition programing is one of the latest and successful methods to feed embryos and recently hatched chicks to prepare chickens with the healthy gut, favorable microbiota, improved immunity, and overall improved growth performance. Currently used materials to feed as early nutrition includes probiotics, prebiotics, exogenous enzymes, amino acids, hormones, vaccines, and drugs. Early feeding to chicks with these nutrients and supplements has been found to improve total digestive tract development, increase growth rate and feed efficiency, reduce post-hatch mortality and morbidity, promote growth of beneficial gut microbiota, improve the immune system and the response to enteric antigens, reduce incidence of developmental skeletal disorders, and increase in muscle development and breast meat yield. Further works are required to fine-tune the *in ovo* feeding technique for application at commercial scale in farm condition, understand the embryonic development and nutrient metabolism process more precisely, and understand how early nutrition affects specific genes responsible for performance, intestinal health, and overall health-related traits in poultry.

AUTHOR CONTRIBUTIONS

AS, SY, and JB wrote this review manuscript. BM reviewed the manuscript and provided critical suggestions and comments. RJ decided a review topic, reviewed the literature and this review manuscript and provided critical review, suggestions, and comments.

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

frontiers in Veterinary Science

REVIEW
published: 17 May 2019
doi: 10.3389/fvets.2019.00136

Antibiotic Application and Resistance in Swine Production in China: Current Situation and Future Perspectives

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To meet increasing demand for animal protein, swine have been raised in large Chinese farms widely, using antibiotics as growth promoter. However, improper use of antibiotics has caused serious environmental and health risks, in particular Antimicrobial resistance (AMR). This paper reviews the consumption of antibiotics in swine production as well as AMR and the development of novel antibiotics or alternatives in China. The estimated application of antibiotics in animal production in China accounted for about 84240 tons in 2013. Overuse and abuse of antibiotics pose a great health risk to people through food-borne antibiotic residues and selection for antibiotic resistance. China unveiled a national plan to tackle antibiotic resistance in August 2016, but more support is needed for the development of new antibiotics or alternatives like plant extracts. Antibiotic resistance has been a major global challenge, so international collaboration between China and Europe is needed.

Keywords: antibiotics, antimicrobial resistance, bacteria, China, human and animal health, swine production

OPEN ACCESS

Edited by:
Rajesh Jha,
University of Hawaii At Manoa,
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Reviewed by:
Xiao Xu,
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *14 December 2018*
Accepted: *10 April 2019*
Published: *17 May 2019*

Citation:
Yang H, Paruch L, Chen X, van Eerde A, Skomedal H, Wang Y, Liu D and Liu Clarke J (2019) Antibiotic Application and Resistance in Swine Production in China: Current Situation and Future Perspectives. Front. Vet. Sci. 6:136. doi: 10.3389/fvets.2019.00136

INTRODUCTION

Over the last decades, China's economy has grown very quickly. The gross domestic product (GDP) increased from 1.21 trillion US$ in 2000 to 10.35 trillion US$ in 2014 (**Figure 1**, World Bank, 2016). During the same period, Chinese production of meat, eggs, and milk has rapidly increased, and this will continue—especially for pork (1–3). Pork is one of the most important sources of animal protein in the country, and its production has jumped from around 40 million tons in 2000 to approximately 56 million tons in 2014 (**Figure 1**, USDA, 2016). The effects of the global financial crisis in 2007 and swine flu in 2011 caused an AMRupt production decline in these 2 years. However, production of swine quickly rebounded in the subsequent years. Concurrently, China's pork consumption increased from 2000 to 2014, with some drops in 2007 and 2011. Since 2012, pork consumption has been slightly higher than production, indicating that the pork demand of Chinese consumers has exceeded the domestic production.

Along with a rapid increase in pork production, both the number and the size of intensive swine farms have grown. The number of big farms with thousands of swine has increased markedly. The percentage of big swine farms, with herd sizes of more than 3,000, increased from 5% in 2003 to 14% in 2010. In the same period, the proportion of small farms, with herd sizes of less than 50, nearly halved, from 71% to 36% (China Animal Industry Yearbook 2004–2011).

Several recent studies detail antibiotic use in animal production (3–6) and the risk this poses in the form of antibiotic resistance (7–9). The current study focuses on the important emerging public health challenges as a result of overuse or abuse of antibiotics in swine production in China. It also outlines the future challenges for the new antibiotics and alternatives.

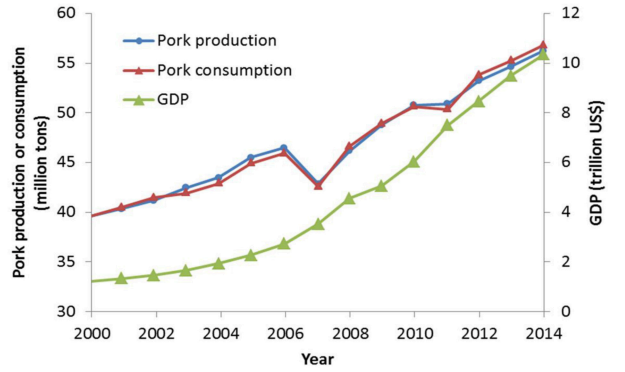

FIGURE 1 | Gross domestic production (GDP), pork production and consumption in China from 2000 to 2014 (data source: World Bank http://data.worldbank.org/country/china and USDA Foreign Agricultural Service http://www.fas.usda.gov/).

ANTIBIOTIC USAGE IN SWINE PRODUCTION IN CHINA

With the shift from small to large swine feeding operations and the increase in overall pork production, there is growing concern about the adverse consequences such as swine health and welfare, disease spreading of large-scale animal production (1). Because the high density of animals in big swine farms exacerbates the risk of quick spread of infectious diseases, farmers in China have responded by using higher amounts of antibiotics. This, in turn, has led to growing concerns regarding overuse and abuse of antibiotics for intensive swine production, especially the health risks (3, 10, 11).

Penicillin was discovered in the 1940s. Since then, antibiotics have changed the treatment of bacterial infections for both humans and animals. Antibiotics were first added to feed for broiler poultry to prevent microbial diseases in the 1940s (12). They were then rapidly used for the same purpose in feed for other food animals, first in the USA and later in other developed countries and developing countries such as China (2). Antibiotics can aid in different ways. When antibiotics are used at low (sub-therapeutic) levels in feed, they can improve growth rate by reducing mortality and disease. Thus, conversion of feed to weight gain becomes more efficient. Antibiotics can further prevent disease at intermediate levels, whereas high (therapeutic) levels of antibiotics are used to treat diseases (13–15). Antibiotics are widely used in animal husbandry as low-cost growth promoters in more than half of the world's countries (7).

As the world's largest pork producer and consumer, China uses a massive amount of antibiotics to support its production (3). Some studies have been conducted to identify the antibiotics used in China's pig farms (16–23). These studies report the extensive use of the major antibiotic classes of sulphonamides, tetracyclines, fluoroquinolones, macrolides, and β-lactams.

Antibiotics have been widely adopted for use in food animals, but reliable data about the quantity and patterns of use (e.g., dose and frequency) for food animals alone are not easily available in China or other developing countries. It is very challenging to make an accurate calculation of antibiotic use in food animals. Studies have adopted different classifications for therapeutic use, nontherapeutic use, or a combination of the two. Most available data lack clear definition of therapeutic vs. nontherapeutic uses, and this ambiguity clearly erodes reliability (24). Based on models developed from American data (25), Krishnasamy et al. (24) estimated that 38.5 million kg of antimicrobials were consumed in China's pork and poultry production in 2012. Among all antibiotics, tetracyclines are the most widely consumed in swine production. Zhang et al. (23) performed a market survey on the usage of the 36 main antibiotics in China including sulfonamides, tetracyclines, fluoroquinolones, macrolides, β-lactams (penicillins and cephalosporins), chloramphenicols, lincomycin, and others. They found that the total amount of antibiotics used for China's swine farming was 48.4 million kg in 2013, which is higher than the result of Krishnasamy et al. (24). Of all antibiotics consumed in China's swine farming, fluoroquinolones and β-lactams contributed more than half.

Moreover, there is a clear geographic heterogeneity for antibiotic consumption in China. Antibiotic consumption hotspots appear in Southwest China (Sichuan), Central China (Hunan), North China (Henan and Hebei) and the southeast coast (Fujian, Guangdong and Guangxi) in China. In particular, Sichuan province has the highest swine density and therefore carries the most serious risks to environment and health (23). Other areas have also seen significant developments in recent years. For example, Xinjiang Uyghur Autonomous Region, the provincial level region with the largest area in Northwest China, is located in the center of the Eurasian continent. It is in the core area of "The Silk Road Economic Belt" and plays an important role in this program. Pork production in Xinjiang increased from 0.025 million tons in 1978 to 0.231 million tons in 2010 (Statistical Yearbook of Xinjiang in 2011). Despite a lack of data on antibiotic consumption in swine farms in Xinjiang, the concentration and detection rate of antibiotic residue in swine manure samples were higher than those of chicken manure and cow dung. The concentration of tetracycline in swine manure was highest, followed by sulfonamides and quinolones (26, 27). Additionally, international trade with Central Asian and European countries is increasing along the Silk Road, which may worsen the spread of antibiotics. In Lake Aibi, 12 species of 14 kinds of antibiotics were detected and detection rates of four kinds of antibiotics were 100% in water samples, with highest average concentration of 54.37 ng L^{-1} (28, 29).

China is tackling the overuse of antibiotics and the AMR problem using different approaches, including educating farmers about AMR caused by excessive use of antibiotics in animal farming, swiftly banning the use of colistin as a feed additive in animal production (30), reducing the list of approved antibiotics for animal application, promoting the use of alternative feed additives such as organic acids (e.g., Selko®-pH, http://selko.com), improving the management of animal husbandry and animal welfare, and law enforcement accompanied by an effective surveillance system [(3), http://www.moa.gov.cn/].

ANTIBIOTIC RESISTANCE AND THE RISK TO HUMAN HEALTH

The overuse and abuse of antibiotics cause environmental pollution, for example the contamination of manure, soil and water (10, 31). Worse, improper use of antibiotics brings risk to human health through food-borne antibiotic residues and selection for AMR, and a greater ability of certain bacteria to resist the effect of antibiotic treatments (7). The causes of AMR are complex, but there is growing scientific evidence suggesting that low-dose, prolonged courses of antibiotic use for animal husbandry accelerated the emergence and spread of resistant bacteria (32–34). In food animal husbandry, AMR can spread not only by direct contact, but also indirectly (**Figure 2**). Direct effects are those that can be causally linked to contact with antibiotic-resistant bacteria from swine. Indirect effects are those that result from contact with resistant organisms that have been spread through food, water, and animal waste application to soil (37).

Many antibiotic classes are used in both swine husbandry and human health care. Therefore, the emergence and spread of resistance to these antibiotics will likely limit the therapeutic options for human diseases. Even worse, this kind of AMR can prolong illness and cause serious disability and ultimately death (32, 33).

In the last decades, AMR has become a global challenge for human health and welfare. In particular, it is a serious problem in China where antibiotics have been overused or misused in livestock husbandry and human health care (38–41). For example, the OqxAB efflux pump, encoded by the genes *oqxA* and *oqxB*, has been found to be one of the mechanisms of plasmid-mediated quinolone resistance (PMQR) (42–44). Zhao et al. (45) investigated the prevalence and dissemination of *oqxAB* in *Escherichia coli* (*E. coli*) isolates from swine, their environment and farmworkers in China. The *oqxA* gene was present in around 39.0% of *E. coli* isolates. About 46.3% of *E. coli* isolates from swine farms were positive for *oqxA*. Approximately 43.9% of *E. coli* isolates from the swine farm environment were also positive. In addition to animal *E. coli* isolates, *oqxAB* was found in 30.3% of human commensal *E. coli* isolates. Because these farmworkers were without previous antimicrobial treatment or hospital admission, this indicated the transmission of *oqxAB* to humans. Compared with results from Sweden (1.8%) and South Korea (0.4%) (46, 47), the prevalence of *oqxAB* in *E. coli* isolates was much higher (39.0%) in China (45).

A further example has been reported by Zhang et al. (48), who researched the occurrence of the *aac* (*3*)*-IV* gene, which confers resistance to apramycin, an antibiotic used in agriculture but not for humans, in Northeast China. Unfortunately, they found workers who carried apramycin resistance genes in all swine farms where apramycin was used as an antibiotic growth promoter. The same was present in swine isolates. Similarly, Ho et al. (49) investigated gentamicin resistance in Hong Kong. They found that 84.1% of human samples and 71.4% of swine samples contained the *aaaC2* gene for gentamicin resistance. Polymyxin resistance was identified as being due to the plasmid-mediated *mcr-1* gene (50). Liu et al. (51) investigated the *mcr-1* gene in swine, pork and inpatients in five provinces in China during the period 2011–2014. They found *mcr-1* in *E. coli* isolates collected from 17.7% of pork samples, 20.23% of swine samples, and 1.40% of inpatient samples with infection. Similar studies have also been conducted in Xinjiang. For example, Xia et al. (52) collected 543 fecal samples from a large-scale swine farm and isolated 454 *E. coli* isolates. They found that 64.5% of the *E. coli* isolates showed resistance to 3–9 antimicrobials, especially to ampicillin and amoxicillin.

THE DEVELOPMENT OF NEW ANTIBIOTICS

Concern about antibiotic resistance has escalated in the last years. In 1986, Sweden became the first country in the world to ban the use of some antibiotics in animal feeds (53). In 2006, European Union (EU) member nations started to ban all antibiotic growth promoters according to EC Regulation No. 1831/2003 (14). As the largest developing country with a growing demand for meat protein, China has not yet completely prohibited the use of antibiotics as growth promoters. Considering the big risk for antibiotic pollution in the environment (soil and water) and potential resistance, more research is urgently needed for the development of new antibiotics or, ideally, alternatives.

New Antibiotics

During the past two decades, efforts to develop new antibiotics have met with some success (54). However, due to their much higher costs compared to the older antibiotics, many have been gradually pulled from the market. Therefore, new antibiotics are still needed to tackle the worsening risk of antibiotic resistance.

Several approaches have been applied to identify new antibiotics or augment currently licensed antibiotics: (1) natural or synthetic compounds as inhibitors of multidrug efflux pumps, (2) small-molecule inhibitors of bacterial transcription factors, and 3) antisense inhibition of multidrug transporter genes using licensed drugs (55–59). As alternatives to antibiotics, use of bacteriophage and plant extracts has also been investigated, which will be discussed in the next section.

By deleting or inactivating specific genes, researchers found some putative new targets, for example reducing the virulence of pathogens (60, 61). Quorum sensing (QS) or other bacterial signaling systems have also been identified as new targets for antibiotic molecules (62, 63). *In-silico* and *in vitro* high-throughput screening of small-molecule and compound libraries have also been increasingly used. Some agents have been in Phase 1 of clinical trials (64). In 2015, Ling et al. (65) discovered a "resistance-free" teixobactin in a screen of uncultured soil bacteria sample. Experiments confirmed no mutants of *Staphylococcus aureus* or *Mycobacterium tuberculosis* resistant to this teixobactin. Hopefully, this study will start an innovative approach to expanding the pool of natural antibiotics (66). Recently, a new class of antibiotics—arylomycins—was reported (67). The arylomycin G0775 showed activity against multi-drug resistant Gram-negative clinical bacterial pathogens by inhibiting the essential bacterial type I signal peptidase (which

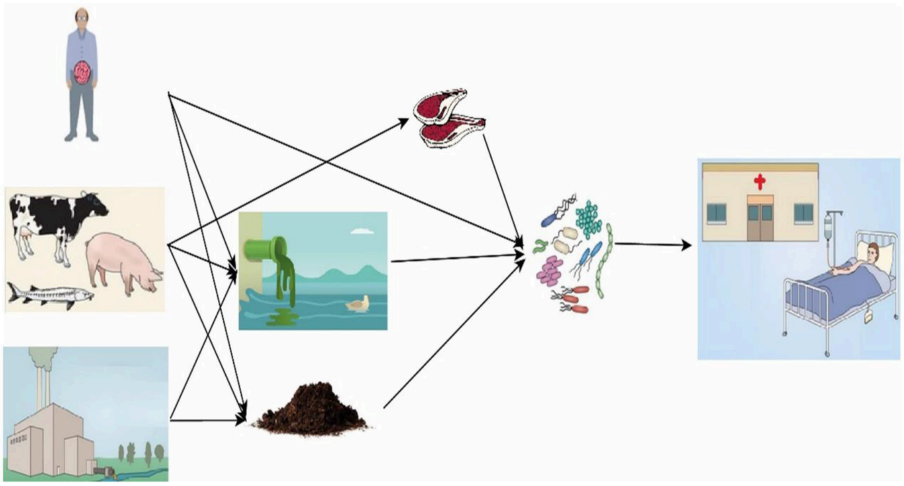

FIGURE 2 | Expected fate, transport, and exposure pathways for antibiotics and the spread of antibiotic resistome. Antibiotics from human and veterinary drugs, growth promoter for animal husbandry and aquaculture, and improper release during pharmaceutical production are released into water and soil. Manure containing antibiotic resistome may be carelessly used for crop production. Antibiotic resistome can remain in meat and the bacteria can be further spread to humans. People take up antibiotics and resistome develops in their guts [modified from Song and guo (35) and Berendonk et al. (36)].

is a novel antibiotic target) through an unknown mechanism as described by Smith et al. (67). Further investigation will hopefully reveal the molecular mechanism underlying this novel class of antibiotics originating from natural products. Efforts will be made to identify and characterize more novel natural products to tackle AMR and problems caused by over-application of antibiotics in swine production.

Plant Extracts—a Promising Alternative

In addition to searching for new antibiotics, alternatives/replacements have received growing attention in the last decades (14, 68). Researchers have explored various kinds of alternatives to animal antibiotics: feeding enzymes, immunity modulating agents, bacteriophages and their lysines, antimicrobial peptides, probiotics, prebiotics, synbiotics, inhibitors targeting pathogenicity, plant extracts and others (14, 69–75). In China, herbs and their extracts have been widely used in traditional medicine for centuries before the introduction of western medicine. Youyou Tu, from the China Academy of Traditional Chinese Medicine in Beijing, was awarded the 2015 Nobel Prize in Physiology or Medicine for her discovery of artemisinin (qinghaosu) extracted from *Artemisia annua* L. (76). Her work was inspired by the Chinese traditional medical book *Prescriptions for Emergencies* by Ge Hong (284–346 CE) (77). Compared to other antibiotic alternatives, therefore, plant extracts have received more attention and support in China.

Natural plant products and their derivatives have been explored for their antimicrobial, anti-inflammatory, anti-oxidative, and anti-parasite properties (78–84) (**Table 1**). A good example is garlic extract, which is widely considered as one of the most effective antibiotic agents (86). In addition, *Areca catechu* is a rich source of compounds with anti- quorum sensing (QS) properties (87). Some studies also found that *P. aeruginosa* genes controlled by QS could be inhibited by the isothiocyanate iberin from horseradish and ajoene from garlic (88, 89). When they are combined with tobramycin, ajoene and horseradish juice extracts function as a synergistic antibacterial (90). Extracts of the genus *Paeonia, one* of the most important sources of drugs in Chinese traditional medicine, can inhibit *C. albicans* growth (91). Extracts from *Fructus psoraleae*, *Folium eucalypti globuli* and *Achillea millefolium,* anti-dermatophitic compounds, have been used to treat different ailments such as dermatomycosis in Chinese traditional medicines (92, 93).

Diarrhea is a common cause of intestinal diseases in children and animals including swine (94). Some studies have been conducted to find plant extracts for inhibiting the proliferation of *E. coli*. Khan et al. (95) found that pathogenic strains of *E. coli* are sensitive to the extracts of three plants (*Acacia nilotica, Syzygium aromaticum* and *Cinnamum zeylanicum*). Herb extracts from *Pulsatilla chinensis*, *Sophora flavescens*, *Phellodendron amurense*, *Radix Astragali* and *Codonopsis pilosula* (Franch) Nannf have been used to treat diarrhea of piglets in Chongqing, Southwest China (96). Because of the influence of harvesting method and other unknown factors (97), plant extracts have been limited by their variability (98). The current high cost also limits the wide use of herb extracts, but the further development of herb extracts may reduce the cost and expand their application in developing countries.

Xinjiang is one of the Chinese regions with high biodiversity. The flora include common bitter beans, *Cynomorium*, *Ephedra*, *Ferula*, liquorice, snow lotus, sea buckthorn, and others. Among these, bitter beans have antibacterial ingredients (99). Horse grass contains alkaloids that, when drunk, can inhibit the function of malignant tumors. Xinjiang *Lithospermum* and liquorice contain glycyrrhizinate, flavonoids and other medicinal ingredients. These plant ingredients have antibacterial effects on *E. coli*, paratyphoid *Salmonella*, *Staphylococcus aureus*, *Bacillus subtilis* and other common pathogens (100).

TABLE 1 | Antibiotic alternatives: plant extracts.

Plant	Effect observed	References
Aged garlic extract, allicin	Improved growth performance, nutrient digestibility, intestinal microbial balance, immune response and meat quality in finishing pigs	(82)
Camellia sinensis	Improved gut health of post-weaning piglets and protection from *E.coli* challenge	(81)
Cinnamon essential oils, Cinnamaldehyde	Antimicrobial activity and improved immune response against e.g., *Salmonella typhimurium* in swine intestine	(83)
Carvacrol, cinnamaldehye, eugenol, etc.	Anti-inflammatory effects on porcine alveolar macrophages	(78)
Capsicum oleoresin, turmeric oleoresin, garlicon	Improved gut health and reduced frequency of diarrhea in weanling pigs	(79, 80)
Agrimonia procera	Growth performance, increased immune response and antioxidative effects in piglets	(84)
Chinese traditional herbal medicine (CTHM)	Beneficial effects on swine growth with improved final live weight, general digestibility and nitrogen retention	(85)

Future Perspective and Conclusions

Global organizations and developed countries have paid increasing attention to tackling the great risks of overuse and abuse of antibiotics and antibiotic resistance (101). For example, the World Health Assembly (WHA) commissioned the WHO to deliver a global action plan on antibiotic resistance in May 2014. The British government sponsored the £10 million Longitude Prize for the best solution for the resistance problem in June 2014. The President's Council of Advisors on Science and Technology in the USA released a report on antibiotic resistance in September 2014.

Slower than many European countries and the USA, China unveiled a national plan to tackle antibiotic resistance in August 2016 (102). The plan highlights the importance of reducing use of antibiotics in China's livestock husbandry. However, the implementation details of the plan are still unclear. Punishment for violations is still lacking. As for many action plans and laws in China, strict implementation is extremely important for reducing the use of antibiotics (103, 104). The plan also emphasizes the development of new antibiotics. As stated above, the high price of new antibiotics and alternatives limits their development (54). In the action plan, the funding source for discovery of new antibiotics or alternatives, for example from government or industry, is still unclear. Antibiotics have been widely overused and abused in Chinese swine farms to prevent diseases. However, it is more important to improve the sanitation and hygiene conditions of swine farms. Rather than using antibiotics, some measures should be applied to improve the health and well-being of swine, in particular reducing animal overcrowding, and controlling facility temperature and ventilation. In addition to the swine farmers, joint efforts from government, academia and veterinary professionals are indispensable.

Antibiotic resistance has become a world-wide challenge and therefore international collaboration is increasingly crucial. International collaboration between the world's largest antibiotics consumers, China and Europe, is indispensable to tackle the AMR problem. The One Health approach is of importance to achieve a sustainable and effective management of AMR by joint efforts of the international community with involvement of all stakeholders.

AUTHOR CONTRIBUTIONS

JC and DL designed the review, contributed to writing and editing. HY, LP, XC, AvE, HS, and YW contributed to writing, while JC is responsible for submission.

FUNDING

This work is supported by Sinograin and Sinograin II projects with JC as the project manager.

ACKNOWLEDGMENTS

This work was supported by the Sinograin project and a pilot project Biotransformation supported by NIBIO core funding. The authors thank Prof. Nicholas Clarke for linguistic correction.

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

frontiers in Veterinary Science

REVIEW
published: 11 June 2019
doi: 10.3389/fvets.2019.00169

Physiological Effects of Dietary Amino Acids on Gut Health and Functions of Swine

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Gut health has significant implications for swine overall health status and nutrient utilization, due to its various functions including digestion and absorption of nutrients, secretion of mucins and immunoglobulins, and selective barrier protection against harmful antigens and pathogens. Both the basic anatomical structure of the gut (such as epithelial cells) and its luminal microbiota play important roles for maintaining gut health and functions. The interactions between epithelial cells and luminal microbiota have significant impact on host nutrition and health through the metabolism of dietary components. Amino acids, which are major nutrients for pigs, are not only obligatory for maintaining the intestinal mucosal mass and integrity, but also for supporting the growth of microorganisms in the gut. Dietary amino acids are the major fuel of the small intestinal mucosa. Particularly, glutamate, glutamine, and aspartate are the major oxidative fuel of the intestine. Emerging evidence shows that arginine activates the mTOR signaling pathway in the small intestine. Utilization of glycine by the small intestinal mucosa to synthesize glutathione is a very important physiological pathway, and the role of glycine as a powerful cytoprotectant has also been recognized. The major end products of methionine and cysteine metabolism are glutathione, homocysteine and taurine, which play important roles in the intestinal immune and anti-oxidative responses. Threonine is highly utilized by the gut and is particularly important for mucin synthesis and maintenance of gut barrier integrity. Moreover, either a deficiency or an excess of dietary threonine can reduce the synthesis of intestinal mucosal proteins and mucins in young pigs. Various new functions of amino acids on gut health and functions have been discovered in recent years. Thus, this review is to provide some up-to-date knowledge for industry application of dietary amino acids in order to enhance swine gut health and functions, and also it is to provide a comprehensive reference for further scientific research in this regard.

Keywords: amino acid, gastrointestinal tract, gut health, gut function, feeding strategy, pig

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *26 September 2018*
Accepted: *16 May 2019*
Published: *11 June 2019*

Citation:
Yang Z and Liao SF (2019) Physiological Effects of Dietary Amino Acids on Gut Health and Functions of Swine. Front. Vet. Sci. 6:169. doi: 10.3389/fvets.2019.00169

INTRODUCTION

The ultimate goal of swine production is to convert various feedstuffs into edible pork for human consumption. Thus, enhancing the efficiency of the bio-transformation of feed mass into swine body mass, especially, the lean mass, is the bottom line for improving the profitability, as well as the sustainability, of swine production (1, 2). During the course of this bio-transformation by the

pig, two initial steps after feed ingestion are feed digestion and nutrient absorption, which mainly take place in the gastrointestinal tract (GI tract or GIT), also known as gut, of the pig. Besides feed digestion and nutrient absorption, pig gut also encompasses a number of other physiological and biochemical features including intermediary nutrient metabolism and energy generation. In addition, the gut is also the largest immune organ in the body with defense mechanisms consisting of barrier function and mucosal immunity (2, 3).

Like other monogastric animals, the GIT of pig harbors several 100 microbial species, contains over 20 hormones, digests and absorbs a vast majority of nutrients, and accounts for roughly 20% of body energy expenditure (3, 4). Thus, both the tissue of the GIT wall and the luminal microbiota play essential roles to maintain a healthy gut and gut functions. The interactions between gut physiology and gut functions occur among the complex features of epithelial mucosa, luminal microbiota, and dietary nutrients (5). Indisputably, a healthy gut in swine is crucial to the overall nutrient metabolism, physiological activities, body wellbeing, and production efficiency at every stage of pig's life (5). Any harmful challenges on gut health can negatively impact swine utilization of dietary nutrients, compromise the whole body health and, consequently, reduce their production performance (6).

Without doubt, a healthy gut is considered as a foundation for swine production. From this regard, the maintenance or enhancement of gut health is essential not only for animal welfare but also for the business profitability of swine production. In the industry practice, formulating ideal diets to enhance the nutritional effects on gut health has been fast becoming a reality (3, 7). For this reason, the primary goal of this review is to summarize some up-to-date knowledge concerning the effects of dietary amino acids (AAs) on swine gut health and functions for animal nutritionists and producers to consider when formulating swine diets, and also to provide a comprehensive reference for animal scientists to use when conducting further research (6) on the nutritional regulation of the gut development, integrity, and functions of swine. To systematically approach the literature, Google Scholar (https://scholar.google.com), the world's largest academic search engine, was employed with key words as "effect of amino acid on gut health of pig or swine" for acquiring those relevant, well-conducted studies, especially that over the last decade. During the literature search and selection process when a specific AA was identified, the name of this AA would have been used as a key word to conduct some further searching and selection.

THE CONCEPTION OF GUT HEALTH

It is generally considered that the primary function of the gut is to digest feed, absorb nutrients, and excrete waste to support animals' lives (4, 5). In order for a pig to well perform this primary function, a healthy gut is without doubt a prerequisite condition before anything else (8). That being said, in the scientific community, what kind of gut is a healthy gut is not an easy question to answer. In the literature, there are many morphological and functional criteria that have been used to describe gut health (5, 7), but there is no single simple definition that has been widely accepted without further questions (2, 9).

Gut Health From Morphology Perspective

Similar to other monogastrics, the GIT of swine consists of five major anatomic sections that are mouth, esophagus, stomach, small intestine, and large intestine. The small intestine, further consisting of duodenum, jejunum and ileum, is a key section that plays a central role for the physiological, biochemical, and immunological functions of the GIT (4). For this reason, most researches studying the gut health of pigs refer to small intestine as GIT or gut of pigs (7). With the same justification, the primary attention of this review has also been given to the health and functions of the small intestine of pigs, especially, the young pigs.

The integrity of anatomical structure is the foundation for the normal functions of the gut. From a morphology perspective, gut structure varies along the GI location, which reflects the functional requirements for digestion and absorption of nutrients, as well as for microbial and chemical defenses (10). The epithelia of small intestine in general consist of a single layer of tall columnar cells called epithelial cells that further include five major types: absorptive enterocytes (the primary ones), Paneth cells (secreting antimicrobial substances), goblet cells (secreting mucins), enteroendocrine cells (secreting GI hormones), and miscrofold or M cells (presenting antigens to the underlying lymphoid cells) (7, 11, 12). All these cells are connected mainly by tight junctions, gap junctions, and desmosomes (7, 11). These connected cells are structurally arranged as villi and crypts. The villi are finger-like projections into the intestinal lumen while the crypts are invaginations between the villi (4).

The surface of each absorptive enterocyte on the villi further has many small projections called microvilli, and all microvilli collectively form an expanded apical surface called brush border. Besides expanding the surface for nutrient absorption, the plasma membrane of the microvilli embeds various enzymes that can help to complete the final stages of nutrient digestion (13). The crypts of the epithelia lie within a lamina propria which is rich in lymphocytes, eosinophils, and plasma cells. The epithelial cells on the villi are continuously replaced by the proliferation of the cells in the crypts and this process is called epithelium turnover. Basically, the cells in the crypts continuously maturate and migrate up to the villi, and after 3–6 days these cells will end up with apoptosis or exfoliation at the villus tips (7, 14). During the migration most of the immature cells differentiate and become mature enterocytes on the villi. Other cells become either enteroendocrine or goblet cells (15).

Villus height (V), crypt depth (C), and V:C ratio are three key morphological indicators of the overall health and functions of small intestine (6, 16). High V may result in a greater absorptive capability for the available nutrients (6). A low C indicates a decreased metabolic cost of epithelium turnover, as crypts function as a villus factory. Deeper crypts indicate faster tissue turnover for villus renewal, which may be needed in response to inflammation caused from pathogens or their toxins (17). A greater V:C ratio suggests increased nutrient absorption

(16), decreased secretion of proinflammatory cytokines, reduced metabolic cost, and improved growth performance (17, 18).

On the top of the epithelial layer is a mucus layer that basically is of viscous secretion from goblet cells (7). The epithelial and mucus layers together constitute the first line of swine defense against the intestinal pathogenic challenges. This line of defense is immediately active and mostly non-specific (10). Because the mucus secreted from goblet cells acts as a physical barrier against foreign substances, the count of goblet cells is a good indicator of potentially higher mucus production and secretion to protect the large area of the epithelial surface (19).

The tight junctions, located in the top of the intercellular structure, are multi protein complexes consisting of transmembrane proteins, such as occludin, claudins, tricellulin, and junctional adhesion molecules (20, 21). Those proteins may interact with the cytosolic peripheral proteins, including zonula occludens protein-1 (ZO-1), ZO-2, and ZO-3 (22), to form a selective physical barrier or para-cellular permeability that prevents the diffusion of molecules with <4 Å diameter in size (11, 21). Thus, the epithelial tight junctions commonly form a strong barrier against the absorption of endotoxins from the colonic lumen into mesenteric circulation (21). Overall, the layer of epithelial cells, together with the mucus layer on top of it and the lamina propria beneath it, provide an optimal micro-environment for chemical digestion, selective permeability, and partly resistance against endotoxins and pathogens (15).

Gut Health From Microbiota Perspective

Modern nutritional researches have learned that besides the normal anatomical structure, as overviewed above, an appropriate luminal microbiota is also an indispensable component for gut health and functions (2, 10). Pig gut is sterile at birth and then colonized by numerous microbes from the dam, feed and environment, starting with lactic acid bacteria, enterobacteria, and streptococci (23), followed by many species of obligate anaerobes (6). Later, the gut microbiota is established as a stable complex micro-ecosystem composed of approximately 10^{14} (or 10^{6}-10^{12}/g of GI content) microorganisms with most of them being bacteria or anaerobic bacteria (roughly 400 to 640 species, representing approximately 140 genera), which symbiose with the pig as host (3, 4, 24). In humans and monogastric animals, the numbers of bacteria increase from 10^{4} cells per gram of digesta in the stomach to 10^{11} cells per gram of digesta in the large intestine (25). In mammals, the gut microbiota is characterized by its high population density, wide diversity, and interaction complexity. In terms of diversity, the gut microbiota consists of a mixture of bacteria, yeasts, protozoa, and virus (4). It is widely believed that almost all of these microorganisms are beneficial to the host, but some of them can be harmful or opportunistically harmful to the health of the host (7).

The activities of gut microbiota have potential effects on host nutrition and health through the metabolism of dietary components and through the interaction with intestinal epithelial cells (25). Studies investigating how bacteria contribute to the development of host intestinal functions have revealed a surprisingly symbiotic relationship between bacteria and the host (10). The gut microbiota salvage energy from otherwise indigestible carbohydrates, and protect the host from pathogens by forming a front line of mucosal defense (26). The commensal microbiota can also direct some postnatal development of the intestine (10). As Stappenbeck et al. (27) reported, certain commensal bacteria may be necessary for the development of intestine to its full absorptive capacity. Some member species in the GI bacterial community also exhibit anti-inflammatory effects on the mucosa. Neish (28) described a mechanism by which some bacterial proteins act as inhibitors of the NF-κB inflammation activation pathway. Overall, the bacterial cells outnumber animal (host) cells by a factor of 10 and have a profound influence on the nutritional, physiological, and immunological processes of the host.

The microbiota in the gut is now regarded as a multicellular, multifunctional organ whose genomes have genetic codes responsible for providing metabolic functions that host has not yet acquired or evolved in its own genome (10). In this regard, a focus on gut health should not forget to support the microbial ecosystem in the gut, to maintain its equilibrium, or to adjust this ecosystem when it is unbalanced (2, 5).

Gut Health From Immunity and Anti-oxidation Perspectives

A well-developed immune system with optimal immune responsiveness is very important for animal's overall health and productive performance (7). Swine gut immune system consists of three lines of defense: barriers, innate immunity, and acquired or adaptive immunity that work together to protect the GIT and the whole body from diseases (29). The resident immune cells (e.g., T, B, and plasma cells), lymphocytes (for acquired immunity), macrophages and cytokines (pro-inflammatory or anti-inflammatory), dendritic cells (for innate immunity), and the related lymphoid tissue associated with GIT all together constitute this largest immune organ in the body (5, 30). Indeed, GIT is a home to more than 70% of all the host's immune cells (30).

The secretion of hydrochloric acid (HCl) by the stomach plays an important role in protecting animal body against the expansion of pathogens that were ingested with feed or water (31). In addition to the acid bath in stomach that causes a several log reduction in microbial counts, there are three component barriers: mucous and mucins, antimicrobial proteins, and secretory IgA. In pigs, the barrier system normally eliminates 99.9% of all infections and that is why it is also known as a "kill zone" level (29). Nonetheless, to any violation in the barrier function, or in the event of pathogenic, antigenic or allergenic challenge, the gut immune system must rapidly and strongly respond to mobilize its innate and adaptive immunities, which is critical in preventing the systemic spread of infection and inflammation (5, 32). The intestinal immune responses could lead to inflammatory responses and secretion of antibodies. As a critical innate immune process, inflammatory responses seek to contain an infection, activate adaptive immunity, repair damaged tissues and return to an immune homeostatic state. However, over inflammation costs more nutrients, and is associated with

increased permeability that may lead to translocation of toxins, allergens, viruses, or even bacteria (5, 33). There is a plethora of interactions between immune cells, as well as with non-immune cells, which help to provide intestinal protection, tolerance and homeostasis (30), and all these related components work together in a coordinated way to prevent disease, maintain homeostasis, and maximize nutrient acquisition in the animal.

During the lifespan of the pig, various reactive oxygen and nitrogen species (ROS and RNS) are ordinarily produced from the aerobic cellular metabolism, which can accumulate under inflammation conditions (34). Some ROS, such as superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2), are significant in the body that can damage cellular macromolecules, such as proteins, lipids, and DNA, and these damages can induce cellular oxidative stress and impair the integrity of mucosal epithelium, which would in turn cause serious problems with intestinal barrier functions. All these problems would lead animals to increased incidences of diseases (35).

Many practical efforts, such as hygiene, chemoprophylaxis, and vaccinations, have been made in prophylactic measures against swine infectious diseases. However, the immune responsiveness and anti-oxidation capacity cannot be maintained only by animal hygiene and vaccinations, but require adequate support from nutritional interventions. Furthermore, the immune alertness and reactivity of the gut can be modulated (oriented or improved) by nutritional components, such as dietary AAs (7). Moreover, the gut microbiota plays a prominent role in the development of gut immune system, particularly the adaptive immunity (2, 7).

Young pigs with early-weaned experience unavoidably face many health challenges or stressors resulted from numerous external and internal factors, such as the sudden change in diets with complex ingredients, anti-nutritional compounds, pathogens, potential toxins, and (or) antigenic molecules (36, 37). Despite a considerable amount of research conducted in the past, post-weaning problems, such as the well-known "growth check," the occurrence of diarrhea, and the nervous signs, often followed by death, are still wide spread in the global swine industry (7). Even though the gut immune system in adult pigs is tightly regulated via a number of molecular mechanisms to prevent excessive activation and inflammation in response to internal stressors (5, 33), the gut immune system in young pigs is anatomically and functionally immature. It is evident that that more research directed toward overcoming the aforementioned problems associated with the immature gut of young pigs will be continued in the foreseeable future (6).

Dietary Factors Influencing Gut Health and Functions

In swine, the overall gut health is influenced by many factors, such as the living environment, feeding strategies, microorganisms (including pathogens) and weaning practice, and is also mediated by some behavioral and psychological stresses (7, 38). Among all these factors, feeding strategies, especially the dietary nutrient components, play critical roles influencing pig gut health and functions (7, 14, 23). The ultimate interactions between gut tissue structure (especially, the epithelial cells) and gut microbiota (including their metabolites) have determinative influence on pig nutrition and whole body health through the metabolism of dietary components (5).

The major nutritional components in swine diets include carbohydrates (including fiber), proteins, AAs, and lipids, and all these components can have positive or negative impact upon gut health (6). It has been shown that the alteration of dietary protein quantity and (or) quality can manipulate the gut structure and functions, as well as the diversity and functions of the gut microbiota (39). Excessive amounts of dietary protein (more than required) can reach the lower GIT, and the fermentation of it can result in the production of various potentially toxic products (such as amines and NH_3) and is often associated with the growth of potential pathogenic bacteria (such as *Clostridium perfringens*) and the reduction of fecal counts of beneficial bifidobacteria, especially in the piglets reared under nutritional and environmental stresses (40).

It is also known that a certain amount of fiber has to be included in diet to maintain the normal physiological functions of swine gut. Taking advantage of the potential prebiotic effects of dietary fiber has been considered as an effective measure to promote gut health and, thereby, minimize the use of anti-microbial growth promoters (AGP) in pigs (41). Dietary inclusion of soluble non-starch polysaccharides can stimulate the growth of commensal microbes in the gut (42). The addition of insoluble fiber sources, such as the husks from cereals, could reduce the excretion of hemolytic *E. coli* and the incidence of diarrhea after weaning (39).

A large number of feed additives have been evaluated for their effects on swine gut health. Organic and inorganic acids have positive effects on swine gut development and health, and in turn on the whole body health and productivity (6). The positive effects of these acids were attributed to various factors including: (1) the anti-microbial activity of non-dissociated organic acids, (2) lowering digesta pH (particularly, in the stomach) and aiding protein digestion, (3) lowering stomach emptying rate, (4) stimulating pancreatic enzyme production and activities in the small intestine, and (5) providing nutrients that are preferred by intestinal tissue thereby enhancing mucosal integrity and function (6, 39).

Essential oils have been used as artificial flavorings and feed preservatives, and some of those essential oils have strong anti-microbial activities (43). Another class of feed additives, exogenous enzymes, have also been utilized in swine diets to improve the digestive function of the gut (44). Jiang et al. (45) reported that combination of essential oils and an enzyme blend effectively improved the ileal morpho-functional aspects, down-regulated its inflammatory reaction, and modulated the fecal microbiology of the weaned piglets.

Due to the global push to eliminate the usage of antibiotics as AGP for pigs, searching for novel alternatives to the in-feed antibiotics to support the industry for profitable and sustainable pork production are currently ongoing. Some in-feed probiotics have been considered by many nutritionists as an ideal alternative to antibiotics (2). For young piglets, probiotics are expected to deliver at least one of the following functions to the gut:

(1) stimulating the development of a healthy microbiota that is predominated by beneficial bacteria, (2) preventing enteric pathogens from colonization, (3) increasing digestive capacity and lowering the luminal pH, (4) improving mucosal immunity, and/or (5) enhancing gut tissue maturation and integrity (39). Prebiotics, another class of feed additives, could also benefit the host in a manner similar to probiotics (46). Combining prebiotics with probiotics may increase the efficacy of probiotic effects on gut development and health in newly-weaned piglets (2).

Moreover, there are also many other feed additives, such as immunoglobulin, ω-3 fatty acids, yeast derived ß-glucans, phytochemicals, and zinc oxide, used in swine diets in order to endorse their positive impacts on gut health and functions (6, 23, 47, 48). However, it is not an intention of this review to cover in details all these feed additives, as well as the aforementioned dietary nutrients. Instead, this review is focused on the beneficial effects of AAs, a very important group of nutrients for pork production. It should be kept in mind that the purpose, or the primary purpose, of inclusion of dietary protein component for swine is to provide individual AAs for the pig to use. In this regard, more and more commercially available feed-grade crystalline free AAs, such as lysine (Lys), methionine (Met), threonine (Thr) and tryptophan (Trp), have been commonly and increasingly supplemented to swine diets in practice.

EFFECTS OF AMINO ACIDS ON GUT HEALTH AND FUNCTIONS

Gut Protein and Amino Acid Metabolism

Gut digestion of dietary protein by the pig begins in the stomach through the biochemical actions with gastric HCl and proteases; however, there was no evidence indicating any absorption of AAs or peptides in the stomach (49, 50). The small intestinal digestion of protein comprises both luminal and mucosal phases. In the lumen, large protein molecules are broken down by the active proteases and peptidases to release oligopeptides as well as free AAs (51). Oligopeptides of more than three AA residues are further hydrolyzed extracellularly by brush border peptidases. It was estimated that the products of protein digestion in small intestinal lumen consists of approximately 20% free AAs and 80% tri- and di-peptides (50). The tri- and di-peptides are further hydrolyzed by both brush border and cytoplasmic peptidases into free AAs, or are absorbed intact and transported into the blood circulation (50, 51).

Comparing with the intact dietary protein, the supplemental crystalline free AAs are absorbed more rapidly and completely than the protein-bound AAs in pigs. However, this rapid absorption could cause a temporary surplus of free AAs and result in an imbalance of available AAs at the sites of protein synthesis. These rapidly absorbed free AAs may be oxidized too quickly for protein accretion (52), and the efficiency of dietary free AA utilization might be reduced when slowly digested protein compose a large portion in the diet (53).

Using dietary non-protein nitrogenous substances, such as urea, ammonium, mucins, as well as the enzymatic secretions and sloughed epithelial cells of the host, the microbiota in pig intestine can also synthesize AAs and proteins for incorporation into bacterial cells (25, 51). It was revealed that the small intestine is also the major site for the microbe-derived AA absorption, and some investigators suggested that the biosynthesis of AAs and proteins by the microbiota in the host GIT partake in the regulation of AA homeostasis of the host (25, 54). However, the intestinal microbiota do not make a significant contribution of AAs to the host since pig exhibits a negative nitrogen balance when fed an AA- or protein-free diet (25).

Some dietary proteins in swine small intestine may escape full enzymatic digestion and flow directly to the large intestine where microorganisms can ferment. The resulting products of the fermentation include mainly microbial proteins but also some small metabolites, such as ammonia, free AAs, urea, methane, and short-chain fatty acids. The amount of free AAs synthesized, however, represents <1% of the total hindgut fermentation products (50).

In terms of gut AA catabolism, there is a substantial breaking down by small-intestinal mucosal cells, as well as by the intestinal microbiota. The major pathways of microbial AA catabolism are deamination and decarboxylation, and the metabolites of the catabolism include ammonia, amines, phenoles, indoles, short- and branched-chain fatty acids, organic acids, and some gaseous compounds (25, 51). These AA metabolites form a highly complex reservoir in the gut, which has significant impact on the physiology of the gut epithelia (55). Some metabolites (e.g., butyrate and indole) are beneficial, while others (e.g., ammonia) exert deleterious effects on the epithelia. It should be pointed out that the gut microbiota can recycle these metabolites for synthesize microbial proteins when needed.

As discussed in section Dietary Factors Influencing Gut Health and Functions above, numerous nutritional factors can influence pig gut health and functions. The content and types of dietary AAs in the intestinal lumen are amongst these factors (14, 56). AAs are obligatory not only for maintaining intestinal mucosal mass and integrity, but also for supporting the growth of luminal microorganisms, and further impacting the health and functions of the gut. Although it was reported that some AA metabolites (e.g., hydrogen sulfide and nitrite) can be taken up by the intestinal cells from the extracellular medium (i.e., luminal content) and exert deleterious effects on intestinal mucosa (25), numerous studies have shown that several AAs, including essential AAs, such as Lys, Met and Thr, and non-essential AAs, such as arginine (Arg), glycine (Gly), cysteine (Cys), glutamate (Glu) and glutamine (Gln), play critical roles in maintaining or promoting gut health and gut functions (56, 57). **Table 1** provides an overview of the research during the last decade concerning the effects of some important AAs (quantitatively) on the health and functions of pig gut. In the following sections of this paper, the current, updated knowledge in the literature regarding the roles of AAs in supporting pig gut health and functions are discussed in more details. When necessary, some desired data from other monogastric animals, such as chickens, are also included.

TABLE 1 | Overview of the research during the last decade concerning the effects of amino acids on pig gut health and functions[a].

Amino acid(s)	Dietary concentrations[b]	Animals examined on	Major effects on gut health observed	References
Amino acid blend (AAB)[c]	1.00% AAB vs. 0.99% alanine	Weaner pigs (24-day-old)	Improved the intestinal morphology, barrier function, and antioxidative capacity; reduced the diarrhea incidence	(58)
Arginine	0.4 vs. 0.0 g/kg; twice daily	Newborn piglets	Showed a beneficial effect on the intestinal barrier system by reducing the trans-epithelial permeability in early rotavirus enteritis	(59)
Arginine	1.0 vs. 0.0%	Weaner pigs (5.3 ± 0.13 kg)	Increased the epithelial villus height and the mucosal vascular endothelial growth factor (VEGF) level of the small intestine	(60)
Arginine	1.0, 0.5, vs. 0.0%	Weaner pigs (21-day-old)	Protecting and enhancing intestinal mucosal barrier function; maintaining intestinal integrity	(61)
Arginine	1.0 vs. 0.0%	Growing pigs (55 kg)	Ameliorated the intestinal abnormalities caused by mycotoxin	(62)
Arginine	1.6, 0.8, vs. 0.0%	Weaner pigs (8.7 ± 0.43 kg)	Suppressed the inflammatory cytokine expression	(63)
Glutamine; glutamine + glutamate	1.00 vs. 0.00%; 0.88 to 0.66 vs. 0.00%	Suckling and nursery Pigs (14–to 21-day-old)	Increased the jejunal villus height by glutamine; increased the jejunal crypt depth by glutamine + glutamate	(64)
Glutamate	1.0 vs. 0.0%	Weaner pigs (5.6 ± 0.51 kg)	Improved intestinal mucosa morphology	(65)
Glutamate	2.0 vs. 0.0%	Growing pigs (55 kg)	Alleviated the adverse effects of mycotoxins on gut structure	(66)
Monosodium glutamate	4.0, 2.0, 1.0, 0.5, vs. 0.0%	Weaner pigs (21-day-old)	Increased jejunal villus height, DNA content, and antioxidative capacity	(67)
Monosodium glutamate	3.0 vs. 0.0%	Growing pigs (25.0 ± 1.3 kg)	Detrimental effects on several physiological and inflammatory parameters measured in the proximal intestine, while exerting some beneficial effects on the distal intestine	(16)
Glutamine	4.4 vs. 0.0%	Weaner pigs (21-day-old)	Improved the intestinal barrier function	(68)
Sulfur amino acids[d]	4.20, 2.90 vs. 1.30 g/kg	Growing piglets (18.6 ± 0.7 kg)	Enhanced the whole-body immune status	(69)
Sulfur amino acids[e]	1.15, 0.94, 0.89, 0.76, vs. 0.65%	Weaner pigs (21-day-old)	Improved intestinal functions via affecting the mucosal antioxidant systems	(70)
Methionine	4.0 vs. 0.0 g/kg	Weaner pigs (21-day-old)	Improved intestinal integrity and oxidative status	(71)
Methionine	0.145 vs. 0.000%	Weaner pigs (7.2 ± 0.97 kg)	Enhanced the duodenum morphology in association with reducing oxidative stress; Improved glutathione production in the mucosa cells	(72)
Cysteine	0.61 vs. 0.00%	Weaner pigs (28-day-old)	Increased the synthesis of mucosal epithelial proteins, such as glutathione and mucin	(73)
N-acetyl cysteine	500 vs. 0 mg/kg	Weaner pigs (14- t0 25-day-old)	Possessing a constructive regulation on the changes of the gut redox status and microbiota in response to weaning stress	(74)
Taurine	0.1 vs. 0.0%	Weaner pigs (5.8 ± 0.58 kg)	Decreased the stimulation of immune response to lipopolysaccharide (LPS); Improved intestinal epithelial barrier function	(75)
Tryptophan	0.4, 0.2, vs. 0.0%	Weaner pigs (7.6 ± 0.04 kg)	Aaltered intestinal microbial composition and diversity; Improved intestinal mucosal barrier function	(76)
Tryptophan	0.2 vs. 0.0%	Weaner pigs (8.9 ± 0.20 kg)	Improved the intestinal development; inhibited intestinal aging	(77)
Tryptophan	0.75, 0.15, vs. 0.00%	Weaner pigs (8.3 ± 0.15 kg)	Negatively affected intestinal morphology and tight junction proteins	(78)
Branched-chain amino acids[f]	Leu (1.38 vs. 1.26%), Ile (0.80 vs 0.60%), Val (1.01 vs. 0.74%)	Weaner pigs (28-day-old)	Enhanced intestinal development, and intestinal expression of amino acid transporters	(79)
Leucine	1.4 vs. 0.0 g/kg	Suckling pigs (7-day-old)	Improved the intestinal development; enhanced the expression of leucine transporters in the jejunum	(80)
Lysine	130, 100, vs. 70%	Young piglets (21.3 ± 0.39 kg)	Enhanced the richness and evenness of the intestinal microbial community	(81)
Threonine	8.5, 7.5, 6.5, 5.8, vs. 5.3 g/kg	Weaner pigs (10–25 kg)	Increased the humoral antibody production and serum specific IgG concentrations	(82)

[a] *In the text, some data on other species including chickens, mice and humans were used. In this table, however, only the studies on pigs are listed.*
[b] *For each study, the last concentration on the list was, in general, of the control group.*
[c] *The AAB included glutamate: glutamine: glycine: arginine: N-acetylcysteine at 5:2:2:1:0.5.*
[d] *Sulfur amino acids contain methionine + cysteine.*
[e] *The specific dietary methionine + cysteine concentrations were 0.83 + 0.32, 0.71 + 0.23, 0.53 + 0.36, 0.49 + 0.27, vs. 0.33 + 0.32, respectively.*
[f] *Branched-chain amino acids, including leucine (Leu), isoleucine (Ile) and valine (Val), were added to meet the recommendations.*

Amino Acid Effects on Gut Morphology Maintenance

Dietary AAs play critical roles in providing fuels for intestinal mucosa and, especially, Glu and Gln are major fuels for small intestine. Glutamate is commonly produced from Gln by glutaminase in the small intestine, and it could be a preferable fuel to Gln for enterocytes when the activity of glutaminase was low (56). The dominant role of Glu as an oxidative fuel may have therapeutic potential for improving the function of the infant gut, due to the high turnover rate of gut epithelial cells in infants (83). However, a recent study in growing pigs (16) showed that dietary supplementation of Glu (in a monosodium form) had detrimental effects on several physiological and inflammatory parameters measured in the proximal intestine, while exerting some beneficial effects on the distal intestine. For example, the dietary Glu supplementation increased the mRNA expression of pro-inflammatory cytokines including tumor necrosis factor α (*TNFα*), interleukin (IL)-1β (*IL-1β*), *IL-6*, *IL-8*, and *IL-10*, as well as the kinase ataxia telangiectasia mutated (*ATM*), in the proximal intestine (duodenum and jejunum), while inhibiting the expression of these pro-inflammatory factors in the distal intestine (ileum and colon) (16).

Similar to Glu, Gln can provide metabolic fuel for the rapidly dividing cells (particularly, the lymphocytes and enterocytes), as well as other epithelial cells of the intestines (17, 56, 84). Dietary Gln supplementation has positive effects on gut development via increasing villus height and V:C ratio, and reducing crypt depth, due to its metabolic fuel function for the gut (6, 17).

Dietary Arg supplementation can attenuate the degree of tissue damage in intestinal ischemia, promote intestinal mucosa healing (85), and reverse intestinal dysfunction (86). Sukhotnik et al. (87) reported in rats that oral Arg supplementation improved the duodenal, jejunal and ileal weights and mucosal cell proliferation, as well as restored the intestinal absorptive function after ischemia. The significant effect of Arg on the growth of GI mucosa in a variety of research animals may be attributed to its unique role over polyamine biosynthesis (88, 89). Notably, polyamines produced by the gut microbiota are associated with intestinal mucosal protection and epithelial cell migration (85). In rats, Hurt et al. (90) also demonstrated that diets supplemented with Arg and Glu helped the maintenance of intestinal tissue oxygenation and/or brush barrier function, and improved systemic nitrogen balance. Arginine is the substrate for nitric oxide (NO) synthesis. Studies showed that either inhibition or overproduction of NO had injurious effects on the guts of pigs and other animals (91, 92).

Sulfur-containing AAs, such as Met and Cys, are also beneficial for maintenance of gut morphology. Luminal microbes are responsible for the extensive catabolism of dietary Met in the gut (56). A study on nursery pigs showed that adding Met in drinking water can improve small intestinal morphology by increasing villous height. Methionine can reduce the bacteria fermentation via improving nutrient digestion and absorption and leaving less substrates for bacteria to use (93). Dietary Met for nursery pigs can enhance the morphology of duodenum in association with reducing oxidative stress and improving glutathione (GSH) production in the mucosa cells (72). Cysteine is extensively utilized by animal gut (56). Bauchart-Thevret et al. (73) concluded that the gut of weanling pigs utilizes 25% of the dietary Cys intake, and that synthesis of mucosal epithelial proteins, such as GSH and mucin, are a major non-oxidative metabolic fate for Cys.

Threonine, with a high utilization rate by the gut, is well involved in intestinal maintenance and functionality (94, 95). It has been reported that Thr is an important component of mucins (40% of the mucus glycoproteins) in the gut (96). Dietary Thr supply is critical for maintaining gut morphology and development because Thr plays a key role in mucin synthesis and barrier integrity maintenance (56, 96, 97). Either deficiency or excess of dietary Thr, however, has adverse effects on the synthesis of intestinal mucosal proteins and mucins in young pigs (96). Chen (98) and Min et al. (96) both reported a positive effect of adequate dietary Thr supplementation on chicken gut morphology, such as villus height, epithelial thickness, number of goblet cells, and crypt depth in three segments of small intestine. Especially, the increasing crypt depth in the Thr-supplemented chickens might provide more surface area for nutrient absorption by increasing enterocyte proliferation and intestinal mucin secretion (96). Most researchers [such as (17)] consider low crypt depth as an indicator of decreasing metabolic cost of intestinal epithelium turnover, while deeper crypts indicating faster epithelium turnover for renewal of the villus as needed in response to the inflammation from pathogens or their toxins. In this particular study, however, Min et al. (96) explained that the deeper crypts indicate increased enterocyte proliferation, increased villus surface area, increased mucin secretion (because goblet cells are mainly present in the crypts) and, therefore, better nutrient absorption.

Amino Acid Effects on Gut Luminal Microbiota

Although the contributions of the *de novo* synthesized microbial AAs to the AA requirements of pigs are still not certain (25), luminal AAs do have a significant impact on the microflora in the small intestine. Glutamate can markedly change the composition of, and increase the diversity of, the intestinal microbial community by promoting the colonization of *Faecalibacterium prausnitzii* and *Roseburia* (99). In addition, it has also been reported in a pig model that the addition of dietary Glu can help to modify the intestinal microbial composition to prevent obesity (99).

The extensive catabolism of dietary Lys in the gut is taken care by the luminal bacteria rather than the enterocytes and, therefore, it is postulated that that dietary Lys restriction can affect gut microbiota (56). Yin et al. (81) firstly reported that Lys restriction enhanced the intestinal richness and evenness of microbial community. Moreover, using a bioinformatics software package, Yin et al. (81) predicted that the altered intestinal microbiota caused by Lys restriction might influence AA metabolism, membrane transport, endocrine system, carbohydrate metabolism, cellular signaling, replication and repair.

Threonine not only regulates the protein homeostasis in the body but also supports the growth of bacteria in the gut (100).

Dong et al. (101) reported that dietary Thr supplementation to a low crude protein diet for laying hens recovered the bacteria diversity caused by the low dietary protein, and increased the abundance of potential beneficial bacteria. One of the explanations for the increased bacteria diversity in this study might be the up-regulation of mucin gene expression by supplementing Thr, because mucins cannot be digested in the small intestine and thereby can reach the cecum, acting as a substrate for saccharolytic bacteria.

Extensive bacteria fermentation of undigested feed components in ceca is responsible for detoxification of harmful substances and prevention of pathogen colonization (102). The major products from bacteria fermentation are short chain fatty acids (a.k.a. volatile fatty acids), which play a key role in maintaining gut health by lowering luminal pH and regulating the microbial composition, especially by stimulating the growth of beneficial bacteria (101). A study on nursery pigs showed that adding a liquid Met analog (an acid form) in drinking water tended to decrease the gastrointestinal pH and the concentrations of cecum acetic acid. However, the total number of lactic acid bacteria and *E. coli* in cecum was not affected (93). Thus, Kaewtapee et al. (93) concluded that this liquid Met analog might enhance nutrient digestion and absorption (due to the increased villus height) and, subsequently, the growth performance. Therefore, less substrates remained for bacteria to use, which results in less metabolites (volatile fatty acids) from the fermentation processes (93).

Amino Acid Effects on Gut Immunological Functions

Studies have shown that some AAs are critical in the maintenance of the immune-physiological functions of the gut (103, 104). Arginine is a central intestinal metabolite, both as a constituent of protein synthesis and as a regulatory molecule limiting intestinal alterations and maintaining gut immune-physiological functions (85, 86, 89). A large number of studies in animals and human have identified the important role of Arg in intestinal immunity (56, 103). Corl et al. (59) reported that early in rotavirus enteritis, Arg has a beneficial effect on the intestinal barrier system of piglets by reducing trans-epithelial permeability via a mammalian target of rapamycin/$p70^{S6k}$-independent mechanism. Another study on pigs showed that supplementing 1% Arg to a mycotoxin-contaminated feed ameliorated the intestinal abnormalities caused by mycotoxin (62). Also, Hurt et al. (90) demonstrated in rodents that diets supplemented with both Arg and Gln enhanced the immunity of gut mucosa and helped maintaining intestinal tissue oxygenation and/or brush barrier function.

Threonine is an essential component of mucus glycoproteins (approximately 40% of the protein) in GIT (96). Threonine supply is critical for maintaining gut immunological functions by participating in mucin synthesis to maintain gut barrier integrity (56, 96, 97). Moreover, Thr has been reported to be an AA with the highest concentration in the γ-globulins of rabbits, horses and humans. Also, the humoral antibody production and serum specific IgG concentrations were all increased in response to the increased intake of true ileal digestible Thr in young pigs (82). All these results indicate that Thr is very important for the protection of gut mucosal barrier and for the immune functions.

As sulfur-containing AAs, Met and Cys have been shown to be beneficial for animal immune system (105–107). Methionine serves as a methyl donor for several important processes, such as DNA methylation and polyamine synthesis (108), which are important for enhancing immune cell proliferation during immune challenge. Cysteine is needed to produce taurine, acting as an antioxidant, as well as a cell membrane stabilizer (108). Taurine is particularly abundant in leucocytes (109). During the immune system stimulation (ISS), utilization of Cys for the production of the compounds involved in immune response, such as taurine and GSH, is increased (108). Rakhshandeh et al. (110) reported that the immune system stimulation by injection of lipopolysaccharide reduced the ratio of whole-body nitrogen to sulfur balance indicating that the sulfur-containing AAs are preferentially preserved for the production of non-protein compounds, such as GSH, to enhance the whole body immune status. This implies that more Met and Cys are needed during the state of immune challenge.

The Anti-inflammatory Effects of Amino Acids

Studies have shown that some AAs can alleviate intestinal inflammation. Using a mouse model, Chau et al. (111) reported that dietary Arg supplementation reduced the expression level of ileal transcript mRNA encoding interleukin-4 (IL-4), a key mediator of intestinal mastocytosis and macromolecular permeability. The data suggested that increasing bioavailable Arg ameliorates intestinal inflammation and pathology. It is likely that the altered activities of Arg-catabolizing enzyme families, arginases and NO synthase (NOS), contribute to ameliorating allergic inflammation. It can be postulated that the activity of arginase through conversion of Arg into ornithine enhances epithelial barrier function. Recent studies have showed that NOS activity can dampen inflammation through regulation of the myeloid and lymphoid cell activation (85). Nitric oxide produced by inducible NOS in inflammatory monocytes and dendritic cells can regulate inflammatory cytokine production, cell differentiation, and survival (85). Modulating the arginase- and NOS-mediated pathways through regulation of the bioavailability of L-Arg or its precursor L-Cit via oral supplementation can provide an efficient and practical strategy to dampen intestinal inflammation and pathology, and regulate the mucosal immunohomeostasis (85).

Ample evidence also demonstrated that Gly has efficacy as an anti-inflammatory and cytoprotective agent (112). While the mechanism responsible for the protective effects of Gly are unclear, it is likely to be multi-factorial involving direct effects on target cells, inhibition of Gly-gated chloride channels, and/or inhibition of inflammatory cell activation. Some studies indicated that Gly has a protective effect in mesenteric

ischemia/reperfusion (IR) injury through the inhibition of apoptosis (113), while others have shown that Gly protection against intestinal IR injury is reached by a mechanism consistent with Gly uptake (114).

The Anti-oxidative Functions of Amino Acids

All AAs are susceptible to oxidation, although their susceptibilities vary considerably (115). Methionine and Cys are the most susceptible to oxidation by ROS. Because of this, they can thus help to defend cells against oxidative stress. Therefore, Met and Cys are considered as endogenous antioxidants (116) functioning through an important antioxidant defense mechanism (117, 118). The anti-oxidative ability of endogenous Met can protect many proteins from oxidative damage. Methionine in the diets for nursery pigs can enhance the duodenum morphology in association with reducing oxidative stress and improving GSH production in mucosa cells (72).

Glutathione is a major cellular antioxidant that functions to detoxify intestinal oxidative stress and injury related to microbe-induced inflammation (73). Several AAs execute antioxidant functions through GSH. Three AAs are needed to synthesize GSH: Gly, Glu and Cys. Glutamine is easily converted to Glu to produce an antioxidant GSH. Therefore, dietary supplementation of Gln may have beneficial effects in reducing the symptoms of inflammatory disorders and may protect the gut against the damaging effect of oxidative stress (84).

Gut Dysfunction Reverse and Detoxification by Amino Acids

Many studies showed that AAs could be used to alleviate the adverse effects of toxins and gut barrier dysfunction. Through a study with 15 growing pigs, Duan et al. (66) concluded that Glu may be useful as a nutritional regulating factor to alleviate the adverse effects of mycotoxins on gut structure (histology, morphology and barrier function) and growth performance, because dietary Glu supplementation partially counteracted the impairments induced by the mycotoxins in the mold-contaminated feed.

Glutamine is a unique nutrient for enterocytes, capable of dual signaling and augmenting the effects of growth factors that govern cellular proliferation and reconstruction after damage (119). Souba et al. (120) suggested that Gln is an important AA in humans for maintenance of gut structure, metabolism, and function especially during critical illness when the gut mucosal barrier is compromised. Kessel et al. (121) reported that enteral feeding of Gln suppressed the injury to the mucous membrane of the small intestine caused by lipopolysacharide endotoxemia in rat. A recent study conducted by Xue et al. (17) in broilers suggested that Gln improved intestinal architecture in the jejunum and ileum during the necrotic enteritis outbreak and recovery and consequently favors intestine structure (increasing villus height and decreasing crypt depth) and functions. In mice, dietary Gln supplementation can block ethanol-induced gut permeability, and protect colonic epithelial tight junctions and adherent junctions (21, 122). Hence, dietary Gln supplementation can maintain gut barrier function and prevent alcohol-induced gut barrier dysfunction (21, 84, 122).

Glycine is not only an essential substrate for synthesizing several important biomolecules (such as glucose and GSH), but is also utilized in the biochemical detoxification via conjugation of endogenous or xenobiotic toxins (123, 124). A study with a rat model suggested that local Gly perfusion diminished the ischemia-reperfusion injury in small intestinal mucosa, as indicated by the increased mucosal protein content, increased mucosal DNA content, and maintenance of mucosal glutaminase activity, during either the pre-ischemia phase or the pre-reperfusion phase (114). Lysine also serves as a partial antagonist of gut serotonin 5-HT4 receptors to reduce stress-related diarrhea as well as anxiety, and may modulate gut motor function (125). Cysteine can modulate local cytokine gene expression, suppress pro-inflammatory and chemotactic gene expression, and promote the expression of pro-apoptotic pathways, in addition to its known anti-oxidant and immunological effects, suggesting that dietary supplementation of Cys may support the recovery of gut mucosal homeostasis (126).

CONCLUSIONS

Overall, AAs are beneficial for maintaining gut health in pigs, especially from the morphology and microbiota perspectives. As summarized above, some AAs provide fuel for the growth and proliferation of intestinal epithelial cells, and others offer nutrients to luminal microbiota for maintaining its diversity and functions. Moreover, the types and levels of dietary AAs can differently or similarly affect the gut structure and functions. Arginine, Gln, Met, and Thr could help with relieving the post-weaning stress of young pigs by improving the immunological functions, anti-inflammatory ability, or anti-oxidant capacity. Glutamate, Gln, and Gly can reverse gut dysfunction under disease conditions and help to reconstruct the gut structure after its damage. Threonine, Arg, Gln, Met and Cys are all beneficial to protecting gut barrier function and maintaining gut mucosal immunity. Furthermore, Glu, Lys, and Thr play important roles in supporting and affecting the growth of bacteria in the intestinal lumen. That being said, the complex mechanisms underlying AAs' effects on gut morphology and functions still warrant further investigation. Considering the global push to ban the usage of antibiotics as AGP for swine production, our current primary effort may be made to explore the specific effects of individual AAs on gut microbiota of young pigs.

AUTHOR CONTRIBUTIONS

SL contributed the conception and structure design to the paper. ZY organized the literature and wrote the first draft of the paper.

Both authors contributed to manuscript revision and approved the final version for submission.

FUNDING

This research was financially supported in part by USDA-NIFA Hatch/Multistate Project 1007691. Some financial and in-kind supports were obtained from the Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany), Ajinomoto Animal Nutrition North America, Inc. (Chicago, IL, USA), and Nutra Blend, LLC. (Neosho, MO, USA).

ACKNOWLEDGMENTS

Contributions to SL's Swine Nutrition Research Program at Mississippi State University from his current and former graduate students, Taiji Wang, Naresh Regmi, Casey Durfey, ZY, Shamimul Hasan, and Rebecca Humphrey are acknowledged.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 February 2020
doi: 10.3389/fvets.2020.00031

Effects of Dietary Grape Seed Meal Bioactive Compounds on the Colonic Microbiota of Weaned Piglets With Dextran Sodium Sulfate-Induced Colitis Used as an Inflammatory Model

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OPEN ACCESS

Edited by:
Vincenzo Tufarelli, University of Bari Aldo Moro, Italy

Reviewed by:
Francesco Vizzarri, University of Bari Aldo Moro, Italy
Inkyung Park, Animal Biosciences and Biotechnology Laboratory (USDA-ARS), United States

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *11 October 2019*
Accepted: *14 January 2020*
Published: *26 February 2020*

Citation:
Grosu IA, Pistol GC, Marin DE, Cişmileanu A, Palade LM and Ţăranu I (2020) Effects of Dietary Grape Seed Meal Bioactive Compounds on the Colonic Microbiota of Weaned Piglets With Dextran Sodium Sulfate-Induced Colitis Used as an Inflammatory Model. Front. Vet. Sci. 7:31. doi: 10.3389/fvets.2020.00031

Microbiota affects host health and plays an important role in dysbiosis. The study examined the effect of diet including grape seed meal (GSM) with its mixture of bioactive compounds on the large intestine microbiota and short-chain fatty acid synthesis in weaned piglets treated with dextran sodium sulfate (DSS) as a model for inflammatory bowel diseases. Twenty-two piglets were included in four experimental groups based on their diet: control, DSS (1 g/kg/b.w.+control diet), GSM (8% grape seed meal inclusion in control diet), and DSS+GSM (1 g/kg/b.w., 8% grape seed meal in control diet). After 30 days, the colon content was isolated and used for microbiota sequencing on an Illumina MiSeq platform. QIIME 1.9.1 pipeline was used to process the raw sequences. Both GSM and DSS alone and in combination affected the diversity indices and *Firmicutes:Bacteroidetes* ratio, with significantly higher values in the DSS-afflicted piglets for *Proteobacteria* phylum, *Roseburia*, *Megasphera* and *CF231* genus, and lower values for *Lactobacillus*. GSM with high-fiber, polyphenol and polyunsaturated fatty acid (PUFA) content increased the production of butyrate and isobutyrate, stimulated the growth of beneficial genera like *Prevotella* and *Megasphaera*, while countering the relative abundance of *Roseburia*, reducing it to half of the DSS value and contributing to the management of the DSS effects.

Keywords: inflammatory bowel diseases, colitis, piglet, grape seed meal, dextran sodium sulfate, microbiota

INTRODUCTION

The intestinal inflammatory bowel disease (IBD) affects the life quality of a large number of people and is a significant problem for public health (1–3). Although it is now known that IBDs are symptoms of an unbalanced inflammatory response between commensal microflora, pathogens, and the host immune system (4), the precise nature of the intestinal microbiota perturbation and the resulting effects remains to be identified. Most of the risk factors implicated in the development of IBD, including diet, stress and anti-inflammatory drugs, can also perturb the

commensal component of the microbiota (5, 6). While the microbiota of healthy hosts shows little shifts in time, the gut microbiota of IBD affecting hosts is not stable. Dysbiosis in IBD do not just change the populations of different microbiota species but is also associated with perturbations of microbial metabolites, like short-chain fatty acids (SCFAs), which can further affect the host (7). There is growing interest to manipulate the gut microbiota for preventative and therapeutic purposes.

In recent years, alternative remedies were studied as promising therapy for IBD, some of the most important ones being the use of natural bioactive compounds with high anti-inflammatory activity such as polyphenols, polyunsaturated fatty acids (PUFAs). Also, SCFAs (acetate, n-propionate, and n-butyrate), which are solely produced by gut microbiota and have shown to ameliorate the disease effects. Studies have demonstrated that dietary polyphenols such as flavonols, stilbenoids, and anthocyanins, or chlorogenic acid derived from tomatoes (8, 9) and blueberries (10) had positive effects in animals with dextran sodium sulfate (DSS)-induced colitis. For example, Scarano et al. (8) demonstrated that mice with DSS-induced colitis fed with tomato diet rich in polyphenols were characterized by a significant "re-shaping" of the gut microbiota in terms of composition when compared to the DSS group, as indicated by a significant increase of the ratio *Bacteroidetes: Firmicutes* as compared with the control. Also, dietary blueberries or broccoli influenced the composition and metabolism of the cecal microbiota and colon morphology in a mice model of IBD (10). Other polyphenol sources found to re-shape the microbiota composition in mice model of IBD are grape seed extract (11) and curcumin (12). In the study of Wang et al. (11), grape seed extract rich in polyphenols increase the abundance of non-pathogenic bacteria in the gut, contributing to the improvement of gut function and IBD symptoms. Also, these dietary bioactive compounds impact the colon positively by affecting the transit time and the production of SCFAs that further affect the pH and enhance the gut barrier properties along with also a protective effect on the colonic mucosa (13). PUFAs have shown to modulate the microbiota dynamics in animal models of IBD. Constantini et al. (14) have demonstrated that ω-3 PUFAs lead to microbiota enrichment with more beneficial bacterial strains. The eicosapentaenoic acid-free fatty acid diet counteracts the DSS-dependent dysbioses of the gut microbiota, facilitating the recovery of a health-promoting layout of the gut microbial ecosystem in mice (15).

Various animal models were used for more than two decades to investigate the pathogenesis and etiology of human IBD to gain indispensable insights into morphological, metabolic, and microbiota changes as well as on other factors associated with the evolution of IBD but also for therapeutic evaluation. The models of chemically induced IBD have used different animal species (mice, rats, and rabbits) (5, 16, 17). Mouse have been considered the most suitable animal model for the relative analogy to human intestine in terms of immune response and inflammatory genes (18).

Recently, pig held an essential place as an animal model due to the similarities they share with humans in terms of gastrointestinal morphology and physiology, which makes them suitable for human studies (7, 19, 20). In particular, pigs are considered to be an excellent large-animal model to study intestinal inflammation in humans (21). Additionally, the pig microbiome is also comparable to humans, facilitating the examination of the relationship between microbial communities, diet, and intestinal health (22). Nutritional interventions, such as ω-3 PUFAs administration, proved to modulate the inflammation and contributed to delaying the onset of experimental DSS-induced IBD in pigs (23).

Using Illumina high-throughput sequencing of the 16S rRNA gene, we aimed in the present study to investigate the capacity of the grape seed meal (GSM) as a dietary rich source of bioactive compounds (polyphenols, ω-6 fatty acids, fibers, etc.) to alleviate the DSS-induced alterations of bacterial diversity and the microbial community composition at the phylum and lower taxonomical levels. Active molecules derived from grape or grape by-products and their effect on IBD have been investigated in the mouse model, but mostly as individual components. In the present study, we investigated the effect of the entire complex of bioactive compounds from grape seed by-product, taken as example the Mediterranean diets that through the diversity of ingredients (fresh vegetables, fruits, nuts, fish, and olive oil) and their high concentration in different bioactive nutrients provided promising results by alleviating IBD symptoms and increasing microbiota diversity. To our knowledge, this is the first study that evaluates the capacity of GSM to modulate the microbiota of DSS-treated piglets as well as the correlations between microbiota composition and the production of colonic SCFAs.

MATERIALS AND METHODS

Animals and Experimental Treatments

Twenty-two TOPIGS-40 hybrid healthy weaned piglets (9.13 ± 0.03 kg average body weight) were individually ear-tagged and randomly assigned to four experimental groups (5–6 piglets/group) based on their initial body weight as follows: (1) Control; (2) DSS; (3) GSM; (4) DSS+GSM.

Control and DSS groups were fed a standard diet based on maize and soybean meal. GSM and DSS+GSM groups were fed the control diet, including 8% dried GSM without interfering with the nutritional requirements of weaning piglets, performance, size, and digestibility. The diets were formulated to meet all nutritional requirements for post-weaning piglets (24) as described by (25). Ingredients and chemical composition of the diets are presented in **Tables 1A–C**. The GSM was provided by a local commercial company (S.C. OLEOMET-SA S.R.L., Bucharest, Romania).

DSS (dextran sulfate 40 sodium salt, MW = 36–50 kDa, Carl Roth GmbH, Germany, 1 g/kg body weight) was orally administered to DSS and DSS+GSM experimental groups for 5 consecutive days. Two cycles of DSS treatment (days 1–5 and 21–26 of the experiment) were used to induce chronic intestinal inflammation in piglets.

All piglets from each experimental group were housed in a large box (a box/group) and every group included mixed sexes. The body weight was recorded at the beginning (day 0) and at the end of the feeding experiment (day 30) for each animal;

Table 1A | Composition and nutrient content of experimental diets (%).

Ingredients (%)	Control diet	GSM diet
Corn	67.47	58.5
Soybean meal	19	18
Gluten	4	4
Milk replacer	5	5
Soya oil	–	2
L Lysine	0.4	0.4
DL Methionine	0.1	0.15
Monocalcium phosphate	1.46	1.33
Feed grade limestone	1.37	1.42
Salt	0.1	0.1
Choline premix	0.1	0.1
Vitamin mineral premix[a]	1.0	1.0
Grape seed meal	–	8
Analyzed composition		
Crude protein (%)	18.42	18.21
Fat (%)	3.03	3.19
Cellulose (%)	3.12	5.8
Lysine (%)	1.2	1.2
Methionine +Cysteine (%)	0.72	0.72
Calcium (%)	0.90	0.90
Phosphorus (%)	0.65	0.65
Metabolizable energy (ME, kcal/kg)	3,248	3,178

[a] *Vitamin–mineral premix/kg diet: (0–18 days): 10,000 UI vit. A; 2,000 vit. D; 30 UI vit. E; 2 mg vit. K; 1.96 mg vit. B1; 3.84 mg vit. B2; 14.85 mg pantothenic ac.; 19.2 mg nicotinic ac.; 2.94 mg vit. B6; 0.98 mg folic ac.; 0.03 mg vit. B12; 0.06 biotin; 24.5 mg vit. C; 40.3 mg Mn; 100 mg Fe; 100 mg Cu; 100 mg Zn; 0.38 I; 0.23 mg Se.*

Table 1B | Antioxidant activity and polyphenols content of experimental diets.

Item	Control diet	GSM diet
DPPH (μM TRE/g sample)	206.89	966.35
Total polyphenols (mg GAE/100 g)	382.93	897.15
Polyphenols composition (μg/mL extract catechin equivalent)		
Hydroxycinnamic acids	318.11	362.25
Flavonols	0	311.12
Isoflavonoids	85.24	122.42
Anthocyanins	0	187.65

Table 1C | Composition in fatty acids of experimental diets.

Polyunsaturated fatty acid content	Control diet	GSM diet
Total PUFA (g/100 g total FAME)	47.58	52.01
Total ω-3 FA (g/100 g total FAME)	2.20	1.45
Total ω-6 FA (g/100 g total FAME)	45.38	50.56
ω-6/ω-3 ratio	20.61	34.88

the feed intake was recorded daily/pen/group. Piglets were fed the experimental diet for 30 days and had free access to food and water all along the experimental period. After 30 days, the piglets were sacrificed, and content from the descending colon was collected from each animal, which was immediately stored at −80°C until further use.

During the whole experimental period, the stool cosinstency was assessed daily. Piglets did not receive veterinary treatments for diarrhea. For each experimental group, the diarrhea incidence was calculated with the following formula adapted after (26): (total number of diarrhea-affected piglets/total number of experimental piglets) × 100%.

Chemical Characterization of the Diets

Feed samples of control and experimental diets were analyzed for nutrient content, dry matter, crude protein, crude fat, crude fiber, and ash according to the International Standard Organization methods [SR ISO 6496/2001, Standardized Bulletin (2010) http://www.asro.ro].

Total polyphenol content was measured and identification of different classes of polyphenols and PUFAs of the diets was carried out by Folin-Ciocalteu reaction, HPLC-DAD-MS, and gas chromatography as described by Taranu et al. (25, 27). Diet antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH, as described previously (28).

Sampling and 16s rRNA Sequencing

Microbial genetic material was extracted from 200 ml colonic content samples using the QIAGEN mini Stool Kit (Qiagen, Dusseldorf, Germany) as described by Grosu et al. (29). The DNA integrity and concentration were verified on gel electrophoresis and Nanodrop Spectrophotometer. The library formation and sequencing of the 16S rRNA gene were carried out using a MiSeq® Reagent Kit V3-V4 on a MiSeq-Illumina® platform using the 300PE approach by BMR Genomics (Padova, Italy).

Microbiota Bioinformatics and Statistical Analysis

The FastQ raw data sequences resulting from the Illumina platform sequencing were further processed using an open reference OTU (operational taxonomic unit) strategy in QIIME (v1.9.1) (30) with default settings. The bacterial OTUs were generating using the UCLUST function with a *de novo* protocol of 97% similarity threshold. Taxonomy was assigned to the resulting representative sequences by comparing against the Greengenes database v13_8 with the help of the UCLUST method, selecting the similarity threshold of 90%. OTUs with a relative abundance of ≤0.005% were removed and were Chimera checked in QIIME with the Blast fragments approach. In order to remove sampling depth heterogeneity, a rarefaction with a cutoff of 23,946, which represents the lowest number of reads from a sample, was performed.

Alpha (within-sample) diversity (estimated with Chao1, observed_otus, PD_whole_tree) and beta (between-sample) diversity (DPCoA) indices were generated using the phylogeny-based unweighted and weighted UniFrac metrics. An OTU-based phylogenetic tree was also generated using FastTree method inside QIIME. A heatmap was also built around the OTU table of the species that were found above a 0.005% relative abundance.

GC Method for SCFAs in Pig Feces

SCFAs (acetic, propionic, butyric and valeric acids) were quantified in water extracts of pig's colon content sample by gas chromatography. Briefly, colon samples were mixed with distilled water in a proportion of 1:2 (w:v), centrifuged at 12,000 g for 25 min and diluted 1:2 with distilled water. A sample volume of 1 μL from the centrifuged extract was injected under split mode into a gas chromatograph (Varian, 430-GC) equipped with a capillary column Elite-FFAP with a length of 30 m, an inner diameter of 320 μm, and a film thickness of 0.25 μm (Perkin Elmer, USA). The carrier gas was hydrogen; flow, 1.5 mL/per min. The injector was set at 250°C, and the split rate was 1:40. The flame ionization detector (FID) was set to 200°C, and the column oven was set to 110°C. The oven temperature was increased to 170°C at a rate of 12°C/min, where it was held for 9.5 min. The analysis time was 10 min. The sample concentration was calculated referring to a standard commercial mixture of volatile fatty acids (CRM46975, Supelco, USA). Results were expressed as μmol/g for total SCFAs and as a percentage for individual SCFA.

Statistical Analysis

The internal statistical method used by QIIME in determining significance between sample groups was performed using the ANOSIM statistical method, a non-parametric method; the significance is determined through permutations. Statistical significance of difference like comparisons between effects was performed under XLstat software package (http://www.xlstat.com) using two-way analysis of variance (ANOVA). The model effects were DSS, GSM, and their interaction (DSS × GSM), in order to evaluate the overall treatment effect. Values of $p < 0.05$ indicated statistically significant differences among the different comparisons. The results are presented as mean ± SEM. The heatmap built on the OTU table for a relative abundance above 0.005% with clustering for OTU ID and treatment was also constructed using XLstat. Additionally, effect sizes were reported for the model effects as described by Lakens (31). Eta squared (η^2) measures the proportion of the total variance in a dependent variable that is associated with the membership of different groups defined by an independent variable. Omega squared (ω^2) is an estimate of how much variance in the response variables are accounted for by the explanatory variables.

RESULTS

Diet Composition

The chemical composition of control and GSM diet is presented in **Tables 1A–C**. GSM experimental diet had an increased content of fibers (cellulose) compared to the control diet (5.80 vs. 3.12%, respectively, **Table 1A**). Also, the GSM diet had a higher concentration of polyphenols and an increased antioxidant activity compared to that of the control diet (**Table 1B**). GSM used in the present study had a total polyphenol content of 5567.22 mg GAE/100 g sample (data not shown). HPLC-DAD–MS analysis showed that GSM was rich in flavonoids (catechins, epicatechins, and procyanidins), the highest concentration being observed for caffeoylquinic acid (57.36 mg/100 g), ferulic acid derivate (34.43 mg/100 g), and dicaffeoylquinic acid (28.85 mg/100 g) (data not shown). Also, our results showed the presence of the antioxidant activity (DPPH) in GSM (5054.71 μM TRE).

The composition in PUFA of the GSM diet was 52.01/100 g of fatty acid methyl esters (FAME) (**Table 1C**) of which the highest proportion was registered for ω-6 fatty acids (50.56 g/100 g FAME) compared to the control diet (47.58 total PUFA and 45.38 g ω-6 fatty acids/100 g FAME). Notably, the ratio of ω-6/ω-3 PUFAs was increased in the GSM diet compared to the control diet (34.88 vs. 20.61, **Table 1C**). The gas chromatography analysis showed that GSM had a high concentration of total PUFAs (65.17 g/100 g sample), with a high content of ω-6 fatty acids especially linoleic acid (63.63 g/100 g, data not shown). GSM contained also an important amount of fibers (37.76%, data not shown).

Table 2 | Diarrhea incidence in experimental groups.

Week of experiment	Experimental group*			
	Control	DSS	GSM	DSS+GSM
Week 1	16.67	20.00	0.00	40.00
Week 2	33.33	60.00	16.67	40.00
Week 3	16.67	80.00	0.00	0.00
Week 4	0.00	40.00	0.00	20.00

**Data represent the percentages of diarrhea-affected animals from total number of animals per experimental group (control group: n = 6; DSS group: n = 5; GSM group: n = 6; DSS+GSM group: n = 5), in all the weeks of the experiment.*

Effects of GSM Diet on Growth Performances and Diarrhea Incidence in DSS-Treated Piglets

After the first DSS challenge, severe diarrhea was observed, in week 2 of the experiment, with 60% of total piglets from the DSS-treated group being affected (**Table 2**). The incidence of diarrhea in the DSS group was also increased in week 3 of the experiment, after the second DSS challenge, and these piglets remained affected until the end of the experiment (week 4, **Table 2**). In DSS-treated piglets receiving GSM diet, the diarrhea incidence was below that of the DSS group, throughout the experiment (**Table 2**).

There were no significant differences for final body weight, average daily gain, and feed intake between treatments (**Table 3**). Regarding feed efficiency (FE), our results showed an increased FE in the DSS group (2.12), while both GSM and DSS+GSM groups had similar FE (1.922 and 2.009, respectively), the best FE being observed for the control group (1.797, **Table 3**). No significant differences in growth and feed intake were found among treatment groups.

Comparison of Richness and Diversity of Gut Microbiota Sequencing

To understand the effect of DSS and GSM on the composition of gut microbiota, we performed 16S rRNA V3–V4 region

Table 3 | The effect of GSM diet on performance of DSS-treated piglets.

	Experimental group*						
	Control	**DSS**	**GSM**	**DSS+GSM**	***p*-value (DSS effect)**	***p*-value (GSM effect)**	***p*-value (DSS × GSM effect)**
ADG (g)	494.1 ± 53.4	435.7 ± 91.0	432.1 ± 23.3	457.1 ± 52	0.693	0.765	0.362
ADFI (g/day/pig)	0.825 ± 0.04	0.925 ± 0.05	0.820 ± 0.04	0.856 ± 0.05	0.358	0.160	0.100
Initial BW (kg)	9.08 ± 0.20	9.00 ± 0.30	9.08 ± 0.30	9.00 ± 0.30	0.943	0.830	0.830
Final BW (kg)	22.92 ± 1.50	21.20 ± 2.40	21.10 ± 0.80	21.80 ± 1.6	0.261	0.922	0.122
FE (feed:gain)	1.797	2.120	1.898	1.873	0.466	0.992	0.889

**Pigs were fed for 30 days with a control diet or a diet including 8% GSM and challenged or not with DSS. Values are represented as the mean ± SEM (Control group, n = 6; DSS group, n = 5; GSM group, n = 6; DSS + GSM group, n = 5); DSS, dextran sulfate; GSM, grape seed meal; ADFI, average daily feed intake; ADG, average daily gain; FE, feed conversion ratio.*

Table 4 | Observed OTUs, PD_Whole_Tree index and Chao 1 mean of the microbiota of piglets treated with DSS and fed with Control or GSM diet.

Experimental group*	**Observed OTUs**	**PD_Whole_Tree index**	**Chao1**
Control	4807.2 ± 188^{a}	258.5 ± 6^{a}	$12{,}766.9 \pm 481^{a}$
DSS	$4375.2 \pm 651^{a,b}$	$241.9 \pm 33^{a,b}$	$10{,}242.4 \pm 407^{a,b}$
GSM	$4022.7 \pm 623^{a,b}$	212.6 ± 30^{b}	$10{,}304.8 \pm 857^{b}$
DSS+GSM	3820.9 ± 495^{b}	196.4 ± 22^{b}	8746.1 ± 901^{b}

**Control and DSS-treated piglets were fed for 30 days with a control diet or a diet containing 8% GSM, as described in the Materials and Methods section. At the end of the experiment, samples of colonic content from all animals (n = 5) were collected and analyzed for identification of microbial groups. Values within a column with different superscript letters are significantly different (p < 0.05).*

sequencing. On the whole, 1,111,323 high-quality sequences and 35,981 distinct operational taxonomic units (OTUs) were identified between all the experimental groups from the usable raw data after the optimization process as follows: (1) control group−4807 OTUs; (2) DSS group−4375 OTUs; (3) GSM group−4022 OTUs; and (4) DSS+GSM group−3820 OTUs.

Based on the sequencing data, the richness of the gut microbiota (Chao1) and the observed OTUs, Chao1, and PD_Whole_Tree indices were decreased after DSS challenge compared to control (**Table 4**, **Figures 1A–C**). Similar results were obtained for the GSM group when compared to the control group for all the three indices (**Table 4**, **Figures 1A–C**).

There were significant differences ($p < 0.05$) between the GSM and control groups at the PD_Whole_Tree (212.6 vs. 258.5, **Figure 1C**) and Chao1 (10304.8 vs. 12766.9, **Figure 1A**) indices. Also, significant decreased values were found for DSS+GSM compared to the control group for all three indices (**Table 4**, **Figures 1A–C**).

In order to compare the overall microbiota structure, β diversity was analyzed using PCoA (principal coordinate analysis) based on three distance matrices, including Euclidean, unweighted_uniFrac, and weighted_uniFrac (**Figure 2**).

The four experimental groups used in our study were separated as four clusters along PC1 (43.26%), suggesting that there were significant differences in the dominant bacterial population among the groups (**Figure 2**).

The results of PCoA showed segregation of samples collected from control and DSS-treated groups especially based on unweighted UniFrac matrix, as demonstrated by the first three principal component scores, which accounted for 43.26%, 16.05%, and 11.31% of total variations.

Bacterial Phyla Abundances in the Colon of DSS-Treated and GSM Diet-Fed Piglets

The total sequence reads used in this study were classified into 16 phyla, and one phylum was noted as unassigned. Overall, the bacterial communities were dominated by bacteria belonging to *Firmicutes* (50.5–60.1%), *Bacteroidetes* (36.1–45.8%), and *Proteobacteria* (1.3–3.49%) phyla, whereas a small percentage (0.01–0.09%) belonged to *Spirochaetes*, *Tenericutes*, and *Euryarchaeota* phyla (**Figure 3**). The constituent ratios of bacteria at the phylum level were different between DSS-treated and control groups, which was consistent with the results of OTU clustering and PCoA (**Figure 3**).

Overall, the relative abundance of *Firmicutes* was reduced by the DSS challenge in a significant way ($p < 0.0001$) when compared to the dietary groups (control and GSM group) without DSS challenge (**Table 5**). The dietary GSM inclusion had a similar relative abundance of *Firmicutes* with the control diet, and in the DSS+GSM group, a slightly lower relative abundance of *Firmicutes* was observed when compared with DSS group alone (50.5 vs. 53.9%, **Figure 4**) and control (50.5 vs. 60.1).

The *Bacteroidetes* phylum increased significantly ($p = 0.0005$) in relative abundance under the effect of DSS as well as under the effect of GSM but to a lesser extent ($p = 0.0332$, η^2 0.11 vs. 0.375, 45.8%, **Table 5**). The *Proteobacteria* relative abundance was also influenced by DSS challenge as well as the GSM treatment increasing significantly ($p = 0.0032$ for DSS effect and 0.0108 for GSM, **Table 5**). In the interaction between DSS and GSM, a lower relative abundance was observed for the *Proteobacteria* phylum.

An interesting aspect of the dietary treatments was that the *Bacteroidetes*/*Firmicutes* observed ratio tended to increase in the DSS group (0.71) and reached the highest value in the DSS+GSM group (0.90) when compared to the other groups. This ratio was similar in both control and GSM dietary groups (0.60 and 0.61).

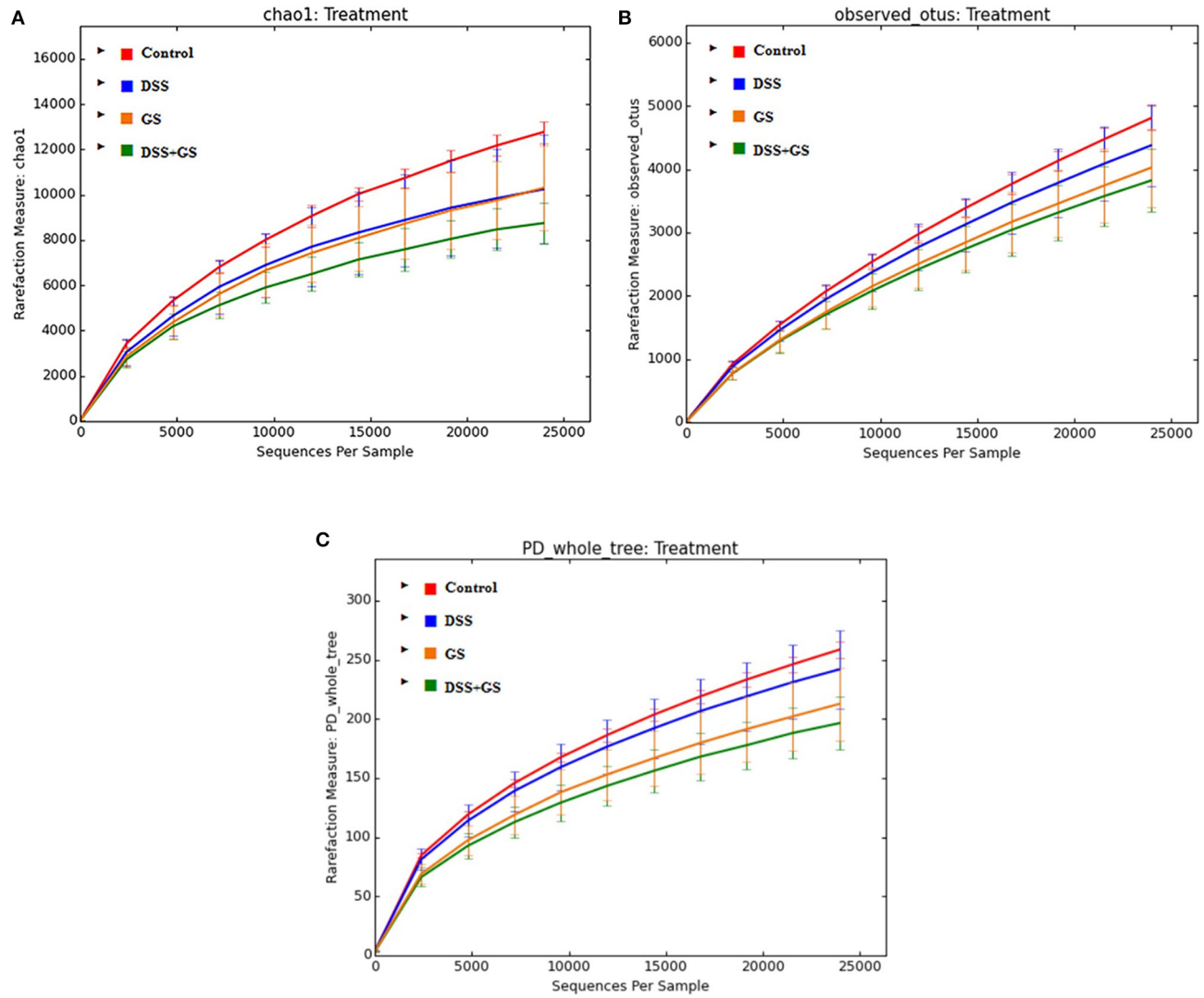

FIGURE 1 | Alpha diversity analysis of dietary groups. The indices are Chao1 **(A)**, observed_otus **(B)**, and PD_whole_tree **(C)**. Control, red; DSS, dextran sodium sulfate, blue; GS, grape seed meal, yellow; DSS+GS, dextran sodium sulfate and grape seed, green.

Microbial Genus Relative Abundances in Gut of DSS-Treated and GSM Diet-Fed Piglets

One hundred forty-nine bacterial taxa were identified as the most frequent species among the groups. Of these, 85 were identified at the genus level, and the remaining 64 could only be classified at the level of family or order taxon.

At the genus level, *Lactobacillus*, *Prevotella*, and *Megasphaera* dominated the colon microbiota among the four dietary groups while genus like *CF231* (a member of *Paraprevotellaceae* family), *Anaerovibrio*, and *Roseburia* have a lower abundance (lower than 4%, **Figure 4**). The highest *Lactobacillus* relative abundance was noticed in the dietary groups not affected by either DSS or GSM, and it was lowered in a significant way ($p < 0.0001$) in the dietary groups affected by DSS or GSM (**Figure 4** and **Table 6**).

For *Prevotella* genus, the dietary GSM inclusion had a significant ($p = 0.0096$) positive effect on its relative abundance. Also, noticeable differences were observed in the interaction between the DSS and GSM with the highest effect size ($\eta^2 = 0.25$, **Figure 4** and **Table 6**). The *Megasphaera* genus relative abundance was stimulated by the DSS and GSM effect in a significant proportion ($p = 0.0076$, $\eta = 0.038$). On the *CF231* genus, DSS had a significant effect on stimulating the bacterial abundance ($p = 0.0008$). The addition of the GSM lowered the relative abundance count significantly ($p < 0.0001$) and in a sizeable way ($\eta^2 = 0.543$) in a manner as to modulate the effect of DSS (**Figure 4** and **Table 6**).

The *Anaerovibrio* genus was influenced significantly ($p = 0.0002$) only by the GSM diet alone, the effect size being

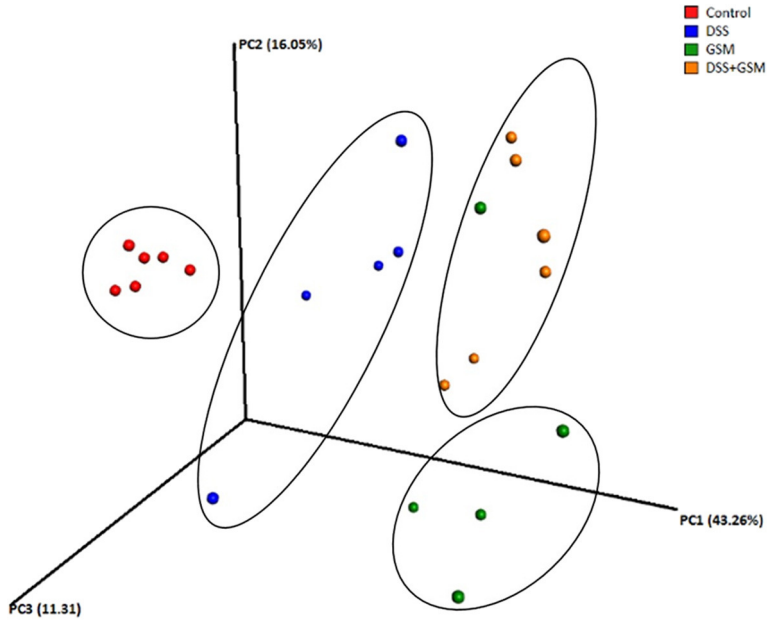

FIGURE 2 | Qualitative principal component analysis based on distance matrix (based on unweighted UniFrac metrics of OTUs). Dietary groups colon piglet samples: control (red), DSS (blue), GSM (yellow), DSS+GSM (green). Ellipses were used to show clustering.

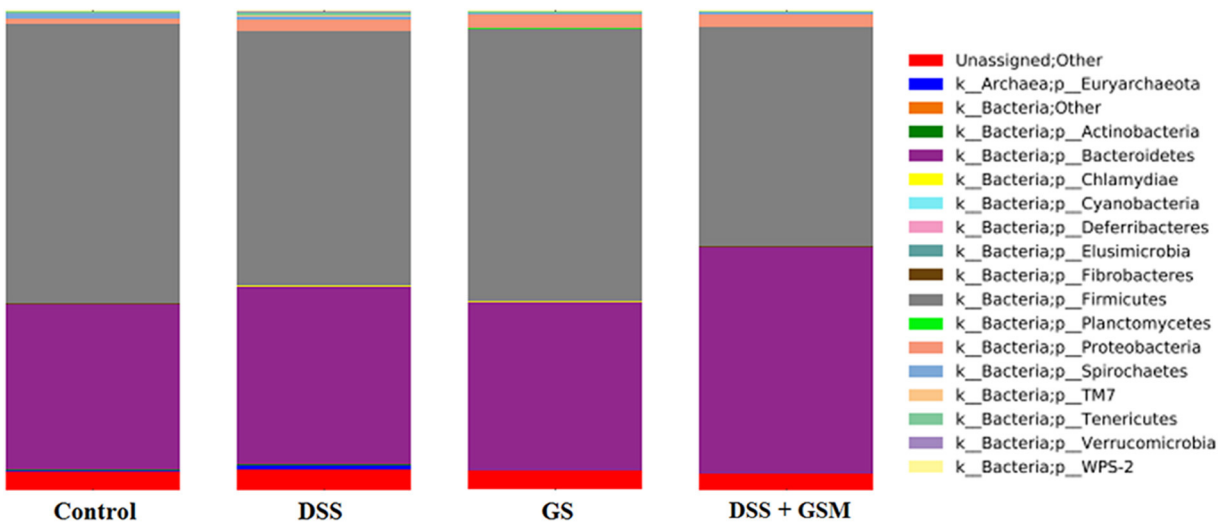

FIGURE 3 | Relative abundance (%) of bacteria phylum as calculated by QIIME. Dietary groups: control, DSS, GSM, and DSS+GSM.

noticeable when compared to the DSS effect (**Table 6**). DSS challenge also significantly increased the relative abundance of bacteria from *Roseburia* genus ($p < 0.0001$) being in contrast with the effect of the GSM diet, which acted by inhibiting the *Roseburia* genus ($p = 0.0001$, $\eta^2 = 0.5$, **Table 6**).

To have a comprehensive image on the dynamics and influence of the DSS and GSM treatments on the microbiota (especially on the most abundant species), we used comparative analysis at the genus level, as shown in the heatmap presented in **Figure 5**.

The higher the abundance of an OTU in a sample, the more intense is the red color at the corresponding position in the heatmap. By default, the OTUs (rows) were clustered by QIIME, and the samples (columns) were presented in the order in which they appear in the OTU table. When observed at the family taxa, a downward trend can be seen for the *Lactobacillaceae*, *Ruminococcaceae*, and *Lachnospiraceae* bacterial families, from control to DSS and DSS+GSM groups while being progressively replaced by *Prevotellaceae* and *Veillonellaceae* families, respectively, for

Table 5 | Effect of GSM diet on microbiota of DSS-treated piglets (*filum*).

	Control	DSS	GSM	DSS+GSM	DSS effect			GSM effect			DSS+GSM effect		
					η^2	*P*-value	ω^2	η^2	*P*-value	ω^2	η^2	*P*-value	ω^2
Firmicutes (r.a)	0.60 ± 0.01	0.54 ± 0.01	0.60 ± 0.01	0.50 ± 0.01	0.607	<0.0001*	0.578	0.053	0.1026	0.034	0.015	0.373	−0.002
Bacteroidetes (r.a)	0.36 ± 0.01	0.38 ± 0.01	0.36 ± 0.01	0.45 ± 0.01	0.375	0.0005*	0.347	0.112	0.0332*	0.089	0.133	0.0217*	0.109
Proteobacteria (r.a.)	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.094	0.0032*	0.085	0.065	0.0108	0.057	0.693	<0.0001*	0.679
Lactobacillus (r.a.)	0.30 ± 0.02	0.12 ± 0.01	0.09 ± 0.01	0.03 ± 0.00	0.285	<0.0001*	0.277	0.535	<0.0001*	0.526	0.074	0.0023*	0.068
Prevotella (r.a.)	0.23 ± 0.01	0.21 ± 0.02	0.22 ± 0.02	0.33 ± 0.00	0.118	0.0378*	0.093	0.197	0.0096*	0.17	0.258	0.0039*	0.23
Megasphaera (r.a.)	0.0003 ± 0.00	0.068 ± 0.00	0.222 ± 0.00	0.211 ± 0.02	0.021	0.0414*	0.016	0.864	<0.0001*	0.856	0.038	0.0076*	0.034
CF231 (r.a.)	0.016 ± 0.00	0.034 ± 0.00	0.01 ± 0.00	0.007 ± 0.0	0.115	0.0008*	0.107	0.543	<0.0001*	0.532	0.215	<0.0001*	0.207
Anaerovibrio (r.a.)	0.004 ± 0.00	0.0073 ± 0.00	0.03 ± 0.00	0.027 ± 0.0	0.00002	0.9769	−0.02	0.553	0.0002*	0.516	0.007	0.6025	−0.017
Roseburia (r.a)	0.015 ± 0.00	0.027 ± 0.00	0.005 ± 0.00	0.013 ± 0.00	0.339	<0.0001*	0.328	0.49	<0.0001*	0.477	0.004	0.2529	0.004

*r.a., relative abundance; η^2, Eta squared; ω^2, Omega squared; Microbiota (filum) relative abundance values are represented as mean with their standard errors. ANOVA (two-way) was performed to analyse the main factors DSS, GSM, and DSS × GSM interaction effects on microbiota incidence. Calculation and effect size magnitudes are given in Materials and Methods section. *P-values lower than 0.05 are statistically significant.*

the same dietary groups (**Figure 5**). The GSM group was characterized by a high abundance of *Anaerovibrio*, *Megasphera*, and *Trembyales* and a lower abundance of *Roseburia*, *CF231*, *Fecalibacterium*, *Lactobacillus*, and *Prevotella*. The genera *Dialister, Acidaminococcus*, and *Faecalibacterium* were also encountered in the colon content of the piglets but in a lower percentage (**Figure 5**).

The Effects of the DSS and GSM Diet on the Fecal SCFA Production

The effects of DSS challenge and GSM diet on SCFA production by anaerobic bacteria are presented in **Table 7**. Although there were no statistical differences in the total SCFA concentration between the experimental groups, statistical differences in concentration were observed in the case of some SCFA.

The acetate proportion was significantly lowered with the addition of GSM ($p < 0.0001$). The effect of the GSM was also felt at the butyrate percentage, it being significantly higher ($p < 0.0001$) compared to control and DSS group. The percentage of valerate was also increased by both the challenge of GSM and that of DSS. The interaction between the effects only seems to affect the propionate levels ($p = 0.044$).

DISCUSSION

The IBD presents a worldwide health concern because of the lack of a cure and definitive therapies to tackle the issue (1, 32). The aim of the present study was to analyze and discuss if nutritional interventions based on bioactive compounds from grape seed could ameliorate and change the microbial composition affected by-product through induced IBD by using the pig as an animal model.

Medication alternatives in IBD such as polyphenolic compounds (17, 33, 34), SCFAs, and PUFAs (35) have been studied lately by many research groups with promising results. The biologic activity and underlying mechanisms have rarely been identified (36). However, Bousenna et al. (17) evaluated a polyphenol-rich grape pomace extract on rats challenged with DSS and observed attenuation in the clinical signs, colon shortening, and limitation of histological lesions usually observed in DSS-induced colon inflammation. Another study carried out by Aboura et al. (37) reported that polyphenol-rich infusion from carob leaves and Opuntia cladodes presented anti-inflammatory effects, counteracted intestinal permeability and colon histological lesions, and decreased DSS-induced pro-inflammatory cytokine expression in mice. Samsami-Kor et al. (38) also showed that resveratrol, a highly studied polyphenol that is abundant in natural sources like grapes, could decrease clinical disease activity index and quality of life in patients with ulcerative colitis (UC) in a randomized, double-blind clinical trial. Increasing SCFA metabolites in the colon via administration of prebiotic high-fiber diets in combination with probiotic bacteria (*Lactobacillus*, *Bifidobacterium,* and *Faecalibacterium*) were also studied for intestinal lesion amelioration, gut barrier improvement, anti-inflammatory effect, and as preventive strategies in the management of DSS in

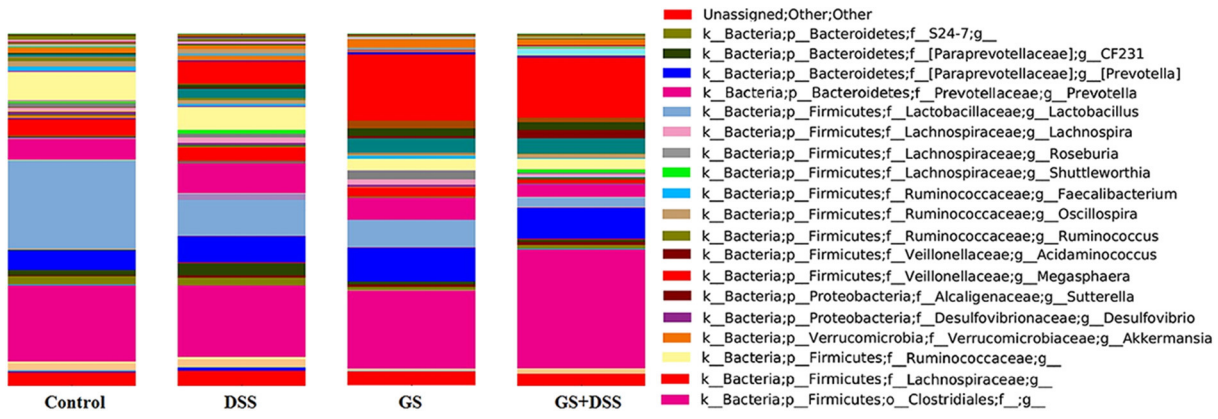

FIGURE 4 | Relative abundances (%) of bacteria genera as calculated by QIIME. Dietary groups: control, DSS, GSM, and DSS+GSM.

mice (39–41). Short-term supplementation with eicosatetraenoic (n-3) free fatty acid was evaluated in a study by Prossomariti et al. (42), improving endoscopic and histological inflammation while also modulating microbiota composition in long-standing UC patients. In both animal and human, gut microbiota participated in different host processes, and an imbalance in its ecological composition may cause systemic and intestinal dysfunction (43). Modulation or aberrations in the gut microbial community have been shown in IBD. The dysbiosis effects associated with IBD have been characterized usually as a perturbation in the ratio of *Bacteroidetes* over *Firmicutes* (44). Modifications of the microbiota at the phylum, genus, and species level are known to occur when DSS is used to induce inflammation (45). Overall, the *Bacteroidetes/Firmicutes* ratios in DSS-afflicted groups were found to be higher than those of the control and associated with dysbiosis (9, 44, 46, 47). In the present study, dramatic changes in overall ratio and diversity of the gut microbiota were observed in DSS-treated pigs receiving GSM diet when compared to the control and other groups. Thus, the ratio of *Bacteroidetes/Firmicutes* significantly increased ($p < 0.05$) in the DSS-challenged group, whereas no difference between GSM and control diet was observed. The administration of the GSM diet to pigs treated with DSS was not able to decrease the *Bacteroidetes/Firmicutes* ratio, which remains higher in comparison with the control.

An increase in the relative abundances of the *Bacteroidetes* phylum and *Bacteroidales* and *Clostridiales* orders and an overall decrease in *Firmicutes* phylum were found by Imhann et al. (48), which were linked to irritable bowel diseases. Similarly, herein, the *Firmicutes* phylum was decreased in a significant way ($p < 0.05$) under DSS effect and was not affected by the addition of GSM into the diet in a significant way. *Clostridium, Roseburia, Acidaminococcus,* and *Escherichia* are often cited as the genera usually found in abundance in irritable bowel diseases (48–52) and DSS treatment, while *Lactobacillus*, *Prevotella*, and *Faecalibacterium* are cited as negatively impacted or inversely correlated with the severity of the disease (33, 53–56). Indeed, in our work, *Roseburia*, *Megasphera*, and *CF231* increased significantly under the DSS presence while *Lactobacillus* registered a significant decrease overall. Interesting is that GSM and especially DSS+GSM treatment progressively decreased the relative abundance of *Lactobacillus*. Dietary GSM was able to counteract the abundance of *Roseburia*, linked in other studies to an increase in abundance and for its role in the onset and progression of IBD dysbiosis (49, 50, 57). The GSM diet also modulated the abundances of *Anaerovibrio* (increasing) and *CF231* (decreasing), which play essential roles in the repair of the intestinal epithelial damage and are constituents of the gut microbiota core (58). Interestingly, the GSM diet alone determined a significant decrease of *Lactobacillus* spp. in comparison with all the other diets, including control; moreover, the DSS-treated pigs that received dietary GSM registered the lowest *Lactobacillus* abundance. Generally, *Lactobacillus* spp. are associated with positive effects in the large intestine, being able to enhance the gut barrier functions, to modulate the immune system, and to compete with pathogen species for the large intestine colonic mucosa (59). The observed reduction in the abundance of *Lactobacillus* spp. in pigs with DSS-induced inflammation or pigs with an intake of GSM diet and dramatically in DSS+GSM groups might be associated with the negative impact that the DSS has on this genus (55) as well as the interaction between *Lactobacillus*, the type of phenolic compounds and their concentration as described in the scientific literature (56, 60). However, the findings are controversial. For example, Ozdal et al. (61) found an increase in *Lactobacillus* abundance with gallic acid, punicalagin, proanthocyanidins, and resveratrol and no effect with (+)-catechin, (−)-epicatechin, and quercetin, while Pastorkova et al. (62), investigating the antimicrobial potential of 15 grape phenolic compounds against yeast and acetic acid bacteria from wine, found that resveratrol, pterostilbene, and luteolin presented the highest antibacterial effect. The grape seed extract was also shown to inhibit the growth of *Streptococcus thermophilus*, *Bifidobacterium lacticus*, *Lactobacillus fermentum*, and *acidophilus* due to their perceived sensitivity to the polyphenol fraction flavan-3-ols (63). Nevertheless, the activity of some biological compounds could be

Table 6 | Effect of GSM diet on microbiota of DSS-treated piglets (*genus*).

	Control	DSS	GSM	DSS+GSM	DSS effect			GSM effect			DSS+GSM effect		
					η^2	*P*-value	ω^2	η^2	*P*-value	ω^2	η^2	*P*-value	ω^2
Lactobacillus (r.a.)	0.30 ± 0.02	0.12 ± 0.01	0.09 ± 0.01	0.03 ± 0.00	0.285	<0.0001*	0.277	0.535	<0.0001*	0.526	0.074	0.0023*	0.068
Prevotella (r.a.)	0.23 ± 0.01	0.21 ± 0.02	0.22 ± 0.02	0.33 ± 0.00	0.118	0.0378*	0.093	0.197	0.0096*	0.17	0.258	0.0039*	0.23
Megasphaera (r.a.)	0.0003 ± 0.00	0.068 ± 0.00	0.222 ± 0.00	0.211 ± 0.02	0.021	0.0414*	0.016	0.864	<0.0001*	0.856	0.038	0.0076*	0.034
CF231 (r.a.)	0.016 ± 0.00	0.034 ± 0.00	0.01 ± 0.00	0.007 ± 0.0	0.115	0.0008*	0.107	0.543	<0.0001*	0.532	0.215	<0.0001*	0.207
Anaerovibrio (r.a.)	0.004 ± 0.00	0.0073 ± 0.00	0.03 ± 0.00	0.027 ± 0.0	0.00002	0.9769	−0.02	0.553	0.0002*	0.516	0.007	0.6025	−0.017
Roseburia (r.a)	0.015 ± 0.00	0.027 ± 0.00	0.005 ± 0.00	0.013 ± 0.00	0.339	<0.0001*	0.328	0.49	<0.0001*	0.477	0.004	0.2529	0.004

*r.a., relative abundance; η^2, Eta squared; ω^2, Omega squared; Microbiota (filum) relative abundance values are represented as mean with their standard errors. ANOVA (two-way) was performed to analyse the main factors DSS, GSM, and DSS × GSM interaction effects on microbiota incidence. Calculation and effect size magnitudes are given in Materials and Methods section. *P-values lower than 0.05 are statistically significant.*

masked by that of others, which constrain the understanding of the exact synergistic effect that could happen (56). In contrast, a significantly higher level of *Prevotella* belonging to *Bacteroidetes* phylum was observed in our study being stimulated by the interaction between DSS and GSM. *Prevotella* genus is considered a commensal, with essential functions in maintaining the gut health of pigs due to its frequent occurrence in the healthy pig gut microbiota, its rare involvement in bacterial infection, and the high butyrate synthesis (64). The involvement of *Prevotella* in the fermentation of plant-derived non-digestible fibers to SCFAs has been observed in piglets (65), allowing them to adapt to new dietary conditions. In human, *Prevotella* has been related to diets rich in vegetables and fruits like vegetarian and Mediterranean diets (66). De Cruz et al. and Slifierz et al. have found interesting results linking the presence of *Prevotella* with remission in Crohn's disease and recovery from chronic effects of DSS-induced colitis (53, 54). Herein, piglets subjected to DSS-induced inflammation (DSS+GSM group) consuming the GSM diet had a higher *Prevotella* in the colon.

GSM effect and DSS challenge alone and in interaction caused a significant ($p < 0.0039$) increase in *Megasphaera* genus abundance. *Megasphaera* including the lactate-utilizing bacteria represents the healthy microbiota of pigs, which maintain the pH balance and play an essential role in the fermentation of a variable part of dl-lactate to butyrate, with some of the highest concentrations of butyrate in comparison to other anaerobic butyrate-producing bacteria belonging to the *Firmicutes* (67). Other studies have also pointed on the beneficial effects of *Lactobacillus*, *Megasphaera*, and their complementarity and association in the production of SCFAs and in promoting intestinal health in pigs (54, 68).

SCFAs produced primarily from the microbial fermentation of dietary fiber appear to be critical mediators of the beneficial effects elicited by the gut microbiome (69). GSM, a by-product of the grape seed oil process, is mainly composed of dietary fibers and polyphenols that offer an ideal substrate for colonic bacteria in their process of colonic fermentation, thus increasing the concentration levels of SCFAs. Indeed, the colonic concentration of butyrate was increased by the GSM diet in both GSM and DSS+GSM groups when compared to control or DSS groups indicative of an increased beneficial microbial activity and a modulatory effect of the GSM. Among the SCFAs, butyrate in particular has been shown to promote commensal bacterial growth (70), provide an energy source for epithelial cells of the host (71), and enhance the overall gut barrier integrity (72–76). A high propionate level was also achieved in the DSS+GSM group comparatively to the rest, which is associated with an overall amelioration and improvement of intestinal barrier function (77).

The bacteria from the phylum *Bacteroidetes* are typically associated with the production of acetate and propionate while the *Firmicutes* phylum [*Megasphaera, Faecalibacterium* (*Prevotella*)] mainly produce butyrates (78). The significantly high levels of butyrate observed from GSM and GSM+DSS were highly correlated with the same species as *Megasphaera*, *Faecalibacterium*, and *[Prevotella]* while the high propionate and acetate concentrations from the GSM+DSS dietary group

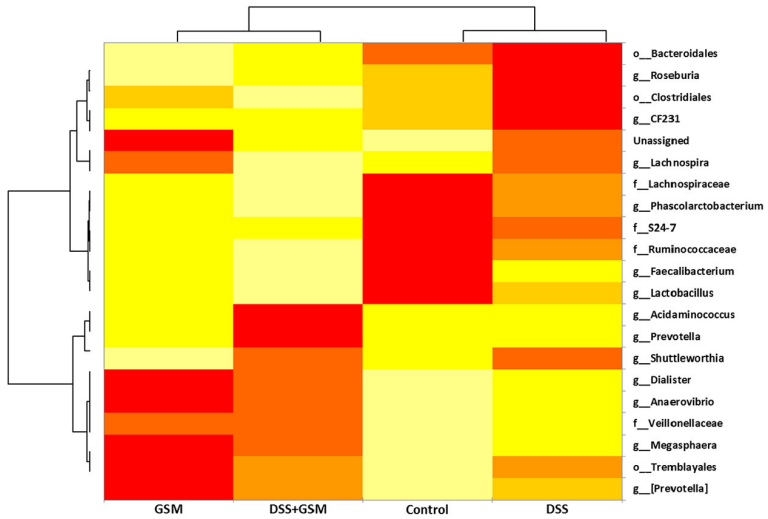

FIGURE 5 | Heatmap of the most abundant genus, family, and order, based on the dietary groups. Clustering between groups and also between taxa was selected. The relative abundance is colored in shades of yellow (low relative abundance) to red (high relative abundance).

Table 7 | The composition in SCFAs of colon content collected from piglets.

Analyte (SCFAs)	Experimental group*						
	Control	DSS	GSM	DSS+GSM	*p*-value (DSS effect)	*p*-value (GSM effect)	*p*-value (DSS × GSM effect)
Total SCFA (mM/g sample)	13.3 ± 2.3	15.8 ± 1.2	12.6 ± 0.5	14.8 ± 0.8	0.060	0.594	0.972
Acetate (%)	53.7 ± 1.8^{a}	52.1 ± 1.2^{a}	48.1 ± 2.2^{a}	42.5 ± 1.1^{b}	0.066	<0.0001	0.386
Propionate (%)	26.1 ± 0.5^{b}	26.3 ± 1.6^{b}	25.0 ± 1.0^{b}	29.0 ± 0.9^{a}	0.058	0.314	0.044
Isobutyrate (%)	1.9 ± 0.3^{b}	2.4 ± 0.3^{b}	2.7 ± 0.2^{a}	2.5 ± 0.3^{a}	0.851	0.051	0.160
Butyrate (%)	13.0 ± 0.9^{b}	12.1 ± 0.3^{b}	16.6 ± 1.0^{a}	16.4 ± 0.3^{a}	0.605	<0.0001	0.898
Isovalerate (%)	1.6 ± 0.2	2.7 ± 0.6	2.2 ± 0.4	1.9 ± 0.6	0.285	0.842	0.099
Valerate (%)	3.6 ± 0.4^{b}	4.4 ± 0.3^{b}	5.3 ± 0.5^{a}	7.6 ± 0.4^{a}	0.002	<0.0001	0.075

**Control and DSS-treated piglets were fed for 30 days with a control diet or a diet containing 8% GSM, as described in Materials and Methods section. At the end of the experiment, samples of colonic content from all animals (n = 5) were collected and analyzed for the composition of SCFAs. Values within a row with different superscript letters are significantly different (p < 0.05).*

were also correlated with members of the Firmicutes phylum (*Roseburia* for propionate and *Shuttleworthia* for acetate). Our results further corroborate with similar findings of other studies that place *Megasphera [Prevotella]*, and *Faecalibacterium* as the most important producers of SCFAs (78). SCFA results confirm the fact that the GSM diet with the high-fiber content contributes, through microbial fermentation, to the significant production of SCFAs with a demonstrated effect on intestinal bowel diseases. In accordance with literature data (11, 79), GSM also provides an excellent matrix for their polyphenol, fiber-rich content, which can further contribute to the amelioration of DSS-induced UC effects by their increased anti-inflammatory and antioxidant capacity, thus improving gastrointestinal health (11, 79).

GSM with high-fiber, -polyphenol, and -PUFA content increased the production of butyrate and isobutyrate, stimulated the growth of beneficial genera like *Prevotella* and *Megasphaera*, while countering the relative abundance of *Roseburia*, reducing it to half of the DSS value and contributing to the management of the DSS effects.

CONCLUSIONS

Our results showed that GSM, a common by-product of grape seed oil processing, which contains significant concentrations of several bioactive compounds, like polyphenols, PUFA, fibers, minerals, etc., had a selective modulatory effect on several bacterial genera in the colon of pigs challenged with DSS. Our study demonstrated that *Bacteroidetes* and *Firmicutes* phyla were the most prevalent bacteria in the colon of pig irrespective of the treatment. DSS challenge affected the colonic bacteria, increasing overall the abundance of *Proteobacteria* phylum and of *Roseburia*, associated with the progression of IBD, and affected the *Bacteroidetes/Firmicutes* ratio contributing to an overall loss in the microbiota species diversity. GSM increased

the production of butyrate and isobutyrate in pigs receiving dietary GSM and stimulated the growth of beneficial genera like *Prevotella* and *Megasphaera* while reducing to half the relative abundance of *Roseburia* registered in the DSS dietary group. GSM is an available raw material source of bioactive compounds that might be used as supplement functional food in IBD. For practical applicability, this dry form of grape seed (meal) could be quickly processed by encapsulation and served along with the daily diet. However, further researches testing other GSM dietary concentrations and their effects are necessary.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ENA database (https://www.ebi.ac.uk/ena/browser/home) under the name PRJEB34923 (ERP117900) | Raw sequences 16s.

ETHICS STATEMENT

The procedures were in agreement with the Romanian Law 206/2004 and the EU Directive 98/58/EC for handling and protection of animals used for experimental purposes. The experimental protocol was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Baloteşti, Romania (Ethical Committee no. 52/2014).

AUTHOR CONTRIBUTIONS

DM, GP, and IŢ realized the design of the experiment. IG and GP performed the DNA extraction. IG analyzed the raw microbiota data. LP and IG performed the statistical analysis. AC analyzed the SCFA content in colon samples. IG, GP, and IŢ wrote the manuscript.

FUNDING

This research was supported by funds from the National Research Project PN-II-RU-TE-2014-4-1287 granted by the Romanian Ministry of Research and Innovation. The publication was supported by funds from the National Research Development Project Projects to finance excellence (PFE)—17/2018-2020 granted by the Romanian Ministry of Research and Innovation.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 09 September 2020
doi: 10.3389/fvets.2020.00573

Synbiotic Effects of Enzyme and Probiotics on Intestinal Health and Growth of Newly Weaned Pigs Challenged With Enterotoxigenic F18$^+$ *Escherichia coli*

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This study aimed to investigate the effect of dietary supplementation with xylanase and probiotics on growth performance and intestinal health of nursery pigs challenged with enterotoxigenic *Escherichia coli* (ETEC). Sixty-four newly weaned pigs (32 barrows and 32 gilts with 7.9 ± 0.4 kg BW) were allotted in a randomized complete block design (2 × 2 factorial). Two factors were ETEC challenge (oral inoculation of saline solution or *E. coli* F18$^+$ at 6 × 10^9 CFU) and synbiotics (none or a combination of xylanase 10,000 XU/kg and *Bacillus* sp. 2 × 10^8 CFU/kg). All pigs were fed experimental diets following NRC (2012) in two phases (P1 for 10 d and P2 for 11 d). The ETEC was orally inoculated on d 7 after weaning. Feed intake and BW were measured on d 7, 10, 15, and 20. On d 20, pigs were euthanized to collect samples to measure gut health parameters and microbiome. Synbiotics increased ($P < 0.05$) ADG in phase 1 and ETEC reduced ($P < 0.05$) ADG and G:F in the post-challenge period. ETEC increased ($P < 0.05$) the fecal score of pigs from d 7 to 13; however, synbiotics reduced ($P < 0.05$) it at d 9 and 11 in challenged pigs. ETEC increased ($P < 0.05$) mucosal MDA, IL-6, Ki-67$^+$, and crypt depth, whereas synbiotics tended to reduce TNFα ($P = 0.093$), protein carbonyl ($P = 0.065$), and IL-6 ($P = 0.064$); reduced ($P < 0.05$) crypt depth and Ki-67$^+$; and increased ($P < 0.05$) villus height. ETEC reduced ($P < 0.05$) the relative abundance of Bacteroidetes and Firmicutes and increased ($P < 0.05$) the relative abundance of Proteobacteria. In conclusion, ETEC challenge reduced growth performance by affecting microbiome, immune response, and oxidative stress in the jejunum. Synbiotics enhanced growth performance by reducing diarrhea, immune response, and oxidative stress in the jejunum.

Keywords: ***Escherichia coli*, growth performance, intestinal health, newly weaned pigs, probiotics, synbiotics, xylanase**

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *05 May 2020*
Accepted: *17 July 2020*
Published: *09 September 2020*

Citation:
Duarte ME, Tyus J and Kim SW (2020) Synbiotic Effects of Enzyme and Probiotics on Intestinal Health and Growth of Newly Weaned Pigs Challenged With Enterotoxigenic F18$^+$ Escherichia coli. Front. Vet. Sci. 7:573. doi: 10.3389/fvets.2020.00573

INTRODUCTION

Weaning is a challenging period for nursery pigs especially with their immune and intestinal functions resulting in reduced growth performance (1). During this period, pigs experience environmental, immunological, psychological, and nutritional challenges (2–4). Consequently, pigs at weaning are highly susceptible to pathogenic microorganism, such as enterotoxigenic

Escherichia coli (ETEC) causing enteric diseases (5, 6). Moreover, the increasing pressure to ban the use of antibiotics as growth promoter (AGP) around the world due to the concern about microbial resistance has been a big challenge to the swine industry and researchers to maintain the gut health and performance of pigs (7–9).

Among these, newly weaned pigs have a low capacity to digest nutrients from plant-based feed because of the immaturity of the gastrointestinal tract (GIT), the different nutritional composition, compared with milk, and the anti-nutritional content that can damage the intestinal epithelial layers (10). Typical plant-based feeds contains ∼2.3–3.8% of xylans, the main non-starch polysaccharides (NSP) (11, 12) increasing digesta viscosity, altering intestinal morphology, and reducing nutrient digestibility (13–16), which can induce a propitious environment for the growing of harmful bacteria, changing the gut associate microbiota in newly weaned pigs (17).

Nutritionally, numerous strategies have been attempted to eliminate or mitigate the effect of these challenges and produce healthy animals. Exogenous enzymes, such as xylanase, have been successfully used to hydrolyze the β-1,4 backbone of xylan, releasing xylan oligosaccharides (XOS) and, consequently, reducing the NSP content and the viscosity of digesta, increasing the digestibility of nutrients (14, 18, 19). Probiotics, such as bacteria from the genus *Bacillus,* are being largely used as an alternative to promote health and performance in the livestock industry. The genus *Bacillus* is well-known for its ability to form spores, produce antimicrobial compounds, and produce exogenous enzymes that are related to the ability to utilize different carbohydrate sources including those derived from plants, such as XOS (5, 20).

It is hypothesized that xylanase combined with *Bacillus* sp. as a synbiotic enhances growth performance of newly weaned pigs challenged with ETEC by enhancing the gut health and modulating the microbiome in the intestine by altering digesta viscosity. Thus, this study aimed to evaluate the supplemental effects of synbiotics on gut health and growth of newly weaned pigs challenged with ETEC.

MATERIALS AND METHODS

Animals, Experimental Design, Additives, and Diets

The experimental procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University following the North Carolina State Animal Care and Use Procedures (REG 10.10.01).

Sixty four newly weaned pigs (32 barrows and 32 gilts) at 21 d of age, with an initial body weight at 7.9 ± 0.4 kg, were allotted in a randomized complete block design in a 2 × 2 factorial arrangement, with ETEC challenge (oral inoculation of saline solution or *E. coli* $F18^+$ at 6×10^9 CFU) and synbiotics (none and a combination of xylanase 10,000 XU/kg and *Bacillus* sp. 2×10^8 CFU/kg) as two factors. Pigs (PIC 337 × Camborough 22) were purchased from a commercial farm in North Carolina, USA. Sows and piglets used in this study were not vaccinated against *E. coli*. The symbiotic used in this study was EnzaPro obtained from BioResource International Inc. (Durham, NC). Each factor had 16 pens ($n = 16$; eight pens with barrows and eight pens with gilts; and four body weight blocks within sex) and pigs were housed individually in a pen.

Pigs were fed the assigned experimental diets meeting the nutritional requirements suggested by NRC (21) for 20 d based on two phases (Phase 1: 10 d; and Phase 2: 10 d). The composition of mash basal diets is shown in **Table 1**.

The quantitation of the xylanase (endo-1,4-β-D-xylanase) activity in feeds was performed using modified XylX6 assay (Megazyme, Wicklow, Ireland) as described by Mangan et al. (22). Xylanase enzymatic activity is calculated relative to a reference standard added to 50 mM Trisodium Citrate pH 6.0 buffer measured at A400. One unit of xylanase activity is defined as the amount of enzyme needed for the release of 1 nmol of reducing sugars per second from 0.5% xylan from Beechwood at 50°C in 50 mM Trisodium Citrate pH 6.0. The xylanase enzymatic activity is shown in **Table 2**. The microbial counting in feeds was conducted at the microbiology lab of BioResource International, Inc.

Experimental Procedures and Sampling

The inoculum of *E. coli* $F18^+$ was prepared to be nalidixic acid resistant and produce heat-stable toxins A (STa) and heat-stable toxins B (STb), using a strain originally resistant to nalidixic acid following our standard protocol as previously described by Cutler et al. (23). The final concentration was 6×10^9 CFU/mL, orally inoculated and divided into two doses.

All pigs were fed the experimental diets for 7 d (pre-challenge period) until ETEC was orally inoculated to 32 pigs on d 7 of the study. The unchallenged group (32 pigs) received an oral administration of sterile physiological saline. The fecal score was recorded by the same trained person from the d 3 to d 7 of the study to analyze the effects of the synbiotic in the pre-challenge period and to confirm that pigs assigned to the challenge group are in normal fecal score before the *E. coli* $F18^+$ inoculation. The fecal score was also recorded from d 8 to d 20 of the study to analyze the effects of ETEC infection (24, 25). The fecal score was recorded using a 1–5 scale: (1) very firm stool, (2) normal firm stool, (3) moderately loose stool, (4) loose, watery stool, and (5) very watery stool.

After 20 d of feeding, 48 pigs, 12 per treatment, were selected based on initial BW (the heaviest and the lightest pigs within sex were excluded of sampling) and euthanized by exsanguination after the penetration of a captive bolt to head in order to remove the gastrointestinal tract for sample collection. Digesta from mid-jejunum (3 m after the duodenojejunal junction) was collected into a 50-mL tube, placed on ice, and carried to the lab for viscosity measurement. Tissues from mid-jejunum were collected, rinsed with 0.9% saline solution, and placed into a 50-mL tube with 10% buffered formaldehyde to be used for histological evaluation to measure villus height, crypt depth, and the ratio of Ki-67 positive cells to total cells in the crypt, as an indicator of the enterocyte proliferation rate (10, 26).

TABLE 1 | Composition of basal diets (as-fed basis).

Ingredient, %	Phase 1	Phase 2
Corn	35.46	36.77
Soybean meal	20.00	25.00
Corn DDGS	10.00	20.00
Whey permeate[a]	18.00	8.00
Poultry meal	5.00	2.00
Fish meal	4.00	0.00
Blood plasma	3.50	2.00
L-Lys HCl	0.48	0.45
DL-Met	0.18	0.11
L-Thr	0.13	0.08
Limestone	0.80	1.40
Vitamin premix[b]	0.03	0.03
Mineral premix[c]	0.15	0.15
Salt	0.22	0.22
Dicalcium phosphate	0.05	0.40
Poultry fat	2.00	3.40
Calculated composition		
ME, kcal/kg	3,436	3,437
Crude protein	24.83	24.05
SID[d] Lys, %	1.50	1.35
SID Met + Cys, %	0.82	0.74
SID Trp, %	0.25	0.23
SID Thr, %	0.88	0.79
Ca, %	0.85	0.81
STTD[e] P, %	0.45	0.40
Analyzed composition		
Dry matter, %	90.36	88.87
Crude protein, %	24.72	24.13
Neutral detergent fiber, %	7.82	9.71
Acid-detergent fiber, %	3.42	4.37
Crude fat, %	4.82	6.16
Ca, %	0.85	0.82
Total P, %	0.68	0.60

[a]*DairyLac80 (International Ingredient Corporation) was used as a source of whey permeate containing (79.3 ± 0.8) % lactose.*
[b]*The vitamin premix provided the following per kilogram of complete diet: 6,613.8 IU of vitamin A as vitamin A acetate, 992.0 IU of vitamin D3, 19.8 IU of vitamin E, 2.64 mg of vitamin K as menadione sodium bisulfate, 0.03 mg of vitamin B12, 4.63 mg of riboflavin, 18.52 mg of D-pantothenic acid as calcium pantothenate, 24.96 mg of niacin, and 0.07 mg of biotin.*
[c]*The trace mineral premix provided the following per kilogram of complete diet: 4.0 mg of Mn as manganous oxide, 165 mg of Fe as ferrous sulfate, 165 mg of Zn as zinc sulfate, 16.5 mg of Cu as copper sulfate, 0.30 mg of I as ethylenediamine di-hydroiodide, and 0.30 mg of Se as sodium selenite.*
[d]*SID, standardized ileal digestible.*
[e]*STTD P, standardized total tract digestible phosphorus.*

TABLE 2 | Xylanase activity and microbial count in the feed.

Treatment	Phase 1	Phase 2
Xylanase, ×1,000 XU/kg of feed		
No symbiotic	0.42	1.62
Synbiotic	13.37	14.49
***Bacillus* sp., $\times 10^8$ CFU/kg of feed**		
No synbiotic	0.00	0.00
Synbiotic	2.00	2.00

Segments of mid-jejunum were longitudinally opened and scraped to collect mucosa. Two samples per pig were placed into 2-mL tubes and stored at −80°C, after snap-freezing in liquid nitrogen immediately after collection. Jejunal mucosa samples (500 mg) were suspended in 1 mL of phosphate-buffered saline (PBS) and homogenized on ice using a tissue homogenizer (Tissuemiser; ThermoFisher Scientific Inc., Waltham, MA, USA). After centrifugation at 14,000 × *g* for 3 min, the supernatant was divided into six sets and stored at −80°C until analysis to measure the concentration of total protein, Tumor necrosis factor-alpha (TNFα), interleukin 6 (IL-6), interleukin 8 (IL-8), protein carbonyl, and malondialdehyde (MDA).

Sample Processing and Analyses

Immediately after collection, digesta from jejunum were divided into two tubes (15 mL) per pig and centrifuged at 1,000 × *g* at 4°C for 10 min. Then, 2 mL from each tube was centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant obtained was transferred to another 1.5-mL tube and kept on ice until measurement. The viscosity was measured using a viscometer (Brookfield Digital Viscometer, Model DV-II Version 2.0, Brookfield Engineering Laboratories Inc., Stoughton, MA), set at 25°C. The viscosity measurement was the average between 45.0/s and 22.5/s shear rates, and the viscosity values were recorded as viscosity in millipascal-seconds (mPa·s) (10, 14).

The concentrations of total protein, TNFα, IL-6, IL-8, MDA, and protein carbonyl were measured by colorimetric methods using commercially available kits according to the instructions of the manufacturers. The absorbance was read using a plate reader (Synergy HT, BioTek Instruments, Winooski, VT) and the Gen5 Data Analysis Software (BioTek Instruments). The concentration was calculated based on the standard curve created from the concentration and absorbance of the respective standard.

Total protein concentration in the mucosa of jejunum was measured using a BCA Protein Assay (23225#, ThermoFisher Scientific Inc. Rockford, IL) following Jang and Kim (26). Before analysis, the samples were diluted (1:60) in PBS to meet the working range for 20–2,000 μg/mL. The absorbance was measured at 562 nm. The total protein concentration was used to normalize the concentrations of TNFα, IL-6, IL-8, MDA, and protein carbonyl.

The concentration of TNFα in the mucosa of the jejunum was measured using the porcine ELISA Kit (PTA00; R&D System Inc. Minneapolis, MN) following Weaver and Kim (27). The working range was 23.4–1,500 pg/mL. Absorbance was read at 450 nm and 540 nm and the TNFα concentration was expressed as pg/mg protein. The concentration of IL-6 in the mucosa of jejunum was determined using the ELISA Kit (P6000B; R&D System Inc.) following Jang and Kim (26). The working range was 18.8–1,200 pg/mL. Absorbance was read at 450 nm and 540 nm, and the IL-6 concentration was expressed as pg/mg protein. The concentration of IL-8 in the mucosa of jejunum was determined

using the ELISA Kit (P8000; R&D System Inc.). Before analysis, the samples were diluted (1:6) in PBS to meet the working range of 62.5–4,000 pg/mL. Absorbance was read at 450 and 540 nm, and the IL-8 concentration was expressed as ng/mg protein.

Malondialdehyde concentration in the mucosa of jejunum was measured using the Thiobarbituric Acid Reactive Substance Assay Kit (STA-330, Cell Biolabs, San Diego, CA) following Duarte et al. (10). The working range was 0.98–125 μmol/L. The absorbance was measured at 532 nm, and the MDA concentration mucosa was expressed as μmol/mg of protein.

Protein carbonyl concentration was measured using the ELISA kit (STA-310, Cell Biolabs) following Zhao and Kim (28). Before the analysis, the sample was diluted to reach a protein concentration of 10 μg/mL. The working range was 0.375–7.5 nmol/mL. The absorbance was read at 450 nm, and the protein carbonyl concentration was expressed as nmol/mg of protein.

Two sections of jejunum per pig fixed in 10% buffered formalin were sent to the North Carolina State University Histology Laboratory (College of Veterinary Medicine, Raleigh, NC). The sections were dehydrated, embedded in paraffin, and stained using hematoxylin and eosin dyes for morphological measurement and Ki-67 immunohistochemistry assay to detect Ki-67 positive cells according to Jang and Kim (26).

Villus height, villus width, and crypt depth were measured using a microscope Olympus CX31 (Lumenera Corporation, Ottawa, Canada) with a camera Infinity 2-2 digital CCD following Kim et al. (29). Lengths of ten well-oriented intact villi and their associated crypts were measured in each slide. The villi length was measured from the top of the villi to the villi-crypt junction, the villi width was measured in the middle of the villi, and the crypt depth was measured from the villi-crypt junction to the bottom of the crypt. Then, the villus height to crypt depth (VH:CD) ratio was calculated. Images of 10 intact crypts from each slide were cropped, and the ImageJS software was used for calculating the percentage of Ki-67 positive cells to total cells in the crypt. All analyses of the intestinal morphology were executed by the same person. The averages of the 10 measurements per pig were calculated and reported as one number per pig.

Jejunal mucosa samples were used to extract DNA content to analyze the microbiome. The DNA extraction was performed using the DNA Stool Mini Kit (#51604, Qiagen; Germantown, Maryland, USA) and following the instructions of the manufacturer. Samples were sent to Mako Medical Laboratories (Raleigh, NC, USA) for microbial sequencing using the 16S rRNA technique following Kim et al. (29). Briefly, the Ion Chef instrument was used to prepare the samples for template and sequencing was performed on the Ion S5 system (ThermoFisher, Inc., Wilmington, DE, USA). Variable regions V2, V3, V4, V6, V7, V8, and V9 of the 16S rRNA gene were amplified with the Ion 16S Metagenomics Kit 113 (ThermoFisher Scientific). Sequences were processed using the Torrent Suite Software (version 5.2.2) (ThermoFisher Scientific) to produce raw unaligned sequence data files for further analysis. Sequence data analysis, alignment to GreenGenes and MicroSeq databases, alpha and beta diversity plot generation, and OTU table generation were performed by the Ion Reporter Software Suite (version 5.2.2) of bioinformatics analysis tools (ThermoFisher Scientific). Samples were analyzed using Ion Reporter's Metagenomics 16S workflow powered by Qiime (version w1.1). The OTU data were transformed to relative abundance before statistical analysis. The OTU data with the relative abundance <0.05% within each level were combined as "Others".

Statistical Analyses

Pigs were allotted in a randomized complete block design using sex and the initial BW as blocks in all measurements to account for the variation of the initial BW and sex dimorphism (30). The experimental unit was the pig, individually housed and fed. The main effects were the factors (ETEC challenge and synbiotics) and their interaction. Factors were handled as fixed effects, and initial BW and sex blocks were handled as random effects. For growth performance and fecal score data, each factor had 16 pigs ($n = 16$; eight barrows and eight gilts; and four body weight blocks within sex). For other data, each factor had 12 pigs ($n = 12$; six barrows and eight gilts; and three body weight blocks within sex). Data were analyzed using the Mixed procedure in SAS version 9.4 (SAS Inc., Cary, NC, USA). The means were separated using the LSMEANS statement in SAS. When an interaction between the factors was significant or tended to be significant, a pairwise comparison was made using the PDIFF option in SAS. Statistical differences were considered significant with $P < 0.05$. Tendency was considered when $0.05 \leq P < 0.10$.

RESULTS

Growth Performance

In the pre-challenge period, the synbiotic tended to increase ($P = 0.059$) the BW of pigs at d 7 after weaning (**Table 3**). At d 10 of the study, 3 days post-challenge, the oral inoculation of ETEC did not affect the BW, whereas it was increased ($P < 0.05$) by the dietary use of synbiotics. However, at d 20 of the study, the BW of pigs challenged with ETEC was reduced ($P < 0.05$), whereas it was not affected by the synbiotic.

At the pre-challenge period (d 0–7) the synbiotic increased ($P < 0.05$) the ADG of pigs. On the post-challenge period (d 7–20), the ADG of pigs challenged with ETEC was reduced ($P < 0.05$). In phase 1 (d 0–10), the ETEC challenge tended to reduce ($P = 0.092$) the ADG, whereas the use of synbiotics increased ($P < 0.05$) it, regardless of the challenge. In phase 2 (d 10–20) and overall, the ADG was reduced ($P < 0.05$) when pigs were challenged with ETEC, whereas it was not affected by the use of synbiotics.

The ADFI was not affected by the use of synbiotics during the pre-challenge period (d 0–7). In the post-challenge period, the ETEC challenge did not affect the ADFI, whereas, the use of synbiotics tended to increase ($P = 0.054$) the ADFI during phase 1 (d 0–10). The ADFI was not affected by the two factors during phase 2 and overall.

At the pre-challenge period (d 0–7) the use of synbiotics tended to increase ($P = 0.066$) the G:F ratio of pigs. However, in the post-challenge period (d 7–20), the G:F ratio of pigs challenged with ETEC was reduced ($P < 0.05$). At phase 1, the G:F ratio was reduced ($P < 0.05$) when pigs were challenged with ETEC, whereas it tended to increase ($P = 0.098$) by the use of

TABLE 3 | Growth performance of pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
BW, kg								
Initial	7.91	7.92	7.90	7.92	0.39	0.929	0.829	0.956
d 7	8.02	8.37	8.09	8.23	0.36	0.776	0.059	0.401
d 10	8.57	9.04	8.31	8.73	0.38	0.122	0.020	0.885
d 20	13.04	13.42	12.08	12.62	0.67	0.022	0.227	0.818
ADG, g/d								
Pre-challenge	15	65	27	44	20	0.768	0.032	0.284
Post-challenge	387	388	307	338	29	0.013	0.517	0.547
Phase 1	66	112	41	80	18	0.092	0.014	0.839
Phase 2	447	438	377	390	33	0.023	0.934	0.652
Overall	257	275	209	235	19	0.019	0.220	0.812
ADFI, g/d								
Pre-challenge	111	140	147	142	15	0.177	0.402	0.235
Post-challenge	459	498	437	466	29	0.289	0.188	0.847
Phase 1	156	199	176	195	16	0.606	0.054	0.457
Phase 2	519	547	494	523	36	0.358	0.281	0.989
Overall	337	373	335	353	21	0.571	0.173	0.638
G:F								
Pre-challenge	0.16	0.47	0.19	0.30	0.12	0.531	0.066	0.370
Post-challenge	0.84	0.78	0.70	0.71	0.04	0.008	0.500	0.385
Phase 1	0.51	0.57	0.29	0.44	0.06	0.010	0.098	0.478
Phase 2	0.87	0.80	0.76	0.73	0.03	0.004	0.126	0.617
Overall	0.76	0.74	0.62	0.66	0.03	0.003	0.811	0.437

[1]*ETEC challenge.*
[2]*Synbiotic.*

synbiotics. In phase 2 and overall, the G:F ratio was reduced ($P < 0.05$) when pigs were challenged with ETEC, whereas it was not affected by the use of synbiotics.

Fecal Score

The fecal score of pigs challenged with ETEC was increased ($P < 0.05$) at d 7, 9, 11, and 13; however, at d 9 and 11, the use of synbiotics reduced ($P < 0.05$) the fecal score of pigs challenged with ETEC (**Figure 1**). The fecal score was not affected by the two factors at d 15, 17, and 20.

Immune and Oxidative Status

The mucosal concentration of MDA was increased ($P < 0.05$) when pigs were challenged with ETEC, whereas it was not affected by the use of synbiotics (**Table 4**). However, the concentration of protein carbonyl was not affected when pigs were challenged with ETEC, whereas it tended to reduce ($P = 0.065$) with the use of synbiotics. The concentration of TNFα was not affected when pigs were challenged with ETEC, whereas, it tended to reduce ($P = 0.093$) with the use of synbiotics. The concentration of IL-6 was increased ($P < 0.05$) when pigs were challenged with ETEC, whereas it tended to decrease ($P = 0.064$) with the use of synbiotics, regardless of the challenge. The concentration of IL-8 was not affected by the factors.

Histomorphology, Immunohistochemistry, and Digesta Viscosity

The enterotoxigenic *E. coli* challenge at d 7 post-weaning reduced ($P < 0.05$) the villus height and VH:CD ratio and increased ($P < 0.05$) the crypt depth and the ratio of Ki-67 positive cells to total cell in the crypt in jejunum of pigs (**Table 5**), whereas, regardless of the ETEC challenge, the use of synbiotics increased ($P < 0.05$) the villus height and VH:CD ratio, reduced ($P < 0.05$) the crypt depth, and tended to reduce ($P = 0.053$) the ratio of Ki-67 positive cells to total cell in the crypt in the jejunum of pigs. The viscosity of the jejunal digesta was not affected by the factors.

Microbiome

At the phylum level (**Figure 2**), the ETEC reduced ($P < 0.05$) the relative abundance of Bacteroidetes and Firmicute and increased ($P < 0.05$) the relative abundance of Proteobacteria. The synbiotic did not affect the relative abundance of microbials at the phylum level. At the family level (**Table 6**), pigs challenged with ETEC tended to reduce the relative abundance of *Clostridiaceae* ($P = 0.067$) and *Prevotellaceae* ($P = 0.069$), and reduced ($P < 0.05$) the relative abundance of *Veillonellaceae*, whereas it tended to increase ($P = 0.063$) the relative abundance of *Helicobacteraceae.* The use of synbiotics did not affect the jejunal mucosa-associated microbiota at the family level. In

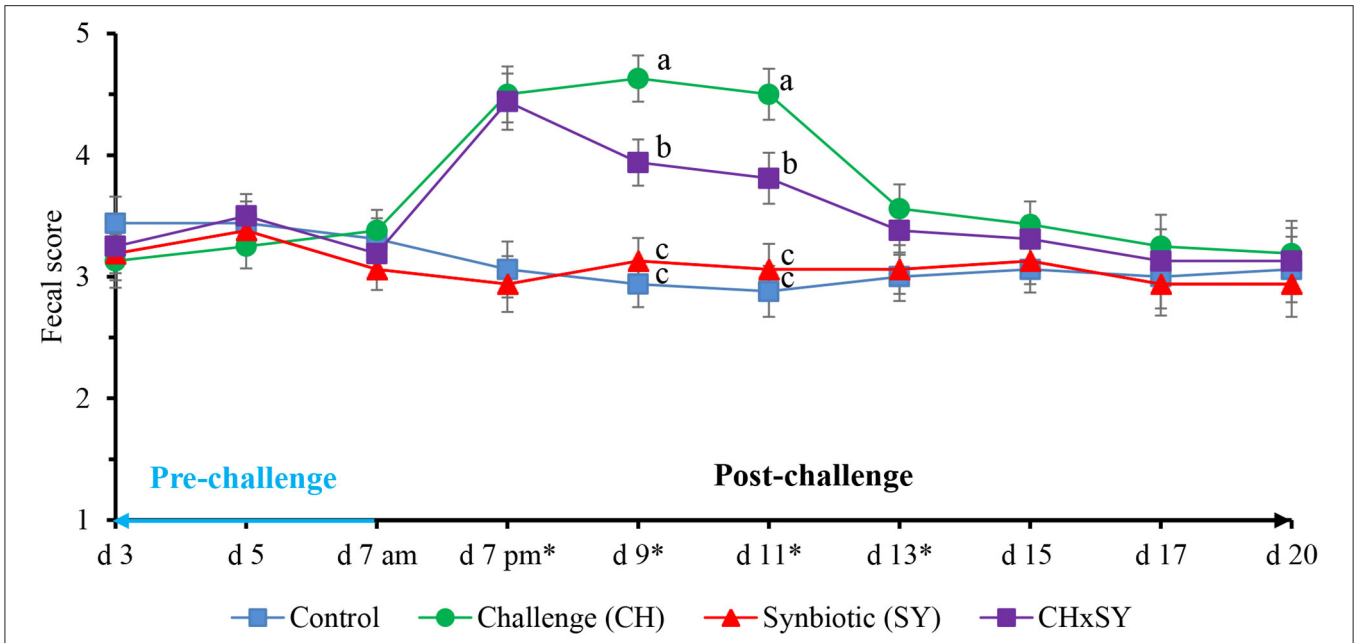

FIGURE 1 | Fecal score of pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY). * **d 7 pm**: CH: ($P < 0.001$), SY: ($P = 0.685$), CH × SY: (0.892); **d 9**: CH: ($P < 0.001$), SY: ($P = 0.124$), CH × SY: ($P < 0.05$); **d 11**: CH: ($P < 0.001$), SY: ($P = 0.236$), CH × SY: ($P < 0.05$); **d 13**: CH: ($P < 0.05$), SY: ($P = 0.718$), CH × SY: ($P = 0.471$). [a,b] Within a column, means without a common superscript letter differ ($P < 0.05$).

TABLE 4 | Oxidative stress and immune parameters in the jejunal mucosa of pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
MDA, μmol/mg of protein	0.24	0.28	0.88	0.76	0.10	<0.001	0.713	0.412
Protein carbonyl, nmol/mg of protein	3.02	2.41	3.24	2.60	0.38	0.529	0.065	0.957
TNFα, pg/mg of protein	0.97	0.86	1.13	0.89	0.11	0.439	0.093	0.645
IL-8, ng/mg of protein	0.49	0.52	0.51	0.53	0.05	0.825	0.546	0.924
IL-6, pg/mg of protein	3.16	2.76	4.98	3.62	0.46	0.006	0.064	0.306

[1]*ETEC challenge.*
[2]*Synbiotic.*

the genus level (**Table 7**), the ETEC challenge reduced ($P < 0.05$) the relative abundance of *Megasphaera, Mitsuokella,* and *Selenomonas* and tended to reduce ($P = 0.060$) the relative abundance of *Helicobacter*, whereas the use of synbiotics did not affect the jejunal mucosa-associated microbiota at the genus level. At the species level (**Table 8**), the ETEC challenge reduced ($P < 0.05$) the relative abundance of *Acidaminococcus fermentans, Selenomonas bovis,* and *Selenomonas lipolytica* and tended to decrease the relative abundance of *Prevotella copri* ($P = 0.096$) and *Roseburia faecis* ($P = 0.079$). Pigs fed synbiotics increased ($P < 0.05$) the relative abundance of *Helicobacter_mastomyrinus* in unchallenged pigs compared with the control group. Pigs challenged with ETEC and fed a diet with synbiotics increased ($P < 0.05$) the relative abundance of *Campylobacter coli* compared with pigs fed synbiotics and not challenged. Pigs challenged with ETEC and fed a diet with a synbiotic tended to increase ($P = 0.075$) the relative abundance of *Campylobacter hyointestinalis* compared with pigs fed synbiotics and not challenged.

There was no effect of the factors on alpha diversity of jejunal mucosa-associated microbiota in pigs estimated with Chao1 richness estimator at the family (**Figure 3A**) and genus levels (**Figure 4A**). At the family level, the Shannon diversity index was not affected by the factors (**Figure 3B**), whereas at the genus level, it tended to be reduced by the ETEC challenge ($P = 0.089$) and the synbiotic ($P = 0.066$), regardless of the challenge (**Figure 4B**). The Simpson diversity index was not affected by the ETEC challenge, whereas it was reduced ($P < 0.05$) by the synbiotic at the family (**Figure 3C**) and genus levels (**Figure 4C**).

DISCUSSION

In this study, pigs were housed individually in order to know the intake of synbiotics affecting intestinal health following

TABLE 5 | Jejunal histomorphology and digesta viscosity of pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
Villus height, μm	405.3	434.1	331.6	408.6	25.5	0.005	0.003	0.153
Villus width, μm	99.7	90.6	95.4	91.4	4.7	0.698	0.153	0.569
Crypt depth, μm	273.6	246.5	306.2	273.9	10.7	0.002	0.003	0.782
VH:CD ratio[3]	1.50	1.80	1.11	1.52	0.09	0.001	0.001	0.534
Ki-67 positive, %[4]	29.95	28.25	35.46	32.34	1.21	0.001	0.053	0.559
Viscosity, mPa·s	1.92	1.90	1.88	1.76	0.07	0.193	0.354	0.465

[1]*ETEC challenge.*
[2]*Synbiotic.*
[3]*Villus height to crypt depth ratio.*
[4]*The ratio of Ki-67 positive cells to total cells in the crypt.*

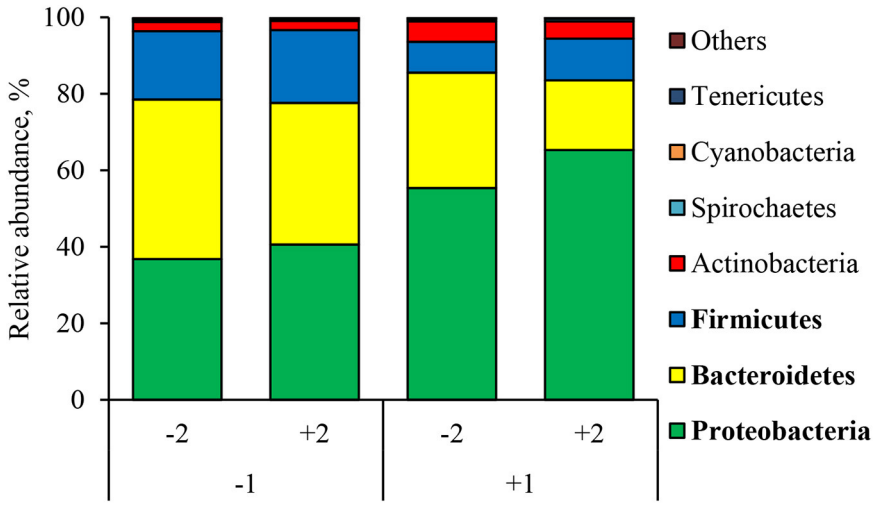

FIGURE 2 | Relative abundance of jejunal mucosa-associated microbiota at the phylum level in pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY). Each pattern represents a particular bacterial phylum. Phylum sequences that did not achieve 0.5% within each phylum were combined as "Others." 1: ETEC challenge (CH); 2: Synbiotic (SY). **Proteobacteria**: CH: ($P < 0.05$), SY: ($P = 0.339$), CH × SY: ($P = 0.668$). **Bacteroidetes**: CH: ($P < 0.05$), SY: ($P = 0.162$), CH × SY: ($P = 0.542$); **Firmicutes**: CH: ($P < 0.05$), SY: ($P = 0.523$), CH × SY: ($P = 0.803$).

procedures previous described (26, 31, 32). The beneficial effects of the synbiotic shown in this study were prominent especially during the period immediately after the weaning when pigs receive the greatest nutritional challenges from plant-based diets, whereas the synbiotic seems to be efficient in enhancing the jejunal histomorphology and reducing the fecal score and the microbial diversity without affecting the growth performance during P2 of this study. Probiotics and prebiotics are shown to be effective to newly weaned pigs because of the immaturity of the intestine and limited digestive capacity of plant-based diets (10, 33). As pigs adapt to plant-based diets, however, pigs develop the intestine to handle fiber and utilize dietary nutrients more efficiently (34–36).

This study confirmed that *E. coli* F18$^+$ can be associated with post-weaning diarrhea (PWD), reducing the growth, modulating the microbiome, and affecting the gut heath of newly weaned pigs as previously reported (5, 25, 37). Enterotoxins (including STa, and STb) from ETEC are a major cause of increased fecal score as shown in this study. The fimbria of the *E. coli* bind to glycoproteins in the microvilli of the intestine of newly weaned pigs by a fimbria receptor interaction causing an interference in the electrolytes fluid that leads to diarrhea by enterotoxin interaction (5, 38–40). The predisposition of newly weaned pigs to PWD caused by ETEC have been related to the psychological, environmental, and physiological stress after weaning, as well as sudden transition from sow's milk to plant-based diets that are solid and less digestible. These stressors disrupt the immune system and the intestinal microbiota leading to intestinal inflammation and PWD (41, 42), consequently reducing growth performance (5, 6, 17). As previously reported (31, 43), the challenge with *E. coli* F18$^+$ in this study reduced growth and feed efficiency without affecting feed intake, which is in agreement with previous studies. Reduced feed efficiency in pigs with *E. coli* infection is related to impaired nutrient

TABLE 6 | Relative abundance of jejunal mucosa-associated microbiota at the family level in pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
Helicobacteraceae	30.13	38.9	43.55	55.41	14.35	0.063	0.196	0.845
Prevotellaceae	42.16	33.89	32.48	21.88	11.52	0.069	0.111	0.841
Lactobacillaceae	4.32	6.70	4.81	5.35	2.07	0.836	0.485	0.661
Veillonellaceae	8.07	6.15	3.19	2.84	1.34	0.003	0.380	0.545
Corynebacteriaceae	2.16	2.24	4.43	4.07	2.01	0.313	0.945	0.913
Campylobacteraceae	2.42	1.46	1.66	1.22	0.69	0.461	0.302	0.698
Lachnospiraceae	1.40	1.60	0.96	1.45	0.40	0.418	0.348	0.695
Succinivibrionaceae	0.77	0.28	1.88	1.24	0.96	0.159	0.444	0.920
Clostridiaceae	1.38	0.82	0.51	0.58	0.30	0.067	0.412	0.294
Ruminococcaceae	0.94	0.95	0.56	0.66	0.33	0.192	0.830	0.859
Eubacteriaceae	0.74	0.78	0.70	0.88	0.33	0.856	0.559	0.705
Porphyromonadaceae	0.74	0.76	0.52	0.67	0.24	0.417	0.665	0.735
Enterobacteriaceae	0.27	1.13	0.32	0.52	0.45	0.522	0.229	0.449
Bacillaceae	0.04	0.13	0.05	0.04	0.05	0.420	0.419	0.357
Others	4.47	4.27	4.38	4.35	1.12	0.999	0.904	0.928

[1]*ETEC challenge.*
[2]*Synbiotic.*

TABLE 7 | Relative abundance of jejunal mucosa-associated microbiota at the genus level in pigs challenged with ETEC (CH) on d 7 post-weaning and fed diets supplemented with a synbiotic (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
Helicobacter	33.34	42.25	49.15	57.52	13.68	0.060	0.289	0.973
Prevotella	42.42	34.70	31.01	24.16	12.05	0.117	0.294	0.949
Lactobacillus	5.77	8.02	5.60	6.04	2.66	0.688	0.616	0.735
Corynebacterium	2.79	2.79	4.89	4.42	2.18	0.398	0.915	0.913
Campylobacter	3.16	1.80	1.96	1.49	0.86	0.367	0.275	0.591
Mitsuokella	2.60	1.19	0.83	0.66	0.68	0.041	0.153	0.27
Selenomonas	1.87	2.39	0.55	0.24	0.52	<0.001	0.818	0.377
Succinivibrio	0.85	0.22	1.72	1.22	0.93	0.208	0.446	0.928
Megasphaera	1.09	0.83	0.35	0.24	0.21	0.002	0.366	0.713
Others	5.30	4.99	3.46	3.82	0.96	0.124	0.975	0.727

[1]*ETEC challenge.*
[2]*Synbiotic.*

absorption (43, 44) and the activation of immune system partitioning nutrients from growth (45).

McLamb et al. (24) previously reported that pigs challenged with ETEC have the immune response activated. According to Loos et al. (46), the secretion of IL-6 in the lumen of the small intestine is stimulated by STa produced by *E. coli* F18$^+$. In this study, the ETEC challenge increased the concentration of IL-6 as previous reported (46, 47). High levels of IL-6 reduce the secretion of growth hormone (48) and damage the intestinal epithelium (49, 50). The challenge, however, did not affect the concentration of IL-8 and TNFα in this study. According to Loos et al. (51), the IL-8 has low expression in response to ETEC. The activation of the immune system in response to the ETEC infection may lead to an exhaustion of the antioxidant mechanism causing the oxidation of cellular protein, lipids, and DNA (52). The results of this study show, on challenged pigs, an increasing level of MDA, a final product of the lipid oxidation, and an indicator of oxidative stress (53, 54). The metabolites from oxidative stress can directly affect the enterocytes' cell wall components, such as lipids and proteins, causing apoptosis and, consequently, reduction of villi length (54, 55). The villi reduction in the challenged pigs leads to increasing the crypt cell proliferation rate, and consequently, increasing the crypt depth which is in accordance

TABLE 8 | Relative abundance of jejunal mucosa-associated microbiota at the species level in pigs challenged (CH) with ETEC on d 7 post-weaning and fed diets supplemented with synbiotics (SY).

Challenged synbiotic	–[1]		+[1]		SEM	*P*-value		
	–[2]	+[2]	–[2]	+[2]		CH	SY	CH × SY
Prevotella copri	39.80	30.59	29.55	20.72	7.94	0.096	0.135	0.974
Helicobacter rappini	16.12	23.06	26.76	27.82	6.97	0.228	0.529	0.643
Helicobacter mastomyrinus	4.12^{b}	13.80^{a}	10.00^{ab}	8.05^{ab}	3.47	0.983	0.193	0.053
Prevotella stercorea	7.39	7.65	6.13	8.42	3.65	0.913	0.561	0.642
Corynebacterium glutamicum	3.61	2.22	4.08	4.60	2.11	0.504	0.838	0.653
Helicobacter equorum	0.07	0.45	2.54	10.07	4.40	0.107	0.287	0.336
Lactobacillus mucosae	2.44	4.18	0.99	2.52	1.51	0.309	0.285	0.942
Prevotella sp.	2.30	1.85	3.59	1.97	1.95	0.648	0.507	0.703
Corynebacterium deserti	2.11	1.17	2.26	2.20	1.15	0.611	0.662	0.705
Lactobacillus kitasatonis	1.18	1.05	1.70	1.85	0.72	0.363	0.984	0.843
Campylobacter upsaliensis	2.31	0.87	1.45	0.33	1.05	0.506	0.230	0.883
Mitsuokella jalaludinii	1.47	0.81	0.86	0.69	0.53	0.360	0.297	0.548
Lactobacillus delbrueckii	1.12	1.01	0.40	0.68	0.43	0.225	0.838	0.653
Selenomonas bovis	1.08	1.50	0.33	0.24	0.29	0.001	0.582	0.384
Dialister succinatiphilus	0.93	0.79	0.63	0.48	0.30	0.247	0.593	0.989
Roseburia faecis	0.90	0.83	0.47	0.54	0.24	0.079	0.986	0.724
Lactobacillus sp.	0.86	0.66	0.35	0.67	0.31	0.430	0.837	0.398
Helicobacter canadensis	1.00	0.13	0.30	1.08	0.70	0.842	0.943	0.202
Prevotella ruminicola	0.64	0.18	1.03	0.27	0.52	0.673	0.296	0.799
Selenomonas lipolytica	0.74	1.00	0.20	0.15	0.22	0.004	0.650	0.491
Mitsuokella multacida	1.12	0.29	0.30	0.37	0.48	0.378	0.361	0.280
Faecalibacterium prausnitzii	0.65	0.62	0.26	0.47	0.16	0.113	0.612	0.464
Succinivibrio dextrinosolvens	0.63	0.25	0.88	0.24	0.48	0.782	0.250	0.774
Campylobacter coli	0.57^{AB}	0.19^{B}	0.28^{AB}	0.63^{A}	0.17	0.637	0.960	0.031
Phascolarctobacterium succinatutens	0.22	0.32	0.27	0.67	0.27	0.243	0.148	0.394
Acidaminococcus fermentans	0.50	0.37	0.16	0.20	0.10	0.036	0.712	0.436
Campylobacter lanienae	0.34	0.15	0.23	0.40	0.11	0.507	0.909	0.113
Lactobacillus amylovorus	0.09	0.17	0.59	0.23	0.21	0.200	0.523	0.319
Campylobacter hyointestinalis	0.30^{A}	0.14^{B}	0.19^{AB}	0.27^{AB}	0.07	0.932	0.568	0.055
Pelomonas puraquae	0.10	0.21	0.16	0.31	0.09	0.375	0.160	0.804
Others	4.25	3.76	3.19	3.27	0.73	0.290	0.782	0.699

[1]*ETEC challenge.*
[2]*Synbiotic.*
[a,b]*Within a row, means without a common superscript letter differ ($P < 0.05$).*
[A,B]*Within a row, means without a common superscript letter differ ($P < 0.10$).*

with previous studies (3, 43, 56). The increased oxidative stress due to the activated immune system may also redirect energy and nutrients from growth to immune response, and to repair the epithelium.

The use of synbiotics, however, was effective to reduce the jejunal mucosal levels of IL-6, TNFα, and protein carbonyl regardless of the ETEC challenge. The reduction of the immune and oxidative stress indicators reduces the epithelial damage and the cell proliferation rate by reducing the deleterious effect of the ETEC on nursery pigs (43, 57–59). These results showed a potential benefit of dietary xylanase and *Bacillus* sp. as a synbiotic on enhancing the gut health and further reducing the jejunal mucosal protein carbonyl concentration. Therefore, this study targeted the investigation of the combinational effects of xylanase and *Bacillus* sp. The synbiotic had beneficial outcomes because xylanase successfully hydrolyzed xylans to XOS in feeds (10, 31, 60, 61), reducing the viscosity of digesta (10, 19) releasing nutrients for digestion (14, 18). Passos et al. (14) reported that dietary supplementation with xylanase showed a linear increase in the ileal digestibility of NDF, indicating the hydrolysis of NSP-releasing oligosaccharides, such as XOS. In addition, *Bacillus* sp. effectively utilizes XOS released by xylanase hydrolysis, further exerting synergetic effects (20) including their antibacterial properties (43, 62, 63).

The synbiotic can selectively affect the growth of microorganisms in the intestine, including those directly added in the diet (64, 65), targeting some metabolic processes and possibly changing the physical-chemical properties of

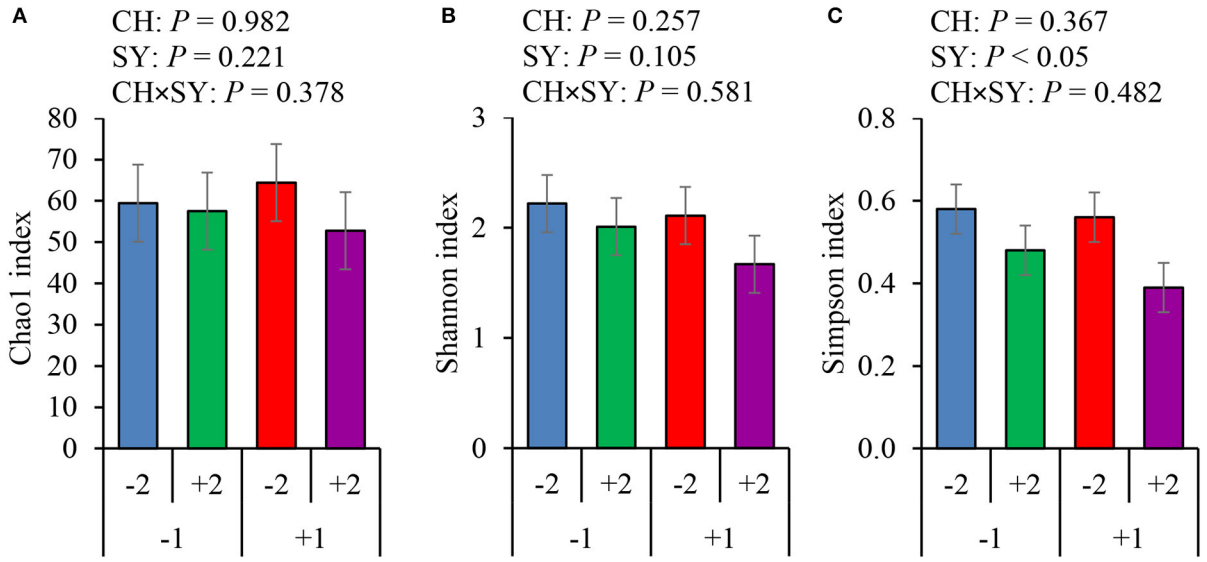

FIGURE 3 | Alpha diversity of jejunal mucosa-associated microbiota at the family level estimated with Chao1 richness **(A)**, Shannon diversity **(B)**, and Simpson diversity **(C)** in pigs challenged (CH) with ETEC on d 7 post-weaned and fed diets supplemented with a synbiotic (SY). 1: ETEC challenge; 2: Synbiotic; SY: synbiotic; CH: challenge; CH × SY: Challenge and synbiotic.

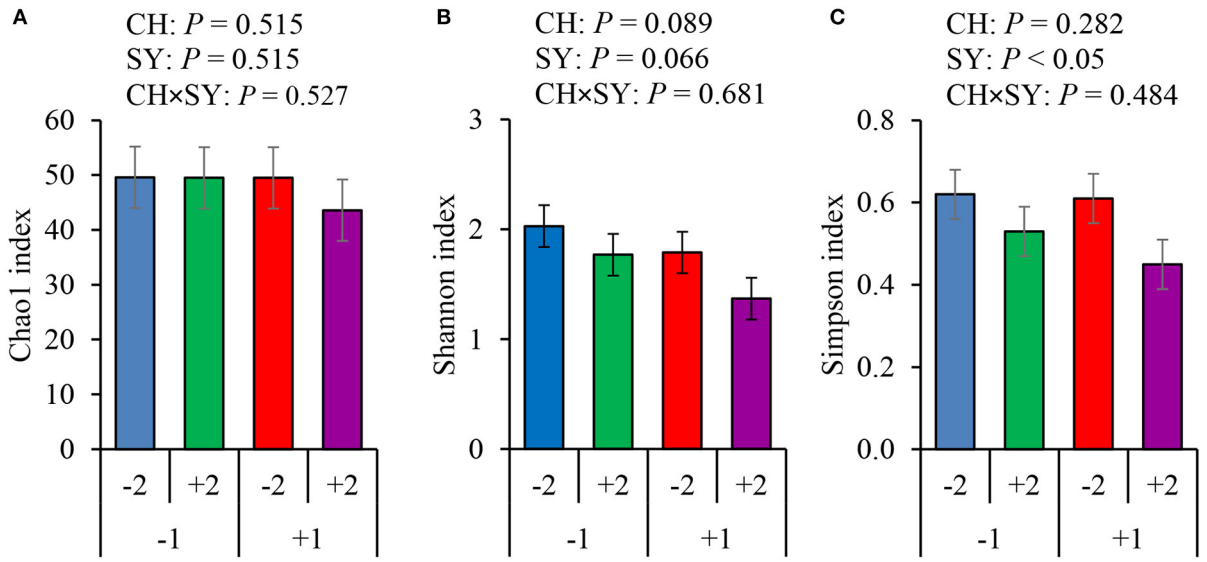

FIGURE 4 | Alpha diversity of jejunal mucosa-associated microbiota at the genus level estimated with Chao1 richness **(A)**, Shannon diversity **(B)**, and Simpson diversity **(C)** in pigs challenged (CH) with ETEC on d 7 post-weaned and fed diets supplemented with a synbiotic (SY). 1: ETEC challenge; 2: Synbiotic; SY: synbiotic; CH: challenge; CH × SY: Challenge and synbiotics.

the digesta (66). This mechanism can promote gut health benefits, such as modulation of gut microbiota by competition and antimicrobial property (67), and consequently, affect the immune system, reduce the oxidative stress, and increase the growth performance of newly weaned pigs (31).

Pigs from the challenge group-fed diets with the synbiotic had reduced diarrhea occurrence earlier than those without synbiotic supplementation. This outcome shows that the dietary supplementation of synbiotics may prevent ETEC from damaging the intestinal epithelium. Although the jejunal digesta viscosity was not affected by either factors in this study, the viscosity can be affected by the ingredient in the diet (19, 31) and the ratio of insoluble to soluble NSP (68). The viscosity observed in this study ranged from 1.8 to 1.9 mPa·s, which is lower than previously reported by Duarte et al. (10) and Passos et al. (14) due to differences in dietary compositions.

The ETEC slightly reduced the microbial diversity index but caused an imbalance in jejunal mucosa-associated microbiota by increasing the relative abundance of Proteobacteria by increasing the family *Helicobacteraceae* and the genus *Helicobacter*, consequently reducing the relative abundance of Bacteroidetes and Firmicutes, *Prevotellaceae*, and *Mitsukella and Selenomonas,* as previously reported by Bin et al. (69) and Pollock et al. (70). The adherence of the ETEC and the production of enterotoxins with the subsequent secretion of fluid to the intestinal lumen (41, 51) create a propitious environment to the growth of proteobacteria (17). The high abundance of *Helicobacteraceae* which belong to the Proteobacteria has been reported to cause a reduction of the mucous layer protection (65), which explains the impact of the challenge on the villus height, immune response, and the oxidative stress status, whereas, *Prevotellaceae*, which belongs to the Bacteroidetes has been related to intestinal mucosa of healthy pigs fed plant-based diets (71, 72).

The synbiotic reduced the diversity of the microbiome without affecting the relative abundance of microbials. *Bacillus* spores, *Lactobacillus acidophilus* and *Pediococcus acidilactici* used as probiotics has been reported to reduce the microbial diversity in pigs (33, 73). According to Poulsen et al. (33), *Bacillus* spores are able to adhere to intestinal epithelium and competitively affect the colonization pattern. Reduction on mucosa-associated microbial diversity have been related to increased inflammatory response (74), even though this was not observed in this study. These results may suggest that the type of the dominant microbials in the jejunal mucosa is more important to affect the intestinal immune response than the microbial diversity. According to Wang et al. (73), the ability of probiotics to reduce the diversity or richness of microbiome can positively affect the growth performance by reducing the deleterious effects of harmful microbes that can affect the immune system, oxidative stress, and intestinal histomorphology. It was confirmed in this study that the synbiotic supplementation increased growth performance, and villus height, reducing diarrhea, immune response, and oxidative stress in nursery pigs.

In conclusion, the ETEC challenge reduced the growth performance of newly weaned pigs by increasing the relative abundance of harmful bacteria, intestinal immune response, intestinal oxidative stress, and crypt depth while reducing the villus height in the small intestine. Dietary supplementation of xylanase and *Bacillus* sp. as a synbiotic enhanced growth performance by increasing the relative abundance of beneficial bacteria in the small intestine, reducing diarrhea, reducing the oxidative stress, and increasing the villus height in the small intestine regardless of the challenge. The synbiotic showed potential benefits on growth performance, reducing diarrhea, immune response, and the oxidative stress status in the small intestine, leading to a protective function on the intestinal epithelium. Therefore, it was demonstrated that the *E. coli* $F18^+$ greatly affects the gut health and growth performance of pigs, whereas the novel synbiotic showed a potential to mitigate the effects of *E. coli* $F18^+$ infection in an AGP-free diet.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The experimental procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University.

AUTHOR CONTRIBUTIONS

SK conceptualized the study and secured the funding. SK and JT designed the study. MD performed the experiments. MD and SK analyzed the data. The manuscript was written and reviewed by MD and SK. SK, MD, and JT discussed the results and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was selected for a research award and funded from North Carolina Biotechnology Center (CFG-8003).

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Conflict of Interest: JT was employed by the company BioResources International.

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

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fvets.2020.575685

Supplementing Synbiotic in Sows' Diets Modifies Beneficially Blood Parameters and Colonic Microbiota Composition and Metabolic Activity in Suckling Piglets

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OPEN ACCESS

Edited by:
Amlan Kumar Patra, West Bengal University of Animal and Fishery Sciences, India

Reviewed by:
Demin Cai, Yangzhou University, China
Sungkwonk Park, Sejong University, South Korea

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *01 July 2020*
Accepted: *04 November 2020*
Published: *30 November 2020*

Citation:
Ma C, Gao Q, Zhang W, Zhu Q, Tang W, Blachier F, Ding H and Kong X (2020) Supplementing Synbiotic in Sows' Diets Modifies Beneficially Blood Parameters and Colonic Microbiota Composition and Metabolic Activity in Suckling Piglets. Front. Vet. Sci. 7:575685. doi: 10.3389/fvets.2020.575685

Nutrients in the maternal diet favor the growth and development of suckling piglets and alter their gut microbiota composition and metabolic activity, thus affecting the hosts. The present study analyzed, in suckling piglets from sows receiving antibiotic or synbiotic supplements from pregnancy to lactation, several biochemical parameters, oxidative/anti-oxidative indices, inflammatory cytokines, and ingestion-related factor levels in plasma, as well as colonic microbiota composition and metabolic activity, and mucosal expression of genes related to the intestinal barrier function. Compared with the control group, maternal synbiotic supplementation decreased ($P < 0.05$) the plasma levels of glucose, AMM, TC, low-density lipoprotein-cholesterol (LDL-C), MDA, H_2O_2, ghrelin, CCK, PP, IL-1β, IL-2, IL-6, TNF-α, Ala, Cys, Tau, and β-AiBA, the levels of propionate and total short-chain fatty acids (SCFAs) in the colonic luminal content, and colonic abundances of *RFN20*, *Anaerostipes*, and *Butyricimonas*; while increased ($P < 0.05$) the plasma levels of urea nitrogen (UN), Ile, Leu, α-AAA, α-ABA, and 1-Mehis, as well as colonic abundances of *Sphingomonas, Anaerovorax, Sharpea,* and *Butyricicoccus*. Compared with the antibiotic group, maternal synbiotic supplementation decreased ($P < 0.05$) the plasma levels of glucose, gastrin, and Ala, as well as abundances of *Pasteurella* and *RFN20* and propionate level in the colonic content. Expression of genes coding for E-cadherin, Occludin, ZO-1, ZO-2, IL-10, and interferon-α were down-regulated in the colonic mucosa. The synbiotic supplementation increased ($P < 0.05$) the plasma levels of UN, Leu, α-ABA, and 1-Mehis, the abundances of *Anaerovorax, Sharpea,* and *Butyricicoccus* and expression of genes coding for E-cadherin, Occludin, ZO-1, ZO-2, IL-10, and interferon-α. Spearman correlation analysis showed that there was a positive correlation between colonic *Anaerostipes* abundance

and acetate and SCFAs levels; whereas a negative correlation between *Fusobacteria* and *Fusobacterium* abundances and acetate level. These findings suggest that synbiotic supplementation in the maternal diet improved nutrient metabolism and intestinal barrier permeability, reduced oxidative stress, and modified colonic microbiota composition and metabolic activity in suckling piglets.

Keywords: biochemical parameters, gut microbiota, metabolites, sows, suckling piglets, synbiotic

INTRODUCTION

Economic benefit in swine farm is directly affected by the survival rate, growth and development, and health of suckling piglets (1). The survival and health of suckling piglets are largely dependent on maternal milk quality (2). Maternal nutrition during lactation is an important factor affecting the quality and quantity of the maternal milk. Therefore, improving maternal nutrient level could help to enhance sows lactating performance and promote the growth and development of piglets.

Gut microbiota is involved in the metabolism, growth, and development of the host (3). Short-chain fatty acids (SCFAs) are products of some specific gut bacteria and could serve as luminal energy substrates in colonocytes (4). In addition, SCFAs exert an anti-inflammatory effect in the gut (5). Microbiota colonization in infant gut begins from their mother's wombs (6) and is affected by diets and other environmental factors (7). Exposure to antibiotics *via* oral administration as a kind medicine (especially the broad-spectrum antibiotics) in newborn animals has a major effect on gut microbiota composition (8). Antibiotics was reported to promote nutrient absorption and increase the piglet growth (9). However, antibiotic overuse leads to drug residues in animals and their products, thus leading to antibiotic resistance and affecting humans health (10). Synbiotics, the mixed additive of prebiotics and probiotics, have shown several beneficial effects in pig production. For instance, several studies showed that dietary synbiotic supplementation improved the intestinal microbiota and growth performance of weaned piglets (11, 12). Therefore, we speculated that synbiotics in the maternal diet could affect the offspring, notably by modifying the gut microbiota and metabolic activity.

Our previous study showed that dietary synbiotic supplementation increased the piglet survival rate by improving the glycolipids absorption and utilization and altering the gut microbiota composition and abundances of sows (13). The present study hypothesizes that maternal synbiotic supplementation may modify beneficially blood indices, gut microbiota composition and metabolic activity, and the mucosal mRNA expression of genes related to the intestinal barrier function. Therefore, the effects of synbiotic supplementation in sows' diets were measured on several parameters in suckling piglets, including plasma biochemical parameters, oxidative/anti-oxidative indices, inflammatory and ingestion-related factors, and free amino acids. In addition, colonic microbiota composition and metabolic activity were measured in piglets, as well as expression of colonic mucosa genes involved in epithelial barrier function and inflammation.

MATERIALS AND METHODS

Experimental Design

The animal experiment was conducted in Hantang Agriculture Co. Ltd., Shimen, Hunan, China. Forty-eight pregnant Bama mini-pigs were selected and randomly allocated into one of three groups (16 sows per group). The sows in the control group were fed a basal diet, those in the antibiotic group were fed a basal diet supplemented with 50 g/t virginiamycin, and those in the synbiotic group were fed a basal diet supplemented with 200 mL/d fermentation broth per animal and 500 g xylo-oligosaccharides (XOS) per ton diet. The fermentation broth was provided by Hunan Lifeng Biotechnology Co. Ltd. and contained $\geq 1.2 \times 10^8$ CFU/g viable *Lactobacillus plantarum* B90 (BNCC1.12934) $\geq 1.0 \times 10^8$ CFU/g and *Saccharomyces cerevisiae* P11 (BNCC2.3854) $\geq 0.2 \times 10^8$ CFU/g. The XOS was provided by Shandong Longlive Biotechnology Co., Ltd., Shandong, China; and contained xylobiose, xylotriose, and xylotetraose at level $\geq$ 35%. The diet composition and nutrient levels for the sows met the Chinese pig local standard (NY-2004), and the premixes for pregnant and lactating sows met the NRC recommended requirements (NRC, 2012) (**Supplementary Table 1**). The experimental period was from mating to weaning (postpartum 21 d). During the trial period, there were four sows returned to estrus in the control group, two sows returned to estrus in the antibiotic group, and three sows returned to estrus in the synbiotic group. The diets were fed twice daily (8:00 a.m. and 5:00 p.m.) fluctuating with the physical condition of the sows throughout the trail, and water was available freely.

Sample Collection and Preparation

At 21 day-old (weaned), the piglets from 12 litters were weighed after fasted for about 12 h and one piglet with middle body weight (BW) per litter was selected. Twelve piglets per group were exsanguinated after electrical stunning (120 V, 200 Hz). Each piglet per group was randomly chosen to collect blood samples from precaval vein into 10 mL heparin coated-tubes and plasma was separated by centrifuging at 3,500 g and 4°C for 10 min and stored at −20°C for further analysis. Colonic contents (middle section) were collected in 10 mL sterile centrifuge tubes and stored immediately at −20°C for subsequent analysis of microbiota composition and metabolites. After washing with cold physiological saline, the colonic mucosal tissues were sampled and immediately frozen in liquid nitrogen (∼2 g), and then stored at −80°C for mRNA analyses.

Determination of Plasma Biochemical Parameters

The plasma levels of albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), ammonia (AMM), aspartate aminotransferase (AST), glucose (GLU), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), total protein (TP), and urea nitrogen (UN) were determined using commercially available kits (F. Hoffmann-La Roche Ltd, Basel, Switzerland) with the Roche automatic biochemical analyzer (Cobas c311, F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Determination of Plasma Oxidative/Anti-oxidative Indices, Inflammatory Cytokines, and Ingestion Related Factors

The plasma levels of catalase (CAT), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC), were determined as per commercially available kit directions (Suzhou keming, Co. Ltd, Jiangsu, China) with Multiscan Spectrum (Tecan, Infinite M200 Pro, Switzerland).

The plasma levels of gastrin, ghrelin, cholecystokinin (CCK), interleukin (IL)-1β, IL-2, IL-6, IL-10, interferon (IFN)-α, insulin-like growth factor (IGF)-1, leptin (LEP), pancreatic polypeptide (PP), peptide YY (PPY), and tumor necrosis factor (TNF)-α were measured according to the Meimian ELISA kit directions (Jiangsu Yutong Biological Technology, Co. Ltd., Jiangsu, China) on Multiscan Spectrum (Tecan, Infinite M200 Pro, Switzerland).

Determination of Plasma Free Amino Acids

Approximately 1.00 mL plasma sample was added into 1.00 mL 8% salicylic acid solution, mixed thoroughly and overnighted at 4°C, and then centrifuged at 8,000 r/min for 10 min to obtain the supernatant. The processed samples were filtered through a 0.45-μm membrane prior to analysis of free amino acids with an automatic AA analyzer (L8900, Hitachi, Tokyo, Japan).

DNA Extraction and 16S rRNA Gene Sequencing

The total genomic DNA of colonic content samples was extracted using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA). The DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3-V4 regions was amplified using the primer 338F (5′-GCACCTAA YTGGGYDTAAAGNG-3′) and 806R (5′-TACNVGGGTATCTA ATCC-3′). The protocol of PCR amplification was conducted according to our previous study (13). The PCR products were successfully separated using 1.2% agarose gel electrophoresis, purified using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN), and further quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified amplicons were then subjected to paired-end (2 × 300) sequencing on an Illumina MiSeq platform (Illumina, San Diego, USA) using the MiSeq Reagent Kit v3 (600 cycles) according to the standard protocol, which was performed by Shanghai Personal Biotechnology Co. Ltd., Shanghai, China. The raw Illumina pair-end read data for all samples are available in the NCBI Sequence Read Archive with accession number PRJNA609410.

Determination of Metabolites in Colonic Contents

The SCFAs in colonic contents were measured with gas chromatography (Agilent Technologies 1206, Santa Clara, CA, USA) according to the previous description (14). The levels of bioamines, indole, and skatole in colonic contents were measured using reverse phase-high performance liquid chromatography

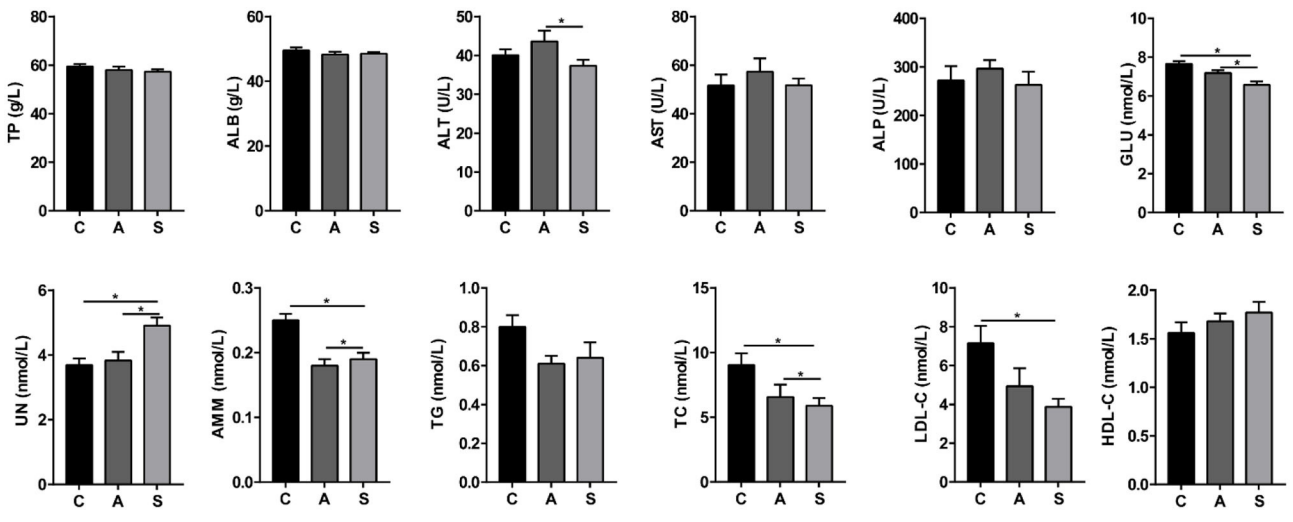

FIGURE 1 | Effect of maternal synbiotic supplementation on plasma biochemical parameters of suckling Bama mini-piglets. C, A, and S present the control group, antibiotic group, and synbiotic group, respectively. The same as below. Data represent the means ± SEM. *indicates statistically significant ($P < 0.05$). $n = 8$ per group.

(Agilent Technologies, Santa Clara, CA, USA) according to a previous study (14).

Determination of mRNA Expression of Genes Related to Intestinal Health

The primers for target genes and reference gene β-actin (listed in **Supplementary Table 2**) were designed using Primer-BLAST. RNA extraction and real-time polymerase chain reaction (RT-PCR) analyses were conducted as a previous report (15). The relative expression level of each target gene was determined by RT-PCR with performing on a 480II system (Roche, LightCycler® 480II, Switzerland) and calculated by the $2^{-\Delta\Delta Ct}$ method (16).

Statistical Analysis

The plasma indices, colonic metabolite levels, and colonic microbiota alpha diversity were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range *post hoc* test with SPSS 22. The microbial community structural variation among samples was performed by the beta diversity analysis (PERMANOVA) (17) and was showed using the partial least squares-discriminant analysis (PLS-DA). The colonic microbiota abundance and overall composition at

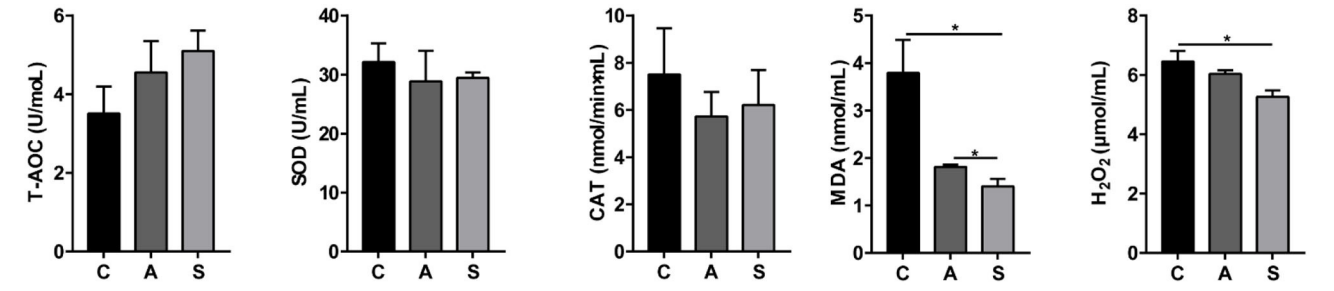

FIGURE 2 | Effect of maternal synbiotic supplementation on plasma oxidative/anti-oxidative levels in suckling Bama mini-piglets. Data represent the means ± SEM. *indicates statistically significant ($P < 0.05$). $n = 8$ per group.

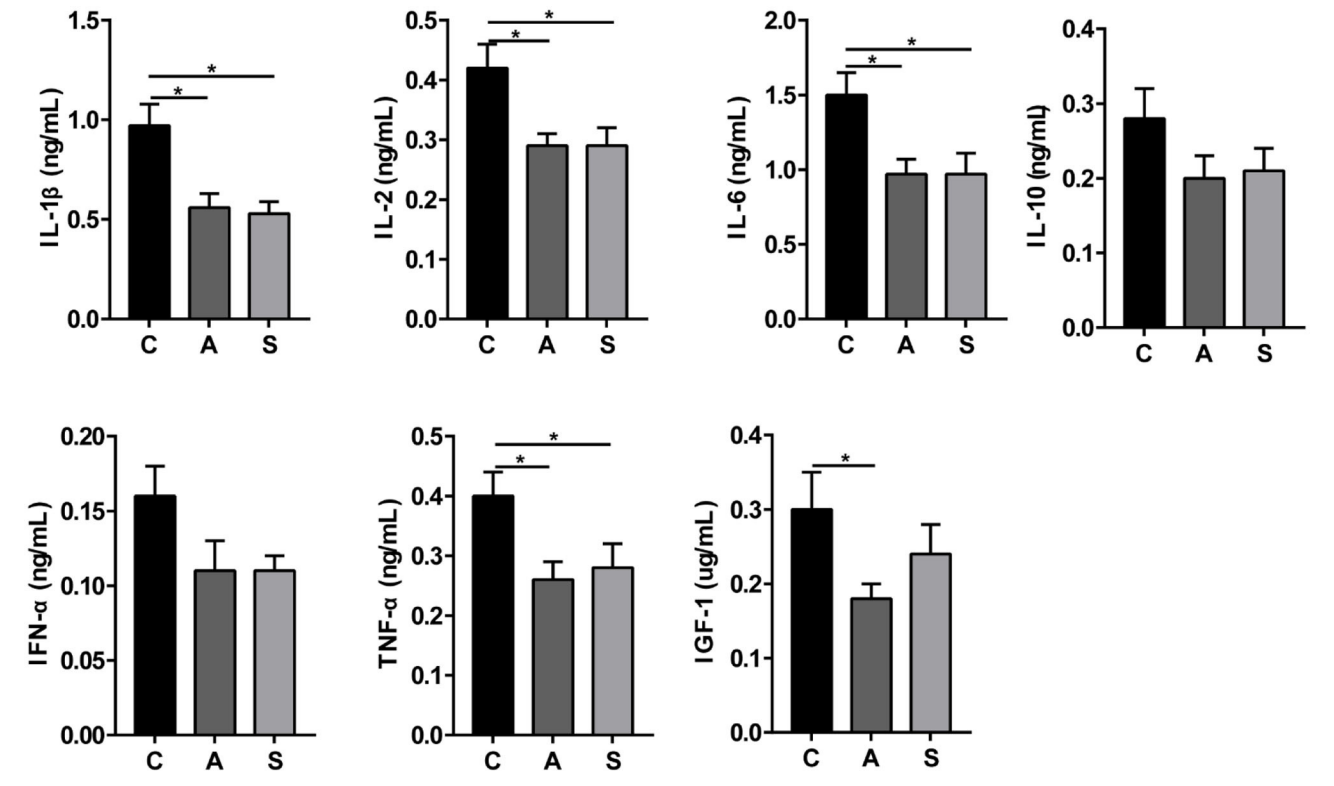

FIGURE 3 | Effect of maternal synbiotic supplementation on plasma inflammatory cytokine levels in suckling Bama mini-piglets. Data represent the means ± SEM. *indicates statistically significant ($P < 0.05$). $n = 8$ per group.

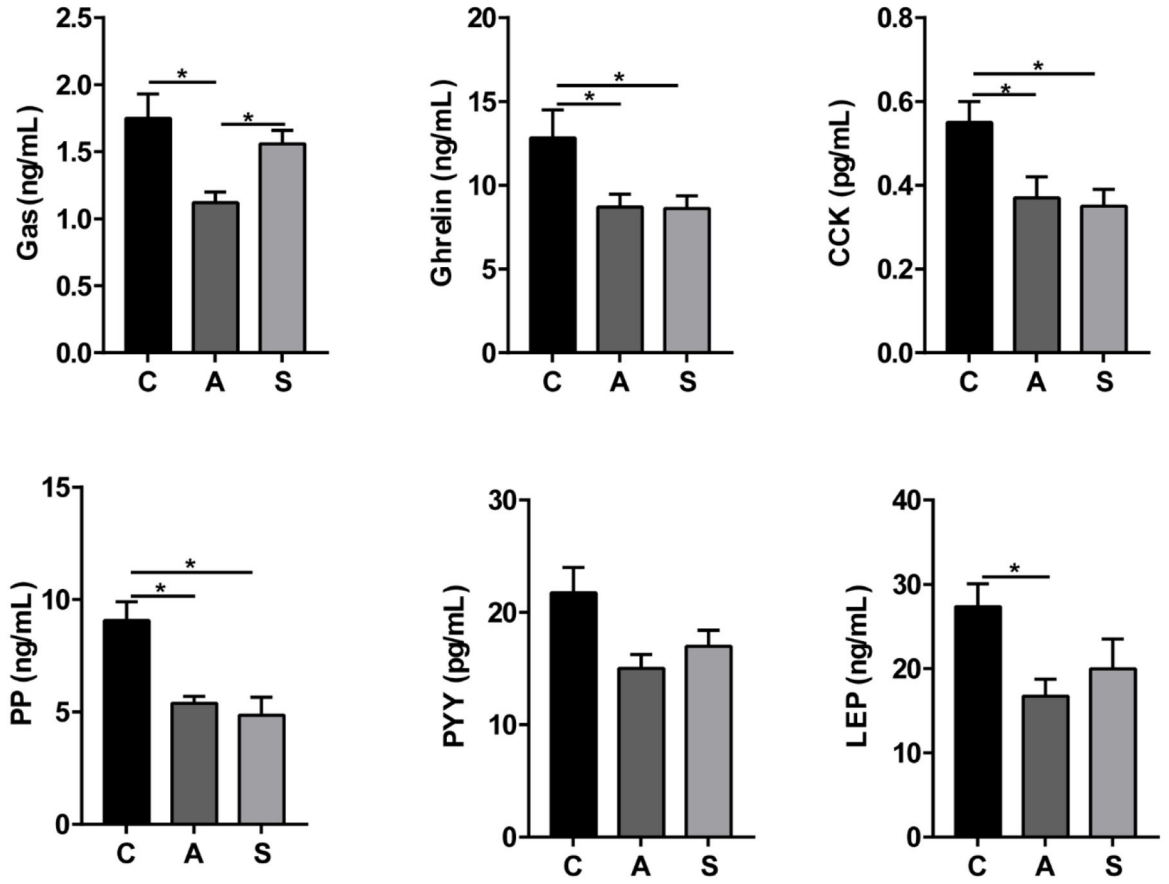

FIGURE 4 | Effect of maternal synbiotic supplementation on plasma ingestion-related factor levels in suckling Bama mini-piglets. Data represent the means ± SEM. *indicates statistically significant ($P < 0.05$). $n = 8$ per group.

phyla and genus levels were analyzed using Metastats (http://metastats.cbcb.umd.edu/) (18). The graph preparation was performed using GraphPad Prism ver7.0 (San Diego, CA, USA). Spearman's correlation between colonic microbiota abundances and metabolite levels was analyzed with the R package. All data were presented as means ± SEM. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Plasma Biochemical Parameters of Piglets

As shown in **Figure 1**, compared with the control group, maternal synbiotic supplementation increased ($P < 0.05$) plasma UN level while decreased ($P < 0.05$) plasma GLU, AMM, TC, and LDL-C levels. Maternal synbiotic supplementation decreased ($P < 0.05$) plasma ALT and GLU levels, increased ($P < 0.05$) UN level, and showed an increased trend in TG level ($P = 0.074$), when compared with the antibiotic group.

Plasma Oxidative/Anti-oxidative Indices, Inflammatory Cytokines, and Ingestion Related Factors of Piglets

As shown in **Figure 2**, compared to the control group, maternal synbiotic supplementation decreased ($P < 0.05$) plasma MDA and H_2O_2 levels and antibiotic supplementation decreased ($P < 0.05$) plasma MDA level. However, the plasma T-AOC, SOD, and CAT indices did not reach statistical significance ($P > 0.05$).

As presented in **Figure 3**, maternal synbiotic supplementation decreased ($P < 0.05$) plasma levels of IL-1β, IL-2, IL-6, and TNF-α; and antibiotic supplementation decreased ($P < 0.05$) plasma levels of IGF-1, IL-1β, IL-2, IL-6, and TNF-α, when compared with the control group.

As listed in **Figure 4**, maternal synbiotic supplementation decreased ($P < 0.05$) plasma ghrelin, CCK, and PP levels and had a decreased trend in LEP level ($P = 0.05$); and maternal antibiotic supplementation decreased ($P < 0.05$) plasma gastrin, ghrelin, CCK, PP, LEP, and SS levels, when compared with the control group. Maternal synbiotic supplementation decreased plasma gastrin ($P < 0.05$) and LEP ($P = 0.05$) levels relative to the antibiotic group.

Plasma Free Amino Acid Levels of Piglets

As shown in **Table 1**, maternal synbiotic supplementation decreased ($P < 0.05$) plasma Ile, Leu, α-AAA, α-ABA, and 1-Mehis levels and antibiotic supplementation decreased ($P < 0.05$) plasma Hypro level, when compared with the control group. The plasma Leu, α-ABA, and 1-Mehis levels in the synbiotic group

TABLE 1 | Effects of maternal synbiotic supplementation on plasma concentrations of free amino acids in suckling Bama mini-piglets (μg/mL; $n = 8$).

Items	Control group	Antibiotic group	Synbiotic group
Ala	29.58 ± 2.90^{a}	28.04 ± 2.83^{a}	13.64 ± 1.5^{b}
Ans	0.87 ± 0.17	0.51 ± 0.03	0.57 ± 0.08
Arg	18.44 ± 1.55	16.06 ± 0.56	18.60 ± 1.60
Asp	2.45 ± 0.54	2.48 ± 0.24	1.96 ± 0.21
Car	7.18 ± 0.36	8.37 ± 0.72	5.86 ± 0.44
Cit	9.28 ± 0.58	8.93 ± 0.66	10.91 ± 0.83
Cys	0.85 ± 0.15^{a}	1.69 ± 0.37^{ab}	1.95 ± 0.21^{b}
Cysthi	3.43 ± 0.33	3.31 ± 0.13	4.12 ± 0.31
$EOHNH_2$	0.56 ± 0.39	1.29 ± 0.56	3.10 ± 0.24
Glu	39.12 ± 9.02	27.93 ± 3.12	21.61 ± 1.97
Gly	31.35 ± 1.92	35.56 ± 2.24	31.80 ± 1.06
His	10.27 ± 0.52	10.74 ± 0.55	11.10 ± 0.69
Hypro	10.52 ± 0.85^{b}	12.88 ± 0.57^{a}	12.08 ± 0.7^{ab}
Ile	15.73 ± 1.98^{b}	16.44 ± 1.5^{ab}	21.23 ± 1.53^{a}
Leu	19.66 ± 1.79^{b}	20.76 ± 1.96^{b}	26.94 ± 1.92^{a}
Lys	20.25 ± 1.69	22.22 ± 2.17	23.60 ± 0.79
Met	3.90 ± 0.46	3.80 ± 0.37	3.97 ± 0.21
Orn	6.76 ± 0.61	7.32 ± 0.65	6.62 ± 0.44
Phe	13.88 ± 0.93	14.68 ± 0.64	15.58 ± 0.40
Pro	16.35 ± 1.14	16.48 ± 1.10	18.37 ± 0.88
Sar	1.09 ± 0.23	1.42 ± 0.43	1.30 ± 0.33
Ser	11.92 ± 1.02	12.47 ± 1.06	10.96 ± 0.82
Tau	9.97 ± 0.36^{a}	9.42 ± 0.75^{ab}	8.16 ± 0.42^{b}
Thr	17.31 ± 1.10	16.7 ± 1.21	15.56 ± 1.20
Tyr	11.31 ± 1.38	11.28 ± 1.00	10.66 ± 0.53
Val	32.45 ± 4.11	32.73 ± 3.56	37.71 ± 2.38
α-AAA	6.86 ± 0.84^{b}	7.86 ± 0.93^{ab}	9.80 ± 0.73^{a}
α-ABA	3.55 ± 0.39^{b}	3.43 ± 0.51^{b}	4.94 ± 0.32^{a}
β-AiBA	0.25 ± 0.04^{a}	0.18 ± 0.02^{ab}	0.40 ± 0.16^{b}
β-Ala	1.00 ± 0.12	1.11 ± 0.14	1.20 ± 0.22
1-Mehis	0.38 ± 0.04^{b}	0.55 ± 0.11^{b}	1.21 ± 0.22^{a}
3-Mehis	2.29 ± 0.12	2.05 ± 0.12	2.23 ± 0.17

Data in the same row with different superscripts differ significantly ($P < 0.05$). Asp: Asp + Asn; Glu: Glu + Gln; α-AAA, L-alpha-aminoadipic acid; α-ABA, DL-alpha-amino-n-butyric acid; β-AiBA, DL-beta-aminoisobutyric acid; β-Ala, beta-alanine; 1-Mehis, L-1-methylhistidine; 3-Mehis, L-3-methylhistidine.

was higher ($P < 0.05$) while plasma Ala level was lower ($P < 0.05$) compared with the antibiotic group.

Diversity of Colonic Microbiota in Piglets

Total 993,960 high-quality reads were generated from 48 colonic content samples, and each sample contained an average of 41,415 reads (range from 31,377 to 57,987). As shown in **Figure 5**, the Chao1, ACE, Simpson, and Shannon indices showed no difference among the three groups ($P > 0.05$). PLS-DA showed that samples from the three groups tended to exhibit a distinct clustering of microbiota composition although there was a partial overlap between the antibiotic group and synbiotic group.

Composition and Abundance of Colonic Microbiota in Piglets

As shown in **Figure 6**, the top five dominant phyla were *Firmicutes* (80.7%), *Proteobacteria* (7.3%), *Bacteroidetes* (6.3%), *Spirochaetes* (2.8%), and *Fusobacteria* (1.4%), which account for > 98% of total colonic bacteria. At phylum level, only *Fusobacteria* relative abundance in the antibiotic group was higher ($P < 0.01$) than that in the control group.

At genus level, *Lactobacillus* (23.2%), *p-75-a5* (3.4%), *Herbaspirillum* (3.3%), *Treponema* (2.5%), and *Oscillospira* (2.5%) were the top dominant genera of colonic microbiota with a clear classification status (**Figure 7**). Further, the abundances of colonic microbiota with a clear classification status of 20 most abundant bacterial genera were analyzed. Relative to the control group, maternal synbiotic supplementation increased ($P < 0.05$) the abundances of *p_Proteobacteria;g_Sphingomonas, p_Firmicutes;g_Anaerovorax, p_Firmicutes;g_Holdemania, p_Firmicutes;g_Sharpea, p_Firmicutes;g_Butyricicoccus*, and *p_Firmicutes;g_Anaerostipes*; while decreased ($P < 0.05$) the abundances of *p_Firmicutes;g_Facklamia, p_Firmicutes;g_RFN20, p_Actinobacteria;g_Arcanobacterium,* and *p_Proteobacteria;g_Brevundimonas*. Maternal antibiotic supplementation decreased ($P < 0.05$) the abundances of *p_Proteobacteria;g_Acinetobacter, p_Firmicutes;g_Facklamia*, *p_Firmicutes;g_Streptococcus,* and *p_Proteobacteria;g_Brevundimonas* while increased ($P < 0.05$) *p_Fusobacteria;g_Fusobacterium* abundance. Compared with the antibiotic group, maternal synbiotic supplementation decreased ($P < 0.05$) the abundances of *p_Proteobacteria;g_Pasteurella* and *p_Firmicutes;g_RFN20*, while increased ($P < 0.01$) the abundances of *p_Firmicutes;g_Anaerovorax, p_Firmicutes;g_Holdemania, p_Firmicutes;g_Sharpea,* and *p_Firmicutes;g_Butyricicoccus*.

Metabolite Levels in Colonic Contents of Piglets

As shown in **Figure 8**, compared with the control group, the levels of propionate, straight-chain fatty acids, and SCFAs were decreased ($P < 0.05$) and spermidine level showed a decreased trend ($P = 0.055$) in the synbiotic group. Moreover, maternal synbiotic supplementation decreased ($P < 0.05$) the propionate level and increased ($P = 0.055$) spermidine level compared with the antibiotic group. The differences in other determined metabolites among the three groups did not present statistically significant ($P > 0.05$) (**Supplementary Figure 1**).

Correlation Between Microbiota and Metabolites in Colonic Content of Piglets

As shown in **Figure 9**, *p_Firmicutes;g_Butyricicoccus* abundance was positively correlated ($P < 0.05$) with isovalerate and branched-chain fatty acid (BCFA) levels, as well as *p_Firmicutes;g_Anaerostipes* abundance with acetate and SCFAs levels. However, a significant negative correlation ($P < 0.05$) was observed between *p_Fusobacteria* and *p_Fusobacteria;g_Fusobacterium* abundances and acetate level. In addition, there was a negative correlation ($P < 0.05$) between *p_Firmicutes;*

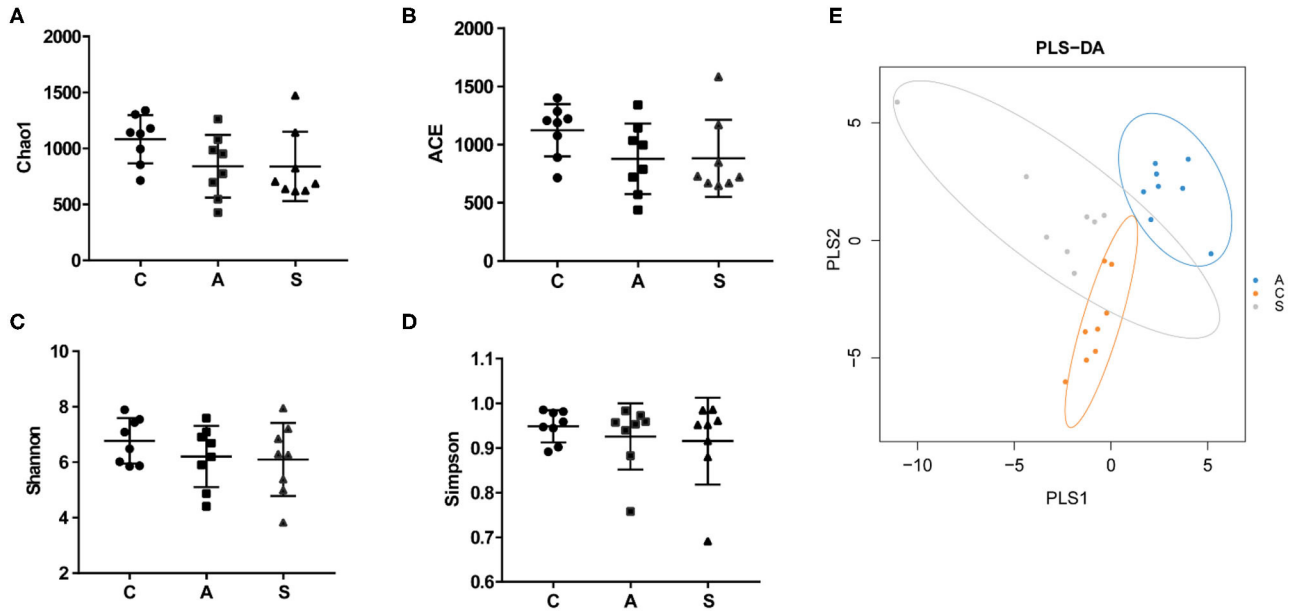

FIGURE 5 | Effect of maternal synbiotic supplementation on alpha diversity of colonic microbiota in suckling Bama mini-piglets. **(A–D)** The microbial diversity is estimated by Chao, ACE, Shannon, and Simpson indices. **(E)** Partial least squares discrimination analysis (PLS-DA) of the colonic microbial community. Data represent the means ± SEM. The data were analyzed by One-way analysis of variance and Duncan's multiple range test. $n = 8$ per group.

g_Facklamia abundance and tryptamine level, as well as *p_Actinobacteria;g_Arcanobacterium* abundance and tryptamine and skatole levels.

mRNA Expression of Genes Related to Intestinal Health in Piglets

As shown in **Figure 10**, maternal synbiotic supplementation up-regulated ($P < 0.05$) the mRNA expression of colonic E-cadherin, Occludin, ZO-1, ZO-2, IL-10, and IFN-α compared with the antibiotic group. Compared with the control group, maternal synbiotic and antibiotic supplementation failed to affect the expression of determined genes.

DISCUSSION

The present study explored the effects of synbiotic supplementation in the maternal diets from pregnancy to lactation on the intestine health of suckling piglets by determining colonic microbiota composition, metabolite levels, and mucosal gene expression, as well as plasma parameters. We found that maternal antibiotic supplementation is counter-productive for the intestinal health based on the measurement of parameters related to the intestinal barrier permeability, whereas synbiotic supplementation improved parameters related to nutrient metabolism and intestinal health.

The piglets utilize efficiently dietary fat when blood TC level decreases. LDL-C transports TC synthesized by the liver to extrahepatic tissue, thus preventing excessive lipid deposition in the liver (19). In the present study, maternal synbiotic supplementation decreased plasma TC and LDL-C levels, suggesting that dietary fat was highly utilized by piglets to favor their growth. Shakeri et al. (20) reported that supplementing synbiotics reduced the blood TC level by altering gut microbiota metabolism. UN is a metabolite of amino acid and/or protein (21), plasma level of which reflects the profiles of protein absorption and utilization in the animal body (22). AMM reflects the liver function and the decrease of plasma AMM level indicates the increase of liver ability for synthesizing urea (23). The present study showed that plasma UN level increased while AMM level decreased in the synbiotic group, suggesting that maternal synbiotic supplementation promoted protein utilization of suckling piglets. These findings suggest that maternal synbiotic supplementation, but not antibiotic supplementation, would enhance the nitrogen metabolism of suckling piglets.

Amino acids (AAs), apart for being an important component of tissue protein, play several important roles in protein metabolism in animals (24). Weanling piglets use branched-chain amino acids (including Ile, Leu, and Val) to maintain their growth and development, especially Leu which contributes to regulate protein synthesis and tissue growth of animals (25). In the present study, maternal synbiotic supplementation increased the plasma Ile and Leu levels in suckling piglets. In addition, previous studies showed that Tau and Cys, main products of Met metabolism, play a vital role in the growth and health of piglets (26). Ala is the main substrate for glucose synthesis in the liver, which can play a role in the body's immune function (27). Tau, mostly found at a high level in animal tissues, has been shown to improve animal lipid metabolism (28). The present study showed that maternal

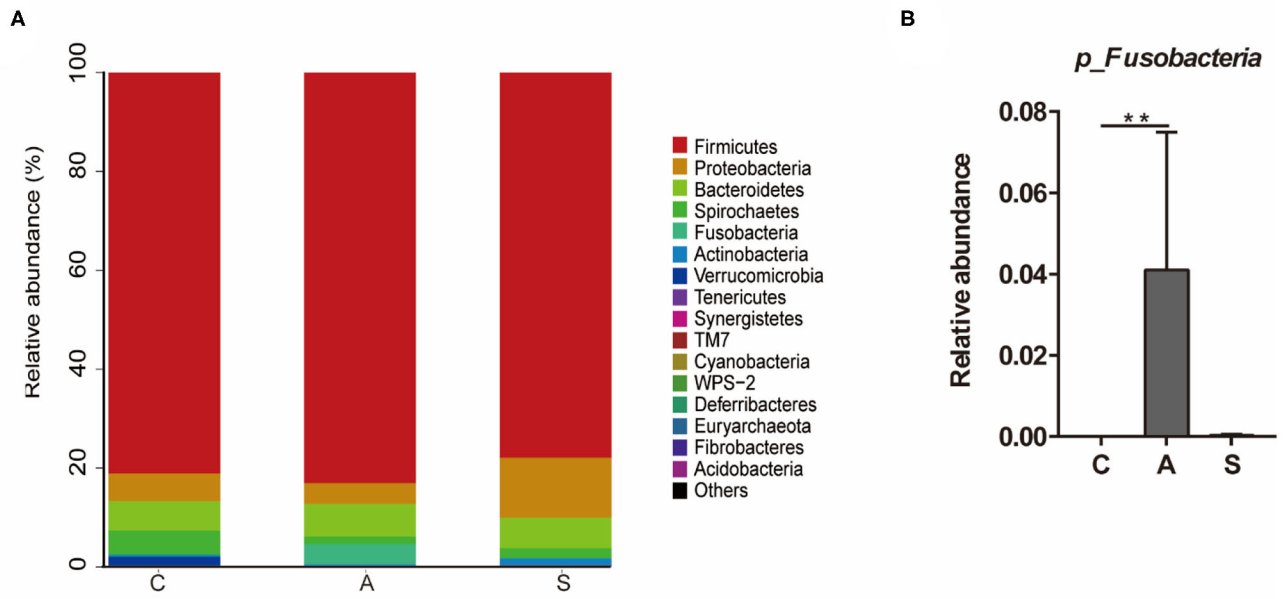

FIGURE 6 | Effect of maternal synbiotic supplementation on the colonic microbial community structure in suckling Bama mini-piglets. Colonic microbiota distributed at the phylum level **(A)** and all phyla were listed. A comparison of relative abundances at the phylum level **(B)** was analyzed by Metastats analysis, and the discrepancy of the top 10 colonic microbiota was listed. Phyla with proportion < 0.001 were grouped in others. $^{**}P < 0.01$. $n = 8$ per group.

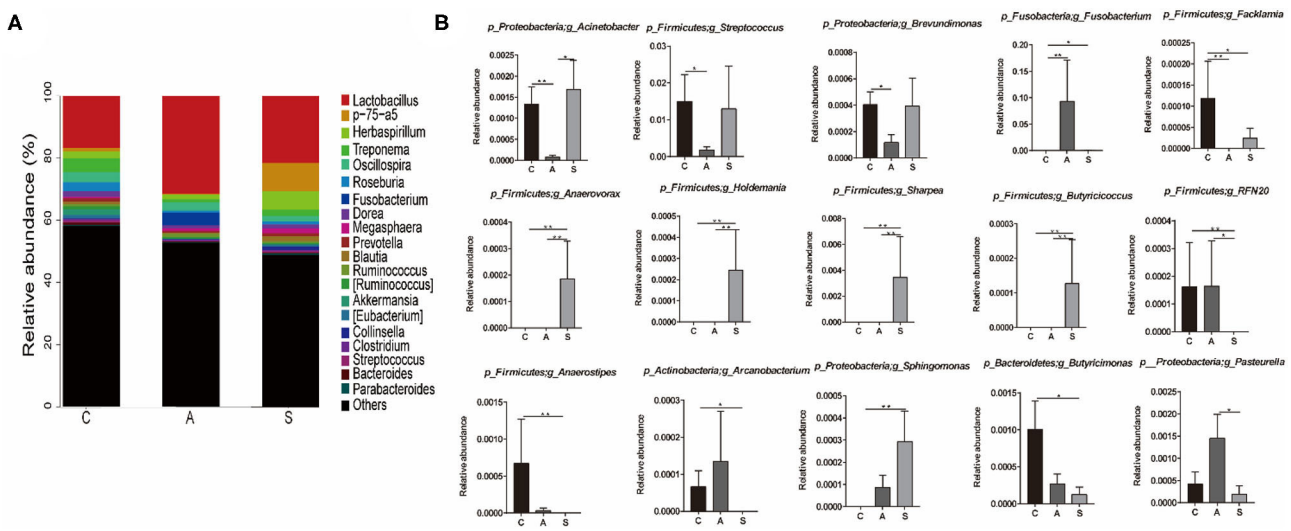

FIGURE 7 | Effect of maternal synbiotic supplementation on the colonic microbial community structure in suckling Bama mini-piglets. Colonic microbiota distributed at the genus level **(A)** and only the top 20 genera were listed. A comparison of relative abundances at the genus level **(B)** was analyzed by Metastats analysis. The 20 most abundant bacterial genera with a clear classification status were presented and compared. $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 8$ per group.

synbiotic supplementation decreased the plasma levels of Tau, Cys, and Ala in piglets, suggesting that dietary synbiotics may modify amino acid metabolism in the offspring. These above-mentioned findings suggested that maternal synbiotic supplementation affects the protein synthesis by altering plasma amino acids levels.

Plasma MDA level reflects lipid peroxidation in the body tissues (29). H_2O_2 is a reactive oxygen species (ROS) that can increase the oxidative stress in tissues (30). A previous study showed that piglets may produce excessive reactive oxygen species thus leading to oxidative stress, which may lead to intestinal barrier dysfunction in weaned piglets (31). Interestingly, we found that maternal synbiotic supplementation decreased plasma MDA and H_2O_2 levels, suggesting that the synbiotics could relieve the oxidative stress exposure to suckling piglets. Among prebiotics, XOS produces SCFAs which

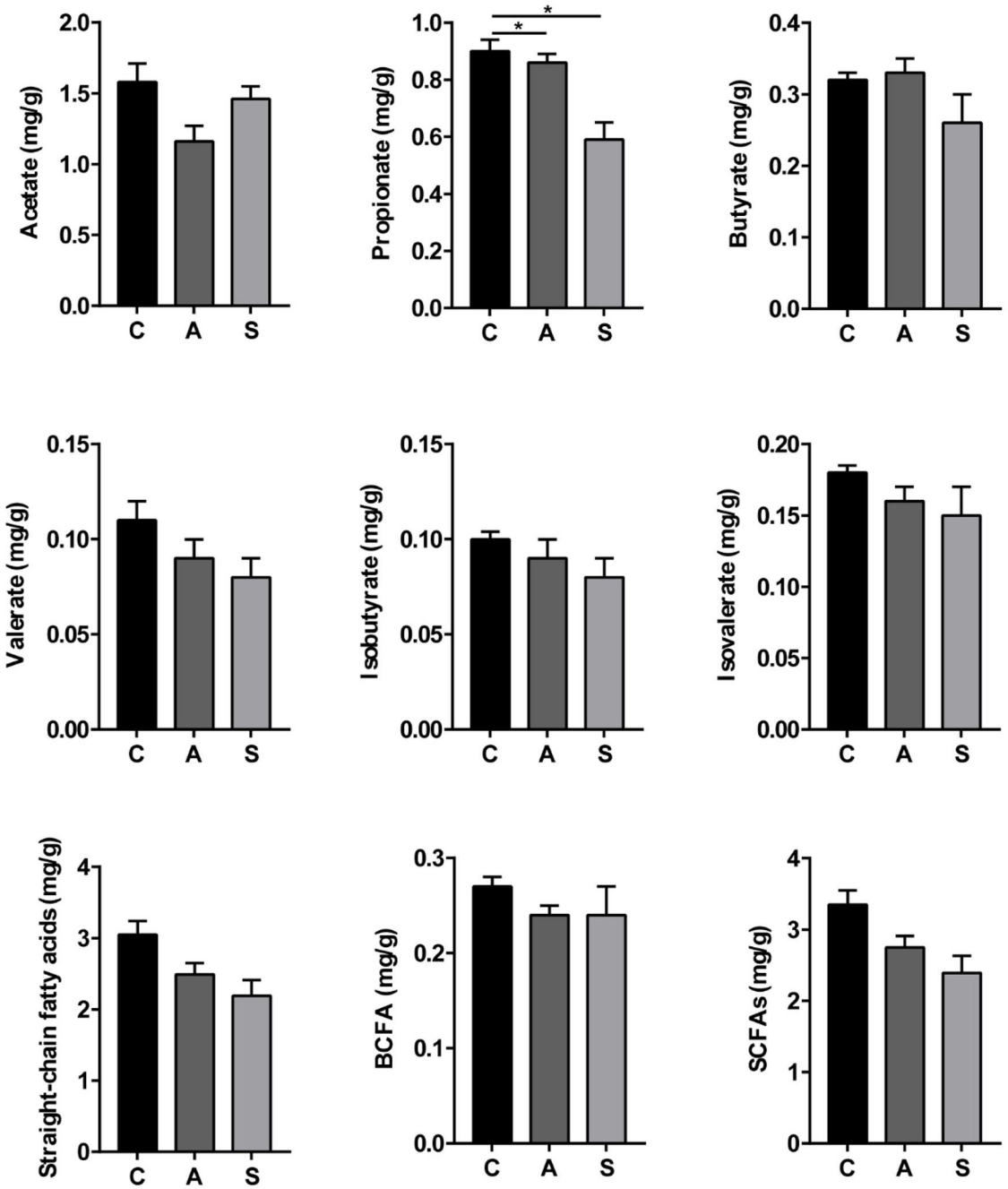

FIGURE 8 | Effect of maternal synbiotic supplementation on colonic short-chain fatty acids levels in suckling Bama mini-piglets. The data were analyzed by Duncan's multiple range test using One-way analysis of variance. Data represent the means ± SEM. *$P < 0.05$. $n = 8$ per group.

may reduce ROS production (32), *Lactobacillus* reduces MDA production (33), and synbiotic addition reduces the MDA level and relieves oxidative stress in tissues (29).

Gut microbiota is involved in nutrient utilization and affects the growth and development of the host (34). Maternal nutrition during pregnancy and lactation modified the gut microbiota composition and health of offspring (35). Gut microbiota diversity was closely related with the host's health (36). The α-diversity of microbiota is decreased, which may be associated with a higher occurrence of low-grade inflammation and some metabolic diseases (37). In the present study, after maternal antibiotic or synbiotic supplementation, the α-diversity of colonic microbiota in piglets did not change, whereas the microbiota composition and abundances changed markedly,

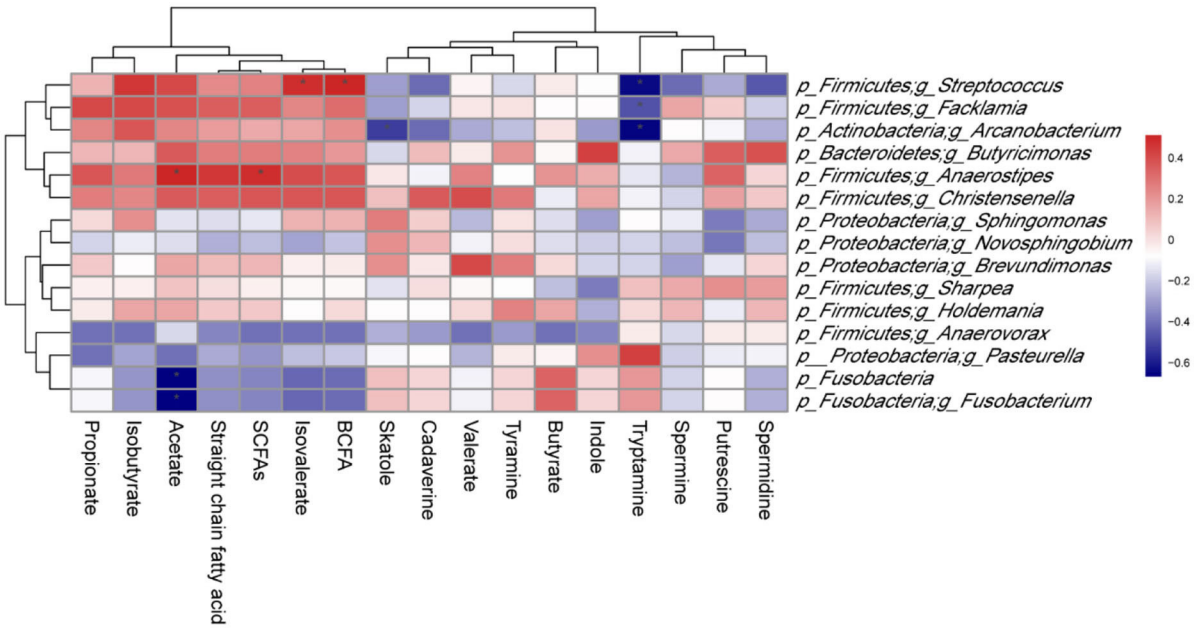

FIGURE 9 | Correlation between colonic microbiota and their metabolites in suckling Bama mini-piglets. Spearman (r) correlations were used, and * means that the correlation is significant. SCFAs, short-chain fatty acids; BCFA, branched-chain fatty acid.

suggesting that maternal synbiotic might not exert a negative effect on suckling piglets.

In the animal gut, the dominant phyla usually includes *Firmicutes*, *Bacteroides*, *Proteobacteria,* and *Fusobacterium* (38). In the present study, the abundances of *Firmicutes*, *Bacteroides*, and *Proteobacteria* accounted for 94.3% of the total sequences. *Firmicutes* plays a vital role in the degradation of polysaccharides and oligosaccharides (39), which involves some key metabolic conversions by the gut microbial community (40). In addition, maternal synbiotic supplementation increased the abundances of *Butyricicoccus* and *Sharpea* belonged to *Firmicutes*. *Butyricicoccus* can reduce the production of pro-inflammatory cytokines to inhibit the host's inflammation (41). We found that maternal synbiotic supplementation increased *Butyricicoccus* abundance, which might reduce the inflammation occurrence of suckling piglets *via* altering gut microbiota composition and abundance. *Sharpea* promotes SCFAs (especially butyrate) and lactate production (42). Our study showed that maternal synbiotic supplementation increased *Sharpea* abundance in the offspring, which may favor inhibition of the proliferation of potential pathogenic bacteria by reducing the gut pH value. Additively, *Fusobacterium* can use glucose as a carbon source, the abundance of which is increased by polysaccharide degradation (43). Several studies reported that *Fusobacterium* might be a contributing factor for inflammation (44), the abundance of which increased in neonatal piglets with diarrhea (45). In the present study, the *Fusobacterium* abundance showed a decreased trend in the synbiotic group, implying that maternal synbiotic supplementation reduced this potential pathogenic bacteria.

Colonic SCFAs can exert crucial effects on intestinal function and health of the host before and after absorption in the blood (46). In addition of providing 60–70% of total energy to colonic cells (47), the SCFAs are associated with the reduction of the host's inflammation (48) and the relieving symptoms of other metabolic diseases (49). Among them, propionate reduces the serum cholesterol level and liver lipogenesis of rats (50). Our study showed that maternal synbiotic supplementation decreased propionate level in the colonic content. These findings suggested that maternal synbiotic supplementation increased certain gut microbiota species and promoted the production of specific metabolites. In addition, colonic *p_Firmicutes;g_Anaerostipes* abundance was positively correlated with acetate and SCFAs levels; and *Fusobacteria* and *p_Fusobacteria;g_Fusobacterium* abundances were negatively correlated with acetate level, suggesting that *Anaerostipes* might promote the SCFAs production while *Fusobacteria* and *Fusobacterium* would diminish them by a underlying mechanism that needs to be determined.

Cytokines can regulate the systemic inflammatory response of the body. The SCFAs promote the migration of leukocytes to the inflammatory site and production of several anti- and pro-inflammatory cytokines, including TNF-α, IL-1β, IL-2, IL-6, and IL-10 (51). Acetate, propionate, and butyrate reduce the production of TNF-α (52), IL-1β, and IL-6 (53). Interestingly, we found that maternal synbiotic supplementation decreased the plasma levels of TNF-α, IL-1β, IL-2, and IL-6 in offspring piglets, suggesting that dietary synbiotics might reduce inflammation in piglets via modifying several bacterial metabolite productions. Additionally, cytokines have the function of regulating immune

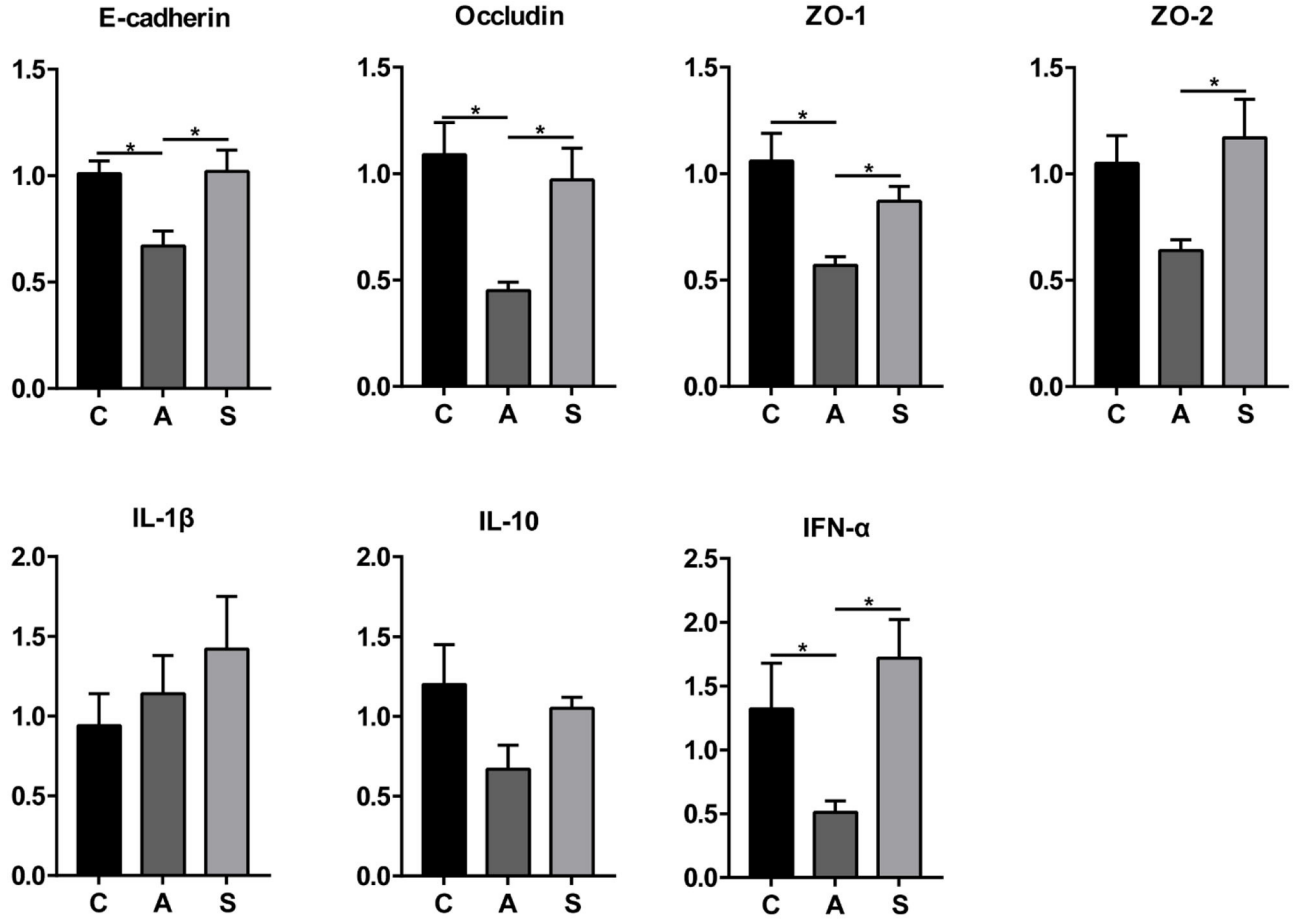

FIGURE 10 | Effect of maternal synbiotic supplementation on mRNA expression of colonic mucosal genes related to the intestinal barrier function in suckling Bama mini-piglets. The data were analyzed by Duncan's multiple range test using One-way analysis of variance. Data showed the means ± SEM. *$P < 0.05$. $n = 8$ per group.

and inflammatory responses and maintaining barrier integrity (54). In the present study, maternal synbiotics up-regulated the mRNA expression of colonic mucosal IFN-α, suggesting that the synbiotic addition in the maternal diets enhances the immune response of suckling piglets via regulating gut microbiota composition and metabolic activity as previously proposed (55).

The SCFAs can modulate hormone secretion (e.g., Leptin) (56) and are involved in modulating the production of Ghrelin (57). CCK can suppress the appetite by acting on the central nervous system (58). Ghrelin can act on appetite (59) and satiety by regulating the gut microbial community of the host. The PP secretion can be stimulated by dietary fat (60). Our study showed that maternal synbiotic supplementation decreased the plasma levels of Ghrelin, CCK, and PP of piglets, suggesting that maternal synbiotic addition might affect plasma hormone secretion of suckling piglet by mediating gut microbiota and their metabolites.

When the intestinal mucosal barrier is damaged, the permeability of which would increase, thus causing intestinal inflammation or other diseases due to harmful substances invading the body tissues (55). Compared with the antibiotic group, dietary synbiotic supplementation up-regulated the mRNA expression of colonic mucosal E-Cadherin, Occludin, ZO-1, and ZO-2, suggesting that the maternal synbiotic administration might improve tight-junction integrity of colonic intestinal epithelial cells via colonic microbiota. Shi et al. (61) found that the mixture of Lactobacillus species increased the colonic mucosal tight-junction proteins and relieved inflammation in antibiotic-supplemented mice by modulating their microbiota structure. Yin et al. (62) also showed that dietary XOS supplementation improved the intestinal barrier by up-regulating ZO-1 expression. Further work is required to explore the dose of synbiotic supplementation in maternal diets presenting an impact on the intestinal permeability in piglets.

In conclusion, maternal synbiotic supplementation from pregnancy to lactation may improve glycolipid and protein metabolism, reduce oxidative stress level, and improve the intestinal health of suckling piglets. Notably, these findings provide a new perspective for manipulating gut microbiota with synbiotic addition to improve the nutrient metabolism and intestine health of offspring. The changes in maternal

milk composition after maternal synbiotic supplementation need further analysis in the future to full interpret the findings of the present study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA609410.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the Institute of Subtropical Agriculture. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

XK designed the experiment. CM, QG, WZ, QZ, HD, and WT carried out the animal trail, and sample collection and analysis. CM and WZ performed the statistical analyses. CM wrote the manuscript. FB and XK revised the manuscript. All authors reviewed this manuscript.

FUNDING

This present study was jointly supported by the National Key Research and Development Project (2018YFD0500404-4), National Natural Science Foundation of China (31772613), STS regional key project of the Chinese Academy of Sciences (KFJ-STS-QYZD-052), Talent Projects of Guangxi Science and Technology Department (AD17195043), and Special Funds for Construction of Innovative Provinces in Hunan Province (2019RS3022).

ACKNOWLEDGMENTS

We thank the staff and postgraduate students of Hunan Provincial Key Laboratory of Animal Nutritional Physiology and Metabolic Process for collecting samples, and technicians from CAS Key Laboratory of Agro-ecological Processes in Subtropical Region for providing technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.575685/full#supplementary-material

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

frontiers in Veterinary Science

REVIEW
published: 12 February 2021
doi: 10.3389/fvets.2021.628258

Intestinal Health of Pigs Upon Weaning: Challenges and Nutritional Intervention

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The primary goal of nursery pig management is making a smooth weaning transition to minimize weaning associated depressed growth and diseases. Weaning causes morphological and functional changes of the small intestine of pigs, where most of the nutrients are being digested and absorbed. While various stressors induce post-weaning growth depression, the abrupt change from milk to solid feed is one of the most apparent challenges to pigs. Feeding functional feed additives may be viable solutions to promote the growth of nursery pigs by enhancing nutrient digestion, intestinal morphology, immune status, and by restoring intestinal balance. The aim of this review was to provide available scientific information on the roles of functional feed additives in enhancing intestinal health and growth during nursery phase. Among many potential functional feed additives, the palatability of the ingredient and the optimum supplemental level are varied, and these should be considered when applying into nursery pig diets. Considering different stressors pigs deal with in the post-weaning period, research on nutritional intervention using a single feed additive or a combination of different additives that can enhance feed intake, increase weight gain, and reduce mortality and morbidity are needed to provide viable solutions for pig producers. Further research in relation to the feed palatability, supplemental level, as well as interactions between different ingredients are needed.

Keywords: feed additives, intestinal health, newly weaned pigs, nutritional intervention, weaning stress

OPEN ACCESS

Edited by:
Domenico Bergero, University of Turin, Italy

Reviewed by:
Francesca Romana Massacci, Istituto Sperimentale di Zooprofilassi dell'Umbria e delle Marche (IZSUM), Italy
Maicon Sbardella, Federal University of Mato Grosso, Brazil

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *11 November 2020*
Accepted: *21 January 2021*
Published: *12 February 2021*

Citation:
Zheng L, Duarte ME, Sevarolli Loftus A and Kim SW (2021) Intestinal Health of Pigs Upon Weaning: Challenges and Nutritional Intervention. Front. Vet. Sci. 8:628258. doi: 10.3389/fvets.2021.628258

INTRODUCTION

Weaning is considered as one of the most critical periods in pig management. It is associated with environmental, social, and dietary stress (1–3), and those various stressors result in low feed intake, body weight loss, and a high incidence of diarrhea, which consequently, can lead to mortality (4, 5). Even though trends for weaning ages at large commercial farms increase to 3–4 weeks of age, pigs are naturally weaned at the age of 12–17 weeks (6, 7). Upon weaning, at typical commercial farms, pigs deal with multiple stressors due to changes such as separation from the sow, relocation with new littermates, and sudden dietary change from sow milk to solid feeds (8). Inadequate feed intake after weaning results in insufficient dietary nutrients utilization and local inflammation (9–11). As a consequence, weaning causes profound changes in the gastrointestinal tract (GIT) of pigs. Intestine is a major site of nutrient digestion and absorption. Intestinal disorders after weaning are caused by alterations in architecture and functions with villus atrophy and crypt hyperplasia and increase in intestinal permeability (12). Moreover, intestinal microbiota disruption and changes are possibly linked to diarrhea and pathogenic infections after weaning (13–16).

Increased research needs and interests in understanding intestinal health in pigs are well-reflected in the number of peer reviewed papers searchable in PubMed (using intestinal health in pigs as keywords in the title or abstract). Since 1960 and until 2005, there have been < 10 papers searched in PubMed, which has been 10 folds increased by 2018 and then 180 papers in 2020. This review focuses on feed additives as nutritional strategies to overcome weaning challenges.

WEANING ASSOCIATED FUNCTIONAL CHANGES IN THE SMALL INTESTINE

Morphological Changes

Enterocytes are composed of villi projecting into the lumen, and a folded cell monolayer structured into crypts in pigs (17). Villi are mainly lined by enterocytes, goblet cells, and enteroendocrine cells, and the crypts are the main site containing stem cells, proliferative and undifferentiated cells, and a subset of differentiated secretory cells (Paneth, goblet and enteroendocrine cells) (18) as shown in **Figure 1**. When stem cells divide, they go through a cell division into a new stem cell and a committed daughter cell (19). The differentiation and maturation of each cell type happens as the cells move either migrate up the crypt–villus axis (enterocytes, mucous, and enteroendocrine cells) or downwards to the bottom of the crypt (Paneth cells) (20). In the mammalian small intestine, active enterocyte proliferation is restricted to the crypts at the base of the villi (21). Stem cells in the crypts undergo cell division and differentiation to form mature absorptive enterocytes, mucus- producing goblet cells, and enteroendocrine cells, and those cells migrate toward the villus tip, where they are discarded into the intestinal lumen (22).

After weaning, a consistent series of intestinal alterations occur. Architectural alterations associated with weaning reported in previous studies are presented in **Table 1**. Within 24 h of weaning, villus height was shown to reduce by 75% compared to pre-weaning status (5). The height reduction of villi is a result of increased cell loss and/or reduced crypt cell production (5). The villus atrophy and the reduction in crypt cell production during the post-weaning period result in loss of mature enterocytes, which could cause a decrease in nutrient absorption (26, 28, 29). Reduced activity of brush-border enzymes, such as lactase and peptidases and nutrient transporters, have been observed to be correlated with shortened villus height (30, 31).

Barrier Function

Tight junction proteins between epithelial cells form the barriers, which closes the paracellular space between epithelial cells regulating permeability through the epithelial layer (32). These proteins consist of transmembrane proteins such as occludin and claudins, as well as cytoplasmic proteins such as zonula occludens (ZO) (33). As a barrier between the luminal and basolateral compartments, tight junction proteins control the passive diffusion of ions and other small solutes, through the paracellular pathway (34). These tight junction proteins serving as a filter to allow important dietary nutrients, electrolytes, and water to translocate from the lumen of the intestine into circulation (35–37). Increases in intestinal permeability can result in inflammatory responses by allowing the entry of toxins, allergenic compounds, or bacteria (38, 39). Intestinal barrier function can be compromised by various factors, such as age, diet, pathogens, and diseases (40, 41).

Weaning induced impaired barrier function of epithelial cells promotes the entering of pathogenic bacteria and allergenic compounds from the lumen into the body (12, 42). Weaning causes compromised paracellular barrier function (2, 43). Active absorption decreases when pigs are weaned at 3 weeks of age or earlier as a process of natural intestinal maturation stimulated by weaning (**Table 2**); however, if pigs are weaned after 3 weeks of age, the active absorption is no more affected by weaning indicating weaning at an early age can disrupt barrier function (43).

Mucosal Immunity at Weaning

Up to 70% of the immune cells are localized in the mucosa and submucosa of the intestine (49, 50). The gut-associated lymphoid tissue (GALT) consists of both isolated and aggregated lymphoid follicles forming Peyer's patches (PP) and mesenteric lymph nodes (51). The induction of intestinal immune reactions starts with antigen presentation by microfold cells (M cells) (52). Lamina propria serves as a mucosal compartment for the regulation of immune responses (predominantly IgA), with few T-cells or dendritic cells, but with myeloid cells with the characteristics of macrophages and granulocytes (53). The production of secretory antibodies, mostly IgA and IgM, is the major defending characteristics of the mucosal immune system. These antibodies are actively transported by immature epithelial cells in the crypts, and immune exclusion is carried out by the generated in cooperation with innate non-specific defense mechanisms (54). Two important periods of maximum exposure to antigens occur immediately after birth and at weaning. At weaning, the abrupt changes in the diet and environment induce alterations in the mucosal immune response (15).

The immune system in the intestine of pigs reaches an adult-like structure at 7-week-old age (55). Conventionally, weaning of pigs is done in the range of 3–4 weeks old, when cytotoxic (CD8+) T cells are primarily absent (55). Weaning also affects the systemic development of innate and adaptive immunity mainly as a consequence of the withdrawal of milk (56). Up- regulated expression of pro-inflammatory cytokines is observed in pigs at weaning (42). Recent studies have shown that pro-inflammatory cytokines, including tumor necrosis factor-α, interferon-γ, interleukin-1β, induce disturbance in intestinal barrier and increase intestinal epithelial permeability (57–59). In addition, inflammation is often associated with intestinal oxidative stress (60, 61). Disruption of cellular redox status can cause excess production of pro-inflammatory cytokines, which could further impair intestinal function (62, 63). The appropriate development of the intestinal immune system and maintaining normal redox state are essential for optimum growth and performance of the pigs. Controlling the intestinal inflammation by the over expression of intestinal pro-inflammatory cytokines may alleviate subsequent intestinal disorders induced by the weaning stress.

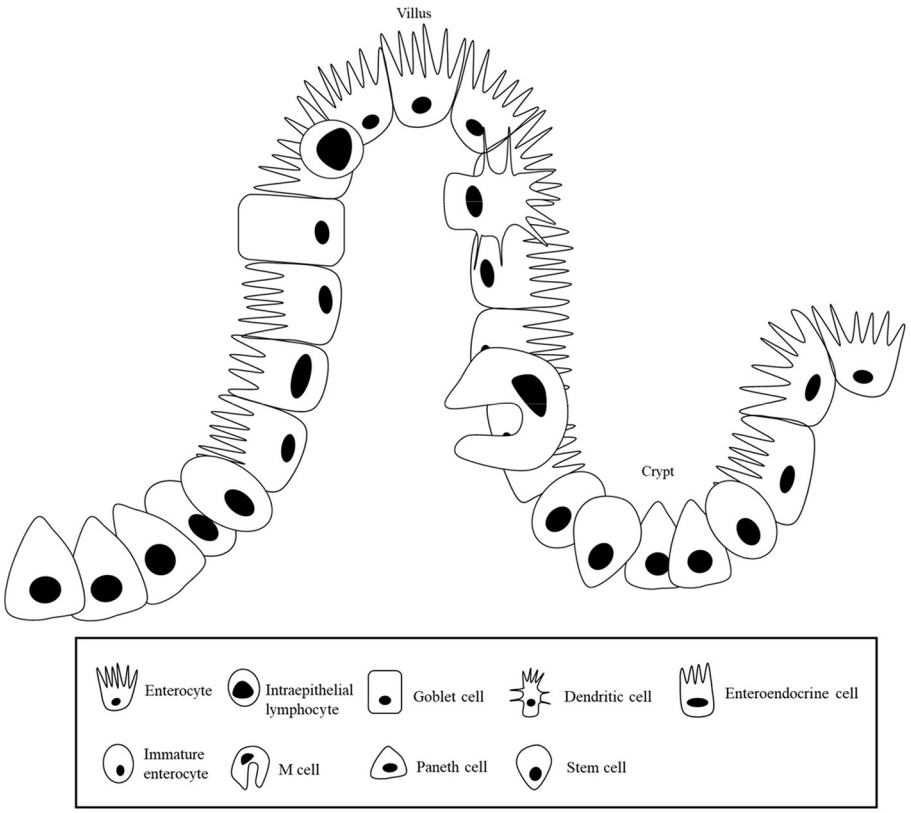

FIGURE 1 | Intestinal epithelial layer.

TABLE 1 | Morphological changes in the small intestine of pigs after weaning.

Weaning age (day)	Intestinal section	Results	References
21	Small intestine	Decreased villus height and increased crypt depth during day 11 post-weaning	(5)
21 or 35	Jejunum	Decreased villus height during day 3 post-weaning when weaned at 21 or 35 day	(23)
14	Small intestine	Decreased villus height to crypt depth ratio at day 7 post-weaning	(24)
28	75% of small intestine	Increased crypt depth at day 5 post-weaning	(25)
26	Small intestine	Decreased villus height at day 2 and 4 and decreased villus height to crypt depth ratio at day 2 and 4 post-weaning	(12)
29	Jejunum	Decreased villus height from day 2 post-weaning with minimal length was observed at day 3 post-weaning and increased crypt depth at day 5 post-weaning	(26)
21	Jejunum	Decreased villus height from day 2 post-weaning and increased crypt depth from day 5 post-weaning.	(27)

Intestinal Microbiota

In pigs, the hindgut is the major site of microbial fermentation, and the microbial population in the small intestine is less diverse than the hindgut (64). The small intestine is a major place for nutrient absorption, and microbiota present in the outer mucosal layer of the small intestine are more susceptible to dietary influence (65, 66). The small intestinal mucosa is frequently exposed to various exogenous antigens and microbial components from feed ingredients. Changes in mucosa-associated microbiota may have enormous effects on host growth and development (14, 16, 67). Most of the past studies are focused on the dietary intervention on luminal and fecal microbiota, few studies evaluated on mucosa-associated microbiota. Post-weaning dietary intervention showed a long lasting effect on mucosa-associated microbiota, but not on digesta in the small intestine (16, 66). The microbial community within the outer layer of the mucosa is closely connected with host tissues, mucosa-associated bacteria are in direct competition

TABLE 2 | Impact of weaning age on intestinal health[a].

Parameter	Weaning age (day)	Experimental period (day)	Early weaning impact	References
Morphology	21 vs. 28	56	ND	(44)
	18 vs. 20	4	↓ Villus height when challenged with ETEC	(45)
	15, 18 vs. 23	35	↑ Lamina propria cell counts	(46)
	28 vs. 49	7	↓ Villus height	(47)
Barrier function	21 vs. 28	56	↑ Expression of tight junction proteins in the jejunum	(44)
	18 vs. 20	4	↓ TER when challenged with ETEC	(45)
	15, 18 vs. 23	35	↓ TER and ↑ mucosal-to-serosal flux of mannitol and inulin	(46)
	28 vs. 49	7	↑ Mucosal-to-serosal transport of horseradish peroxidase	(47)
Mucosal immunity	21 vs. 28	56	ND	(44)
	18 vs. 20	4	↑ Mast cell activation when challenged with ETEC in pigs weaned at day 20, but not at 18	(45)
	15, 18 vs. 23	35	↑ Numbers of mast cells, corticotrophin-releasing factor, and cortisol levels	(46)
Microbiota	14, 21, 28, vs. 42	7	↓ Microbial diversity and richness	(48)

[a]*ND, no differences were observed; ETEC, Enterotoxigenic F18 E. coli; TER, transepithelial electrical resistance.*

with substrates with the host (68). Distinct microbial populations present throughout the gastrointestinal tract due to the different physicochemical conditions and substrate availability (69, 70). The fecal microbiota is distinctly different from that of the luminal of the small intestine. The similarity index of the fecal microbiota and luminal microbiota of the large intestine was 0.75, whereas it was only 0.38 when comparing the fecal and luminal microbiota of the small intestine (69). Mucosa-associated microbiota of cecum was distinctively different from that of the digesta in the cecum (64). From the outer mucosal layer into the lumen, a rapid declining oxygen gradient exists, which generating a distinct microenvironment between mucosal tissue and lumen (71). Mucosa-associated microbiota provides a line of defense against pathogens and modulates the immune status of the host (54, 72–74). The microbiota induces production of IgA by the mucosal immune system, which is secreted into the lumen to limit bacterial colonization and prevent penetration of bacteria through the epithelial layer (54, 75–77).

At weaning, the abrupt changes in the diet and environment induce alterations in the intestinal microbiota (15, 78). During the weaning transition, a major shift in the dominant genus (*Bacteroides* to *Prevotella*) was observed (79). Yang et al. (80) compared microbiota composition of healthy and diarrheic piglets and found the diarrheic piglets had an altered competitive relationship between *Prevotella* and *Escherichia* before weaning and had lower relative abundances of five genera that play key roles in nutrient metabolism (*Bacteroides*, *Ruminococcus*, *Bulleidia,* and *Treponema*) than healthy piglets after weaning. In a similar study (81), diarrheic pigs had a lower *Bacteroidales*, the fiber-degrader family, than non-diarrheic pigs during weaning, which was considered as a biomarker of diarrhea. Reductions in *Lactobacilli* is one of the most evident change after weaning (78). It was postulated the alterations in the composition and activity of the GIT microbial community is correlated with pathogenic infections after weaning (4, 82). A lower stability of the microbial community structure was observed in the ileal digesta of weaned pigs than that of unweaned pigs (78). The intestinal bacterial community composition was shown to become stable at 6 months of age (69). **Table 2** summarizes the impact of weaning age on intestinal structure and function.

NUTRITIONAL INTERVENTION

To assist in overcoming the weaning-associated intestinal dysfunction and depressed growth, effective dietary strategies need to be explored. Feed additives including protein hydrolysates, emulsifiers, prebiotics, probiotics, feed enzymes, nucleotides, organic acids, phytogenic feed additives, immunoglobulin-containing compounds, and/or mycotoxin deactivators are commonly used in the nursery pig diets to promote growth and intestinal health (see **Table 3**). The following session reviews the effects of selected feed additives.

Protein Hydrolysates

Protein hydrolysates are produced from a variety of protein sources by chemical, microbial or enzymatic hydrolysis to eliminate or reduce anti-nutritional factors (127). Typical protein hydrolysates used in animal diets are animal protein hydrolysates (such as salmon viscera and porcine intestines) and plant protein hydrolysates (such as soybean protein hydrolysates) (128). Through the production of protein hydrolysates, anti-nutritional factors are totally or partially hydrolyzed, which make those hydrolysates a high-quality protein source for nursery pigs (129–131). Digestion of protein is mainly completed in the small intestine (132). After weaning, decreased enzymatic activity of peptidases (aminopeptidase N and dipeptidylpeptidase IV) were detected (26). Improvements in crude protein digestibility by soy protein hydrolysates supplementation have been reported in nursery pigs (133–135). Blood plasma is a commonly used animal protein hydrolysate in nursery pig diets. It has been shown to increase growth performance (136), enhance intestinal barrier function (121), and modify intestinal immune function

TABLE 3 | Selected feed additives targeting intestinal health of newly weaned pigs with additional references.

Feed additive	Initial body weight or age	Feeding duration (day)	Observations	References
Fermented soybean meal	5.5 ± 0.2 kg	28	Improved growth efficiency and reduced diarrhea	(83)
	35 day	30	Increased nutrient digestibility, and positively affected fecal microflora by increasing lactic acid bacteria and decreasing *Escherichia coli* count	(84)
	35 day	35	Increased ADG and final body weight, and reduced serum urea nitrogen, increased serum immunoglobulin (Ig) G, IgM and IgA, and increased villus height of duodenum, jejunum, and ileum	(85)
	5.97 ± 0.14 kg	15	Modulated the expression of genes related to inflammatory response and anti-oxidant activity leading to a reduction on serum cortisol after lipopolysaccharide challenge	(86)
Fermented soybean protein	5.8 ± 0.9 kg	28	Improved ADG, ADFI, FCR, and increased digestibility of dry matter, gross energy, crude protein, fat, Ca, P, and increased villus height of duodenum, jejunum, and ileum	(87)
Emulsifiers	6.0 ± 0.2 kg	14	Positively affected fat digestibility	(88)
	7.9 ± 1.0 kg	35	Increased ADG, digestibility of dry matter, gross energy, and crude fat, and decreased serum triglyceride concentration	(89)
	7.2 ± 0.1 kg	19	Increased villus height of duodenum and jejunum, enhanced barrier function and positively affected fat digestibility	(90)
Probiotics	7.7 ± 1.1 kg	21	Increased feed intake, ADG, and increased digestibility of nitrogen and phosphorus	(91)
	7.6 ± 0.6 kg	42	Improved ADG and FCR during 14-day post-weaning, increased protein digestibility, increased villus height of jejunum and ileum, and increased expression of tight junction proteins when added into a low crude protein diet.	(92)
	21 day	16	Modulated intestinal microbiota by increasing *Firmicutes* phylum in the ileum and increased *Actinobacteria* phylum which includes *Bifidobacteria* in the colon	(93)
	8.4 ± 0.2 kg	28	Microbial shifts in the porcine gut in response to diets containing *E. faecalis* were similar to the response to which containing antibiotics	(67)
Prebiotics	6.3 ± 0.3 kg	28	Increased growth efficiency, increased digestibility of dry matter and affected *Bifidobacteria* concentrations	(94)
	6.13 ± 0.13 kg	14	Selectively stimulated the number of *Lactobacilli* whereas suppressed *E. coli* and *Sreptococcus. suis* and improved intestinal barrier function	(95)
	5.65 ± 0.27 kg	21	Upregulated the expression of TLR4 and calprotectin protein alleviating inflammation in the intestine and decreased diarrhea incidence challenged with enterotoxigenic *E. coli*	(96)
	4.72 ± 0.23 kg	21	Increased apparent digestibility of crude protein, calcium, and phosphorus, and decreased the incidence of diarrhea, increased the fecal shedding of *Lactobacillus* reduced *E. coli,* and improved small intestinal morphology and enhanced the growth performance	(97)
	4.9 ± 0.3 kg	14	Reduced incidence of diarrhea when challenged with *E. coli*. K88	(98)
Synbiotics	4.8 ± 0.6 kg	24	Reduced diarrhea, and increased intestinal microbial diversity when challenged with *E. coli* K88	(99)
	7.19 ± 0.45 kg	28	Improved ADG and FCR, increased digestibility of dry matter and crude protein, and increased the fecal abundance of *Lactobacillus* spp. and reduced *Enterobacteriaceae* counts	(100)
	8.09 ± 0.25 kg	28	Modulated the microbiota by increasing *Ruminococcaceae* and *Lachnospiraceae* and decreasing *Erysipelotrichaceae* and *Prevotellaceae.* Enhanced intestinal fermentation by increasing the concentration of acetate in feces	(101)
Xylanase	10.7 ± 1.2 kg	21	Increased ADG, and digestibility of dry matter and gross energy, and reduced digesta viscosity, and reduced inflammatory response	(102)
	7.2 ± 0.4 kg	24	Enhanced growth performance and gut morphology, reduced digesta viscosity, reduced intestinal oxidative stress and the enterocyte proliferation	(103)
	7.5 ± 0.1 kg	19	Increased digestibility of gross energy and total non-starch polysaccharide by increasing the digestibility of arabinoxylan. Reduced pro-inflammatory digesta viscosity, and improved intestinal barrier function	(104)
Phytase	28 day	42	Increased ADG, ADFI, and growth efficiency, and increased digestibility of minerals	(105)
	6.27 ± 0.01 kg	35	Enhanced growth performance and feed energy efficiency	(106)
Protease	6.3 ± 0.5 kg	14	Improved ADG, ADFI, FCR, reduced diarrhea, increased digestibility of crude protein, enhanced intestinal morphology, and increased nutrient transport efficiency	(107)
	8.3 ± 0.63 kg	21	Improved growth performance and reduced fecal score. Improved digestibility of dry matter, gross energy, crude protein, and phosphorus. Reduced ammonia nitrogen in cecum and colon and total volatile fatty acid in ileum and colon. Reduced the *E. coli* and increased *Lactobacillus* count in the colon	(108)

(Continued)

TABLE 3 | Continued

Feed additive	Initial body weight or age	Feeding duration (day)	Observations	References
	6.42 ± 0.12 kg	42	Enhanced growth performance and digestibility of dry matter, and nitrogen. Reduced blood creatinine and fecal NH_3	(109)
Nucleotides	4.8 ± 0.4 kg	21	Improved ADFI, positively affected ADG, and positively enhanced villus structure	(110)
	7.3 ± 0.1 kg	28	Improved ADG and ADFI	(111)
	7.3 ± 0.3 kg	42	Increased final body weight, ADG, and growth efficiency, and increased digestibility of dry mater and energy	(112)
Organic acids	7.2 ± 0.2 kg	42	Improved ADG and FCR, increased villus height, increased acetic and propionic acid concentrations, and altered microbial community structure	(113)
	6.3 ± 0.6 kg	14	Reduced inflammatory cytokines and altered microbial community composition	(114)
	8.63 ± 1.56 kg	28	Improved ADG and FCR. Reduced diarrhea score by reducing *E. coli* count in feces. Improved digestibility of dry matter, ether extract, total carbohydrates, fiber, and phosphorus and improved intestinal morphology	(115)
Phytogenic feed additives	21 day	11	Reduced diarrhea and inflammation when challenged with *E. coli*	(116)
	7.4 ± 1.3 kg	35	Increased post-weaning feed intake	(117)
	8.4 ± 1.6 kg	35	Increased weight gain, improved fecal consistency, and increased digestibility of dry matter and crude protein	(118)
	8.2 ± 2.3 kg	22	Decreased pro-inflammatory cytokines	(119)
	25 day	42	Increased growth efficiency and increased nutrient digestibility	(120)
Blood plasma	5.5 ± 0.1 kg	14	Reduced diarrhea and decreased pro-inflammatory cytokines	(121)
	6.0 ± 0.1 kg	14	Increased growth efficiency and reduced activation of the immune system	(122)
	6.8 ± 0.1 kg	12	Improved ADG, ADFI, and growth efficiency	(10)
Mycotoxin deactivators	8.2 ± 0.1 kg	34	Reduced oxidative stress and immune activation	(123)
	9.9 kg	27	Improved body weight, ADFI, and FCR	(124)
	6.0 ± 0.3 kg	35	Improved body weight, ADG, and ADFI	(125)
	9.1 ± 0.1 kg	42	Improved body weight, and ADG. Reduced TNFα, and 8-OHdG	(126)

(122) when fed to newly weaned pigs (further information see 3.9). Additionally, some peptides derived from protein hydrolysis especially milk and soy protein possess various biological functions including antimicrobial, antihypertensive, and immunomodulatory activities (86, 128, 137, 138).

Soy Protein Hydrolysates

Soybean meal is one of the most commonly used ingredients in animal feed; however, digestive disturbances are often observed when it is fed to young animals especially newly weaned pigs (139–141). Soybean meal contains various anti-nutritional factors including trypsin inhibitors, lectins, indigestible carbohydrate complexes, and soybean globulins (130, 139, 142, 143). Trypsin inhibitors and lectins can be inactivated by proper heat treatment and fat extraction (140, 144). However, the presence of indigestible carbohydrate complexes, antigenic soybean globulins, and residual trypsin inhibitor limits its use in young pig diets (139, 144, 145). Glycinin and β-conglycinin, antigenic proteins, are the major anti-nutritional factors that cause allergic responses in young animals (139, 146, 147). These proteins can cause hypersensitivity that induce abnormal intestinal morphological change and diarrhea when fed to young pigs (139, 148, 149). Fermented soybean meal using microorganisms such as *Aspergillus oryzae*, *Bacillus subtilis*, and *L. casei* and enzyme-treated soybean meal are shown to have reduced anti-nutritional factors and increased concentrations of CP and AA than conventional soybean meal (83, 150). Through the microbial fermentation or enzymatic treatment of soybean meal, the antigenic proteins are hydrolyzed into small size peptides and the glycosidic bonds in the carbohydrate fraction in soybean meal are broken down by enzymes produced by fungus and bacteria, or by a mixture of enzymes (129, 151). Fermented and enzyme-treated soybean meal have been shown to improve growth performance and feed efficiency of nursery pigs when partially replaced conventional soybean meal in the diets (83, 84). Soy oligopeptides, a soy protein hydrolysate, was shown to improve amino acid absorption compared to an intact soy protein or corresponding amino acid mixtures in a human study (152). Amino acid absorption in the portal blood from a soy protein hydrolysate was more efficient than the constituent amino acids from an amino acid mixture and those from an intact soy protein in rats (153). In addition, enhanced intestinal morphology was observed when fed soy protein hydrolysates to nursery pigs (85, 87). Despite the improved nutritional values, the bitter taste of soy hydrolysates resulting from the hydrolysis of soy proteins has been a major problem

in food applications (154, 155). The hydrophobic amino acids are shown to be involved in the bitter taste of various peptides (156). Concealed hydrophobic side chains in the interior of the protein are released with the protein hydrolysis which elucidates bitterness (157, 158). Therefore, the feed palatability testing may be necessary to ascertain if soy hydrolysates can promote growth of pigs without negatively affecting feed intake of nursery pigs.

Emulsifiers

Animal fats and vegetable oils are commonly added to meet energy concentration in the diet. To be absorbed in the gastrointestinal tract, dietary fat has to be emulsified by detergent action of the endogenous emulsifiers (such as bile salts) and hydrolyzed by lipase into fatty acids and mono- and diglycerides. Sow's milk contains ∼40% fat on a dry matter basis (159, 160); whereas, typical nursery diets include fat from 3 to 6% as a maximum level (161). Digestibility of fat from sow's milk in suckling pigs is over 90%; however, digestibility of fat from solid feed in newly weaned pigs is as low as 73% (162, 163) and increases gradually return to the preweaning level ranging from 4 to 6 weeks post-weaning (23, 164). The form of the milk fat presents as micelles and consequently aid digestion (165) by pancreatic lipase, whereas fat in solid diets is not in an easily accessible form. The synthesis of hepatic bile acid is low at weaning in pigs (166). Therefore, the emulsification process is a rate-limiting step in the digestion of dietary fat during this period.

Lysophospholipids

Phospholipids, nature's principal surface-active agents, performs as an excellent emulsifying agent. The main constituents of the phospholipid mixture are phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid (167). The majority of the phospholipid in the small intestine is derived from bile with a smaller component coming from the diet. Phospholipase A2, a pancreatic enzyme secreted in bile, hydrolyzes the ester bond at the sn-2 position of the phospholipid, yielding a free fatty acid and lysophospholipids with a different head group, which are then incorporated into micelles for subsequent absorption (168–170). On a commercial scale, lysophospholipids are often produced by the modification of soybean phospholipids (chemical or enzymatic methods) using phospholipase A2 which yields a mixture of lysophospholipids with different head groups depending on the source of the phospholipids (e.g., lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylethanolamine, and lysophosphatidic acid) (170, 171). Hydrophilic-lipophilic balance (HLB) values are assigned to emulsifiers from 0 to 20, and higher values are assigned to those are more hydrophilic. Soybean lysophospholipids have an HLB value of 19 (172), whereas the native soybean phospholipids have values of 5 (173). In addition, lysophospholipids have been reported to involve in various biological processes such as cell growth, proliferation and differentiation mediated by specific G-protein coupled receptors (174–176). Lysophospholipids supplemented in the diet showed to increase crypt cell mitosis and enhance villus morphology in broiler chickens (177). Lysophospholipids involve in epithelial cell restitution via cytoskeletal remodeling with activation of actin filament redistribution and stress fiber formation (178). It showed to reduce mucosal damage and inflammation by increasing epithelial cell restitution when induced colitis in rats (179). In broiler chickens, lysophospholipids increased crypt cell mitosis (180), and enhanced villus morphology (177).

Prebiotics

One of the most frequently employed product is prebiotics (181). Prebiotics has been widely used for improving beneficial microbial populations in the intestines. The definition of prebiotics was first introduced by Gibson and Roberfroid (182) as "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 host health." This concept has been refined during the past 20 years, and the definition to date was defined by Bindels et al. (183) as "a prebiotic is a non-digestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host." Bindels et al. (183) indicated the metabolic benefits attributed to prebiotics do not require a selective fermentation, which was mentioned in the earlier concept. The revised definition instead focused on the concept of ecological and functional characteristics of the microbiota to be relevant for host physiology, such as ecosystem diversity, and the support of broad microbial consortia. Many studies focusing on prebiotics such as inulin, fructooligosaccharides, galactooligosaccharides, and mannanoligosaccharides, proved the link between prebiotics consumption and restoring intestinal balance (184–187). Additionally, regardless of bacterial fermentation, prebiotic oligosaccharides (such as fructooligosaccharides and galactooligosaccharides) were shown to exert an anti-inflammatory effect or have an anti-adhesive activity to inhibit binding pathogens (188, 189). Studies with fructooligosaccharides showed that supplementing with fructooligosaccharides caused a shift in intestinal microbial composition via modulating short-chain fatty acids production, which provides substrates and promotes normal proliferation and differentiation of intestinal cells (190, 191).

Fermented Rice Bran Extracts

Rice bran, a co-product obtained during rice milling process, is rich in cell wall materials such as hemicellulose and cellulose containing neutral detergent fiber in the range of 19–34% (192, 193). The high fiber content is a major limitation of its use in young animal diets especially in newly weaned pigs. Defatting, fermentation, and enzymatic treatment (193–195) have been applied to improve the nutritional value of rice bran. Prebiotic properties of rice bran were reported in studies with mice (196) and pigs (94). Glucooligosaccharides, one of the emerging prebiotics was shown to be assimilated by *Bifidobacterium* species, but not by pathogenic species including *Clostridium* and *Salmonella* (197). Rice bran oligosaccharides, mainly composed of glucooligosaccharides, was reported to possess prebiotic potential (193, 198). The rice bran glucooligosaccharides was

shown to be able to promote the growth of *Lactobacillus* species, which was not hydrolyzed by human intestinal conditions.

Probiotics

Probiotics is defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition" (199). Prebiotics and probiotics exert their beneficial effects in a similar manner, through the modulations in the intestinal microbiota. Probiotics affect the microbiota via beneficial microorganisms, whereas prebiotics alter the microbiota by the supply of a substrate. Cultures commonly used in feed are lactic acid bacteria, Bacillus and yeasts (200). The beneficial microbes play an important role in maintaining the host health. They reduce the colonization and invasion of pathogens, maintain epithelial integrity, and enhance immune function (201, 202). Probiotics used in pig diets showed beneficial effects including reduced diarrhea incidence and improved in growth performance (13, 203). The combinational use of prebiotics and probiotics as synbiotics beneficially affects the microenvironment of the intestines to improve the survival and colonization of live beneficial microorganisms in the GIT (204–206).

Postbiotics

Postbiotics is relatively new term in animal science and collectively refers to bioactive compounds produced by probiotic microorganisms during a fermentation process (207, 208). Postbiotics, in fact, has been used in animal production in different terms including bacterial extracts and yeast culture. Postbiotics often includes microbial cell contents and cell wall. Fermentation products of *Saccharomyces cerevisiae*, also called yeast culture, have long been used in animal feeds to enhance appetite of lactating animals (104, 209–211), but more recently to enhance intestinal health of nursery pigs by bioactive compounds in fermentation products (212, 213). Yeast culture includes residual yeast cell wall fragments, and various products from yeast fermentation such as organic acids, nucleotides, vitamins, and amino acids (104). Yeast cell wall fragments have also used as postbiotics to modulate intestinal immune status and health (2, 126, 214). Selected bioactive compounds in postbiotics are proposed to alter microbiota composition (215). Selected postbiotics could also be investigated for their synergistic benefits with the use of probiotics.

Feed Enzymes

The major goal of the use of feed enzymes is to eliminate anti-nutritional factors to better utilize nutrients in the feed (200, 216). Carbohydrase has been widely used for their roles in breaking down non-starch polysaccharides (NSP) present in most vegetable ingredients (217, 218). The use of NSP enzymes showed to improve the growth performance of nursery pigs by enhancing intestinal health, nutrient digestibility (192, 194, 195). Chen et al. (102) evaluated supplemental effects of xylanase fed to nursery pigs with or without 30% corn distillers' dried grains with solubles (DDGS) as a source of NSP. The supplementation of 30% DDGS increased digesta viscosity, reduced the digestibility of dry matter and gross energy, and increased intestinal inflammation, whereas the supplementation of xylanase alleviated the negative effects on growth performance by feeding high-level DDGS by reducing digesta viscosity, improving nutrient digestibility, and reducing inflammatory response. In addition, xylo-oligosaccharides generated in the small intestine from xylans by xylanase hydrolysis could be potential prebiotics for lactogenic bacteria which warrants further research.

Protease breaks down peptide bonds in protein and polypeptides. Specific protease can target allergenic proteins in legume seed meals, such as glycinin and β-conglycinin causing gut inflammation, diarrhea and growth reduction (108). Duarte et al. (103) and Chen et al. (219) showed supplemental protease reduced gut inflammation and improvement protein digestibility and feed efficiency in nursery pigs. Phytase catalyzes the phytate hydrolysis and releases phosphorous and phytate-bound nutrients (220). The use of phytase increased phosphorus digestibility, bone characteristics, and growth performance (105, 221). More recently elevated dose of phytase so called superdosing of phytase (often more than 10-folds of typical dose levels) has received attention and applied in pig production. It is hypothesized that typical supplementation level of phytase would not completely hydrolyze phytate in the stomach and superdosing of phytase would provide opportunities of complete hydrolysis of phytate in the stomach. Complete hydrolysis of phytate not only provides available phosphates along with release of other essential minerals but also free inositol for their potential function in insulin sensitivity and carbohydrate metabolism.

Nucleotides

Nucleotides are bioactive molecules that play important roles in metabolic, structural and regulatory functions (222). The milk of sow contain large concentration of nucleotides during 28-day lactation (223) that supplies the needs of the piglets. At weaning, the requirement of nucleotides increases for immune response and the intestinal recovery, whereas the endogenous synthesis is insufficient to meet the requirements (224, 225) and the weaning diet has low concentration compared with milk (226). Therefore, exogenous sources of nucleotides can be used to supply this demand and alleviate the effects of the weaning stress (110, 111, 223, 226). Sauer et al. (226) reported that dietary nucleotides positively affect the intestinal morphology, the immune response, the hepatic function and the microbiota. The consumption of nucleotides can improve the feed efficiency of nursery pigs by reducing the immune response and the oxidative stress status, whereas increasing the villus height and the energy digestibility (110, 111). The effect of dietary nucleotide on modulating the immune system and the microbiota suggested that it can be used to prevent post-weaning diarrhea in pigs as confirmed by Wiseman (225). According to Li et al. (112) dietary nucleotides can reduce diarrhea caused by enterotoxigenic *E. coli* by modulating the microbiota and enhancing the immune response of weaning pigs. Some of unsolved questions include the types and profiles of nucleotides for the effectiveness. Commercially available nucleotide supplements are typically obtained from yeast extracts providing combination of adenosine-5-monophosphate

(AMP), cytidine-5-monophosphate (CMP), guanosine-5-monophosphate (GMP), and uridine-5-monophosphate (UMP). Some others source nucleotides from bacterial fermentation extensively including inosine-5-monophosphate (IMP). Ideal ratio among nucleotides and functional uniqueness of IMP warrant future investigations.

Organic Acids and Acidifiers

Organic acids have been used in the pig diets to decrease gastric pH (227), prevent pathogenic bacterial growth (228), improve nutrient digestion (229), and improve growth performance (230). Gastric pH in weaned pigs ranges between 2.6 and 5.0, whereas the optimum gastric pH for vegetable protein digestion is in the range of 2.0–3.5. Inclusion of organic acids such as fumaric and citric acids are shown to have beneficial effects in newly weaned pigs (231, 232). Organic acids can modulate the intestinal microbiota by inhibiting the pH-sensitive microbial without affecting the lactic acid bacteria (233, 234). According to Ren et al. (235) 1% formic and propionic acid mixture can reduce the inflammatory response of weaning pigs challenged with enterotoxigenic *E. coli*. Current challenges with organic acids, however, are their effectiveness affecting luminal pH at a realistic supplementation level without affecting appetite or feed intake of nursery pigs. Recent advances to overcome these challenges include encapsulation or coating technologies.

Phytobiotics and Phytogenic Feed Additives

The major biological functions of phytogenic feed additives (PFA) include improve feed palatability, stimulation of digestive enzyme secretions, microbiota modulation, antimicrobial, anti-inflammatory, and antioxidant activity (116, 117, 119, 236, 237). The PFA are reported to improve piglets' post-weaning feed intake and growth performance when added into sow diets. A mixture of phytogenic compounds (anethol, cinnamaldehyde, and eugenol) used as feed additive for sows during late gestation and lactation showed to increase post-weaning feed intake and growth rate of piglets (117). The three compounds were detected in amniotic fluid and the positive effects on post-weaning performance were attributed to the maternal exposure to the flavor of the phytogenic compounds. Li et al. (118) evaluated the effects of essential oil (a mixture of thymol and cinnamaldehyde) supplemented in feeds for nursery pigs with or without antibiotic growth promotors. The supplementation of thymol and cinnamaldehyde increased growth of pigs during 35-day post-weaning period, and the effect was similar to feeding antibiotics. In the same study, improved dry matter and crude protein digestibility were detected by the essential oil supplementation. Similar beneficial effects of PFA on nutrient digestibility in s nursery pigs were reported in other studies (120). The potential mechanisms of improving nutrient digestibility may be partially due to the stimulation of digestive enzymes activities and stimulation of bile secretion by phytogenic compounds (238). Beneficial effects on intestinal morphological changes may provide further information on promoting growth performance; however, the results obtained from different studies have not been consistent (239) where PFA reduced feed intake possibly due to strong aroma from oregano extracts. Commercial products often mask the aroma from PFA by encapsulation or coating which are practical for the feed application of PFA.

Immunoglobulin-Containing Compounds

Under the commercial production systems, pigs are usually weaned at 3–4 weeks of age, whereas this is early stage of their life when the ability of pigs to produce immunoglobulins is not fully developed (55). The addition of immunoglobulins-containing compounds in the post-weaning diets may be beneficial. Immunoglobulin-rich product, blood plasma, has been shown to have beneficial effects on increasing post-weaning feed intake and growth rate, and reducing post-weaning diarrhea (121, 122, 240). Furthermore, in disease challenge studies with *E. coli*, blood plasma is reported to maintain intestinal barrier function, increase antibody production, and decrease pro-inflammatory cytokine expression (241, 242). In addition, supplementation of blood plasma is reported to alleviate negative impact on growth performance by feeding mycotoxin contaminated feed (10). However, despite its high nutritional value, the availability of amino acid (especially lysine) can be reduced with excessive heating treatment during manufacturing process of blood plasma (240). Additionally, increasing biosecurity concerns using blood plasma has limited its application in swine diets (24, 25).

Mycotoxin Deactivators

Among the mycotoxins identified (∼300–400), aflatoxins, fumonisins, ochratoxin A, trichothecenes such as deoxynivalenol (DON), and zearalenone are some of the mycotoxins that can significantly affect animals' health (27, 243). Impact of major mycotoxins on nursery pigs are summarized in **Table 4**. Previous studies have shown that young pigs are especially susceptible to trichothecenes (especially DON), and fumonisins due to their negative effects on intestines (252, 253). Consumption of DON-contaminated feed can decrease feed intake, impair intestinal barrier function, and increase intestinal inflammatory response in pigs (123, 254–256). Exposure to DON causes epithelial injuries and compromise barrier function by decreasing tight junction proteins expression and can modulate immune response by increasing the susceptibility to enteric infections (257–259). Commonly used methods include adsorbents (binding agents), enzymatic or microbial detoxification, purified enzymes, and/or "bio-protection" method using substances such as plant ingredients. Absorbents can absorb certain mycotoxins such as aflatoxin, but it does not work at the same extent to other mycotoxins. Murugesan et al. (27), in a study comparing the adsorption capacity of different commercially available mycotoxin binder products, showed that tested products have poor adsorption for DON. Alternative strategies such as enzymatic or microbial detoxification, where mycotoxins are catabolized or cleaved to less or non-toxic compounds are much more effective compared to using binding agents (27, 260). Holanda and Kim (123) reported that yeast-based detoxifiers with functional components can improve detoxifying

TABLE 4 | Impact of mycotoxins on nursery pigs and regulatory limit of major mycotoxins.

Initial body weight or age	Mycotoxin type and contamination level	Experimental period (day)	Impact	Reference
11.4 ± 0.1 kg	Aflatoxins - 140 or 280 μg/kg	28	Decreased weight gain and altered humoral and cellular immune responses	(244)
14.2 ± 3.0 kg	Aflatoxins - 250 or 500 μg/kg	70	Reduced ADG and ADFI	(245)
27 day	Deoxynivalenol - 3.2 mg/kg	34	Reduced ADG during the last 13 day	(123)
10.3 ± 0.2 kg	Deoxynivalenol - 4 mg/kg	21	Reduced ADG, ADFI, and growth efficiency	(246)
8.9 kg	Fumonisins - 7.2, 14.7, 21.9, 32.7, or 35.1 mg/kg	28	Decreased ADG, ADFI, and growth efficiency increased the serum sphinganine-to-sphingosine ratio	(247)
28 day	Fumonisins - 3.7 mg/kg	28	Increased the serum sphinganine-to-sphingosine ratio and altered heart and intestine morphology	(248)
12–14 kg	Orchratoxin A - 800 μg/kg	84	Decreased BW and increased kidney weight	(249)
21 day	Zearalenone - 1 mg/kg	22	Had no effect on growth performance; however negative effect was shown on genital organs and serum hormones in gilts	(250)
10.4 ± 1.2 kg	Zearalenone - 1.1, 2.0 or 3.2 mg/kg	18	Negatively affected immune function in gilts	(251)
21 day	Aflatoxins - 180 ug/kg; Fumonisins - 9 mg/kg; Deoxynivalenol - 1 mg/kg	48	Reduced BW, ADG, ADFI, and growth efficiency	(2)
6.8 ± 0.1 kg	Aflatoxins - 2,778 μg/kg; Fumonisins - 170 mg/kg; Zearalenone - 1 mg/kg	33	Reduced ADG	(10)

Regulatory limit of major mycotoxins in finished feed of young pigs (mg/kg)[a]

Region	Aflatoxins	Deoxynivalenol	Fumonisins	Zearalenone	Ochratoxin A
United States	0.02	1	20	Not defined	Not defined
European Union	0.02	0.9	5	0.1	0.05

[a] *United States regulatory limit according to the Food and Drug Administration Regulatory Guidance for Toxins and Contaminants. European Union regulatory limit according to the European Commission Directive 2003/100/EC and the European Commission Recommendation 2006/576/EC.*

properties in newly-weaned pigs fed DON contaminated feed (3.2 mg/kg), potentially by increasing adsorption capacity, improving immune function, and enhancing intestinal health. Fumonisins disrupt the synthesis of sphingolipids-containing cell membrane because they have a chemical structure that is similar to that of the sphingoid bases deoxysphinganine (261), key enzymes involved in sphingolipid biosynthesis (262). This dysregulation of sphingolipid biosynthesis causes accumulation of the sphingoid bases (sphinganine and sphingosine), and their metabolites (261, 263). Negative impact of fumonisins include porcine pulmonary edema, damages to gastrointestinal structure, and reduction in growth performance (254, 264, 265). In a study evaluated effects of different commercial products on mitigating fumonisins negative effects during nursery phase showed a bentonite and yeast-based product alleviated negative impact of fumonisin (50–60 mg/kg) on growth performance (124). Different regulations on maximum levels of mycotoxins for young pigs have been established by different countries; however, previous studies have shown that the contamination levels below the regulatory limits showed negative effects on growth performance and immune function (see **Table 4**). Furthermore, information on the regulatory limits on some of the major mycotoxins (i.e., zearalenone and ochratoxin A) and co-contamination of multiple mycotoxins are not available. The co-contamination with multiple mycotoxins in feed can cause more adverse effects than a single mycotoxin due to the additive or synergistic interaction (266). Additionally, limited practice on mitigating chronic exposure to low-dose mycotoxins may negatively impact production efficiency. Understanding the prevalence of mycotoxins in the feed and applying effective interventions are critical to ensure young pigs' health.

CONCLUSIONS

At weaning, pigs deal with multiple stressors such as separation from the sow, a new environment, separation from littermates and cohabitation with new pigs, and the abrupt change of diet types from liquid sow milk to solid feeds. Weaning causes morphological and functional changes of the small intestine of pigs where most of the nutrients are being digested and absorbed. These changes can result in severe diarrhea and even cause mortality. In addition, due to the increasing feed safety concerns, volatile price of specialty feedstuffs, and regulatory changes on using certain feed additives (i.e., antibiotics and zinc oxide), some of the commonly used feedstuffs and additives in the nursery diets have been limited for their use. Alternative nutritional strategies aligning with these changes have been tried to combat the weaning challenges.

In order to minimize weaning-associated depressed growth, the need for developing effective nutritional strategies is critical. Functional feed additives that have a positive influence on enhancing intestinal health will aid in amelioration of the depressed growth and intestinal dysfunction associated

with weaning stress. The functional feed additives such as protein hydrolysates, emulsifiers, prebiotics, probiotics, postbiotics, enzymes, nucleotides, organic acids, phytogenic feed additives, immunoglobulin-containing compounds, and mycotoxin deactivators were evaluated their roles in promoting intestinal health and growth of nursery pigs to allow better nutritional management during the crucial post-weaning period. The evaluations on how these feed additives affect the intestinal architectural structure, intestinal barrier function, mucosal immunity, and intestinal microbial community can provide valuable information to formulate optimized nursery diets. Combinational uses of these feed additives as synbiotics, could provide further benefits to nursery pigs.

AUTHOR CONTRIBUTIONS

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

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