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To my mother in heaven

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Part I:

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List of abbreviations

CB	Cobb
RS	Ross
M	Male
F	Female
CO	Control diet
PO	Probiotic-supplemented diet
PY	Phytobiotic-supplemented diet
NSP	Non-starch polysaccharides
GC	Goblet cells
SCFA	Short-chain fatty acids
BCFA	Branched chain fatty acids
VH	Villus height
VW	Villus width
CD	Crypt depth
V/C	Villus height to crypt depth ratio
VSA	Villus surface area
IL	Interleukin
TNF-α	Tumor necrosis factor alpha
IFN-γ	Interferon gamma
TGF-β2	Transforming growth factor beta 2
CLDN5	Claudin 5
MUC2	Mucin 2

1. Chapter 1: General introduction

Gut microbiota of broilers plays a crucial role in maintaining gut health. Gut microbiota directly or indirectly (via metabolites) interacts with intestinal epithelial cells, communicates with immune cells and modulates cell proliferation and barrier function (Mahapatro et al., 2021). Modification of gut microbiota by feed additives such as probiotics and phytobiotics has been shown to improve animal health and performance (Park et al., 2020; Lee et al., 2015). Probiotics, such as *Bacillus* spp.

, are commonly used in broiler diets due to their ability to remain stable and viable during the feed manufacturing processes, storage, and passage through the gastrointestinal tract (Zentek and Goodarzi Boroojeni, 2020; Goodarzi Boroojeni et al., 2016). Adding *Bacillus* spp. in broiler diet has been shown to inhibit intestinal pathogens, diminish gut inflammation and alter gut morphology, which finally lead to an improved growth performance (Park et al., 2020; Song et al., 2014). Phytobiotics are a diverse group of plant-based products that have shown positive effects on gut health. Important bioactive compounds of phytobiotics are polyphenols, specifically procyanidins, which possess potent anti-oxidant, anti-inflammatory, and anti-bacterial properties (Viveros et al., 2011). The potential effects of probiotics and phytobiotics on gut health of broilers have been extensively studied and great deal of variation in measured effects have been observed, even with the same strain of probiotic or type of phytobiotic product. Changes in gut microbiota and immune response by host and environmental factors may vary how the birds respond to feed additives (Broom and Kogut, 2018; Kers et al., 2018).

Gut microbiota and immunological trait of broilers can be influenced by host-related factors including age, breed, sex, and intestinal location. Co-development of gut microbiota and host immune system occurs concurrently with broiler growth. Over time, bacterial composition, diversity, and richness evolve in the gut, while the immune system develops its functions dealing with luminal stimuli and become mature as broiler ages (Kohl, 2012). Modern broilers have been selectively bred for desirable performance such as increased growth rate, feed efficiency, and meat yield. However, studies have shown that the genetic makeup of modern broiler chickens may still affect their immune response to certain challenges (Mayahi et al., 2016; Cheema et al., 2003). Distinct gut microbial composition and metabolites have been suggested to be the reasons for differences in immune response between broiler breeds (Richards et al., 2019; Hong et al., 2012). Sex-related variations in their physiology and growth performance of broilers may also alter their microbial composition and immune system (Dalgaard et al., 2021; Lumpkins et al., 2008). As a result, variations in intrinsic factors such as age, breed, sex, and extrinsic factors including diet may have different impacts on the gut microbiota and immune system. Broilers have been found to harbor distinct microbial

communities along the gastrointestinal tract. The distal part of the small intestine (e.g., ileum) and caecum are important sites for microbial fermentation as evidenced by an increase in bacterial density and metabolite production distally along the gut (Rehman et al., 2007). However, the ileum and caecum have different physiological functionality in the gut, thereby creating different environment for diverse bacterial communities (Zhou et al., 2021; Brisbin et al., 2008). To address this issue, the present PhD thesis was conducted to investigate the impact of these factors on bacterial activity, immunological traits, and gut morphology in broilers, with the first part focusing on the ileum and the second part on the caeca. Furthermore, the regional-specific changes observed were further discussed to provide insights into the possible underlying mechanisms.

Hypothesis

The hypothesis was that host-related factors, including age, breed, and sex, and dietary supplementation with *Bacillus*-based probiotics and procyanidin-rich phytobiotics affect the gut microbial activity, immunological traits, and morphology of broilers in region-specific manners. Therefore, alterations in microbial metabolites and mucosal cytokines by these factors were expected differently in the ileum and caecum of broilers.

2. Chapter 2: Literature review

2.1. Gut microbiota and microbial metabolites of broilers

A wide range of microorganisms are located throughout the chicken's gastrointestinal tract from the crop to the ceca. In general, bacterial density typically increase distally along the gut (Zhou et al., 2021). The crop, a diverticulum of the esophagus, serves as a temporary storage site for ingested feed and also acts as a natural fed-batch fermenter. The microbial fermentation within the crop is largely dependent on the dietary substrates and amount, as well as its surrounding conditions such as pH and oxygen levels (Classen et al., 2016). The gizzard and proventriculus are primarily responsible for grinding and digesting feed, but they have a limited role in bacterial fermentation (Józefiak et al., 2007). In the small intestine, bacterial fermentation is limited due to rapid motility and the luminal environment such as the presence of digestive secretions that restrict bacterial growth (Kastl et al., 2020). However, compared to the duodenum and jejunum, the ileum appears to be an important site for microbial fermentation, as shown by an increase in bacterial density distally along the small intestine (Meimandipour et al., 2011; Rehman et al., 2007). The caeca, located between the small and large intestine, offer a favorable environment for microbial fermentation due to their relatively anaerobic conditions and long retention time of digesta (Oakley et al., 2014b). Moreover, the presence of high concentrations of mucins in the caecum, which are secreted in the upper gastrointestinal tract, can be utilized by caecal microbiota as an energy source and enhance their growth (Oakley et al., 2014b).

Gut microbiota possesses diverse metabolic activities to utilize dietary substances and release metabolites into the gut. It is well known that dietary protein and carbohydrates influence the metabolic activity and taxonomic profile of the gut microbiota (Apajalahti and Vienola, 2016). The primary metabolites from carbohydrate fermentation include short-chain fatty acids (SCFA) and lactate, whereas branch-chain fatty acids (BCFA), biogenic amines, and ammonia are the main products of protein fermentation (Qaisrani et al., 2015). These bacterial metabolites, especially SCFA, have various functions in the gut, including promoting cell proliferation, altering mucus production, and modulating immune response of the host. For example, butyrate acts as an energy source for the intestinal epithelium, while acetate and propionate can regulate intestinal cell activity (Zou et al., 2019; Liu et al., 2017; Hosseini et al., 2011). Moreover, SCFA play a role in cellular signaling related to cytokine production by directly binding to certain receptors such as free fatty acid receptor (or G protein-coupled receptors) and/ or by regulating target cell epigenetics after they were taken up into the intestinal cells (Liu et al., 2021). In contrast, putrefactive metabolites such as some biogenic

amines and ammonia are toxic to chickens and at high concentrations, may have detrimental impacts on chicken growth and performance (Apajalahti and Vienola, 2016).

2.1.1. Development of gut microbiota

Gut microbiota of broiler chickens begins to develop immediately after hatching. Newly hatched chicks are exposed to microbes from the eggshell surface and environment, resulting in a rapid increase in bacterial density in the gastrointestinal tract of broiler chicks. Apajalahti et al. (2004) found that the bacterial density in the ileum and caecum of broiler chicks reached 10^8 and 10^{10} cells/g of digesta, respectively, one day after hatching. Within one week of age, this bacterial density increased to a maximum of 10^9 and 10^{11} cells/g of digesta in the respective locations. As broiler chickens age, their gut microbial community becomes more complex, with increasing diversity in terms of bacterial species and richness (Zhou et al., 2021; Lu et al., 2003; Van Der Wielen et al., 2002). However, the change in microbial composition varies in different intestinal regions. Using the 16S rDNA gene-based polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique, Van Der Wielen et al. (2002) observed that microbial composition in the ileum and caecum of broilers varies significantly after day 4 of age. The microbial community in the ileum of broilers was found to be lower in richness and diversity than that in the caecum (Mohd Shaufi et al., 2015; Meimandipour et al., 2011). Furthermore, bacterial metabolites such as SCFA and lactate vary along the intestinal tract of broilers. Meimandipour et al. (2011) showed that acetate concentration was very low in the duodenum, jejunum, and ileum of broilers, while that of propionate and butyrate were not detectable in these parts. In contrast, caecum showed higher concentration of SCFA, especially acetate, than the small intestine. Lactate was present in both the small intestine and caecum, but more predominant in the small intestine. However, concentration of these metabolites can vary with age and may be influenced by factors such as bacterial composition and available substrates (nutrients) in the gut (Liao et al., 2020; Meimandipour et al., 2011).

2.1.2. Gut microbiota in commercial broilers

Genetic background of broilers has been identified as a significant factor that affects composition of their intestinal microbiota (Richards et al., 2019; Schokker et al., 2015). Modern broilers have been selectively bred for high performance through intensive breeding programs. Commercial modern broilers such as Ross and Cobb have shown improved performance with nearly identical body weight, feed intake and feed conversion ratio (Pascalau et al., 2017). However, despite their similar performance, these broilers exhibit distinct gut microbial populations and immune responses to specific challenges. Richards-Rios et al. (2020; 2019) analyzed the changes in microbial composition in the ileum and caecum of Ross, Cobb, and Hubbard, over a 42-day period using 16S rRNA sequencing. The study showed that microbial

composition in the ileum differed between breeds from day 3 of age and continued to vary until day 42, whereas the caecal microbiota varied between breeds only during the first few days of age and remained consistent afterward. These microbial variations could be linked to the genetic diversity of the broilers (Schokker et al., 2015). The genetic influences alter gut anatomy and physiology, creating a specific environment for microbial colonization, resulting in distinct microbial compositions between broiler breeds (Schokker et al., 2015).

2.1.3. Sex influences on gut microbiota of broilers

Gut microbiota has been found to be different for male and female broilers. Lumpkins et al. (2008) reported a distinct separation of bacterial communities in the ileum of male and female broilers by 16S rDNA sequencing, with less than 30% similarity between populations. They also found that the amount of lactic acid bacteria was higher in males than in females. Similarly, Humer et al. (2015) observed that male broilers had higher lactate and lower acetic acid concentration in the ileum compared with females. Several studies also showed the sex-difference in microbial composition in the caecum of broilers. Lee et al. (2017) reported that female broilers had an increased abundance of anaerobic *Firmicutes* in the caecum, while male broilers had a higher abundance of *Bacteroidetes*. A similar abundance pattern of these bacteria has also been observed in humans, with women having a higher *Firmicutes* to *Bacteroidetes* ratio than men (Razavi et al., 2019). This ratio has been associated with the efficiency of energy harvesting in both humans (Razavi et al., 2019) and broilers (Zhu et al., 2019). Therefore, alterations in microbial composition between male and female broilers could be linked to their different growth performance. Furthermore, these two dominant bacteria have different metabolic pathways for carbohydrate fermentation, with *Firmicutes* mainly producing butyrate and *Bacteroidetes* contributing to acetate and propionate production (Resch et al., 2021). These bacterial metabolites can affect gut morphology by altering cell proliferation, mucus production and epithelial integrity, which may result in a distinct luminal environment, hence altering gut microbial composition between the sexes (Kayama and Takeda, 2020).

2.2. Gut immune system of broilers

Gut immune system of broilers comprises two primary immune mechanisms: innate immunity and adaptive immunity. Innate immunity is the first line of defense against infection and offers a rapid immune response. It consists of physical and chemical barriers, such as mucosal epithelium and mucus layer, as well as cellular and humoral components including phagocytes, heterophils, dendritic cells, natural killer cells, complements, and antibacterial proteins (Alkie et al., 2019). The adaptive immunity is established after the initial exposure to antigens, developing a delayed and specific immune response. The adaptive immune system is categorized into cell-mediated immunity and humoral immunity, which involve T and B

lymphocytes, respectively (Rodrigues et al., 2021). In the gut of broilers, T and B lymphocytes are located in the gut-associated lymphoid tissue (GALT), including the bursa of Fabricius, caecal tonsils and Meckel's diverticulum as well as Peyer's patches, the epithelium (intraepithelial lymphocytes), and lamina propria (Bar-Shira et al., 2003). B lymphocytes produce antibodies, which play a crucial role in inducing the elimination of extracellular pathogens, while T lymphocytes are the main cells involved in eliminating intracellular pathogens. T lymphocytes can be further divided into helper, cytotoxic, and regulatory T lymphocytes based on their functions and the secretion of cytokines and chemokines (Bar-Shira et al., 2003). Cytokines are proteins secreted by a wide range of cells and have important roles in the activation and regulation of immune cell proliferation. Generally, cytokines are classified into four broad groups (Lee et al., 2019): Th1 cytokines (e.g., IL-2, IL-8, IL-12, IL-18, TNFα, and IFN-γ), Th2 cytokines (e.g., IL-4), Th17 (e.g., IL-17α) and regulatory T (Treg) cytokines (e.g., IL-10 and TGF-β2), as well as pleiotropic cytokines (e.g., IL-1β and IL-6). These cytokines can bind to cell surface receptors and act as signaling molecules between cells, facilitating communication between the immune system and other systems in the body, thereby contributing to the maintenance of immune homeostasis.

2.2.1. Age-associated alterations in gut immune system

The development of gut immune system of broiler chickens is a dynamic process involving the interactions between gut microbiota and the host immune system. Song et al. (2021) reported temporal changes in cytokine expression in the ileum of broilers, with most cytokines being expressed at very low levels during the first week after hatch, but gradually upregulating and reaching their peak around day 30 of age. Furthermore, genes related to T cell receptors and antibody production (e.g. Immunoglobulin A) were also upregulated in the ileum with age, thus reflecting the maturation of cellular immune function (Song et al., 2021). In the caecum of broilers, the expression of several cytokines was initially low during the first week and most of them showed high variability with age, particularly during the first three weeks of life (Crhanova et al., 2011; Bar-Shira and Friedman, 2006). These show presence of functional but immature immune cells in both ileum and caecum during the first week of age. Despite this, cytokine expression, particularly IL-2, was higher in the caecum than other parts of the small intestine and colon of broilers, suggesting that the caecum is more active immunologically during the first week of age (Bar-Shira et al., 2003). The initial colonization of gut microbiota during the first few days of life is crucial for programming of the innate immune functions, while development of the adaptive immune functions occurs towards the end of the first week post-hatch (Rodrigues et al., 2021). Microorganisms and their metabolic products can interact with enterocytes and immune cells, and subsequently trigger intracellular signaling, leading to

cytokine and chemokine synthesis and secretion (Mahapatro et al., 2021). Thus, gut immune system of broilers is constantly responding and adapting to the changes in gut microbiota.

2.2.2. Variations in gut immunological traits among different broiler breeds

Different breeds of broiler chickens have been shown to exhibit varying levels of disease susceptibility, which may be associated with differences in their immune systems. Hong et al. (2012) showed breed-specific differences in the expression of immune mediators, including antimicrobial compounds (β-defensins and gallinacin) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF) in the crop and jejunum, even under non-disease challenge conditions. These differences in immune system expression may have contributed to observed differences in disease susceptibility to enteric pathogens. For example, Ross broilers were more resistant to necrotic enteritis caused by *Clostridium perfringens* and *Eimeria maxima* compared with Cobb broilers, as evidenced by fewer intestinal lesions and better growth performance (Jang et al., 2013; Hong et al., 2012). Emam et al. (2014) also found differences in immune responses against sheep red blood cells and *Brucella abortus* antigen in commercial broiler breeds, i.e. Ross and Cobb, and indigenous chickens. The study showed that after immunization of chickens with these antigens, the antibody titer of Immunoglobulin M and Immunoglobulin Y in the serum was higher in Ross compared with Cobb and indigenous chickens. Differences in the genetic background of these chickens (breeds) may have been responsible for the observed variations in immune response (Emam et al., 2014). The distinct gut commensal bacteria among broiler breeds may also affect the immune system, leading to differences in susceptibility to certain diseases (Kim et al., 2015).

2.2.3. Impact of sex on gut immunological traits

Biological differences between males and females can affect susceptibility to certain infectious diseases. Leitner et al. (1989) investigate the immune differences in the antibody response against various bacterial, virus, and protein antigens between male and female broilers. The study showed higher levels of antibody production and T cell proliferation, and improved survival rate in females compared to males (Leitner et al., 1989). It is believed that sex hormones, such as estrogen and testosterone, play a role in modulating the immune system of broilers (Dalgaard et al., 2021). Females have higher level of estrogen and males have higher level of testosterone (Younis et al., 2023). Estrogen has been found to enhance immune function through cytokine production, cell activation, and proliferation, while testosterone has a suppressive effect on lymphocyte proliferation (Fransen et al., 2017). Dalgaard et al. (2021) reported that administration of exogenous estrogen enhanced the development of bursa of Fabricius, as well as the proliferation and functions of chicken lymphocytes, while exogenous testosterone was found to suppress the development of the bursa in young chicks.

2.3. Impacts of *Bacillus*-based probiotics on gut health of broilers

Probiotics refer to living microorganisms that confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2001). Among the probiotic species commonly used in poultry production, *Bacillus* spp. are considered one of the most promising probiotic species for poultry production due to their stability and viability during feed processing and in the gut (Zentek and Goodarzi Boroojeni, 2020; Goodarzi Boroojeni et al., 2016). The beneficial effects of *Bacillus* in broilers may be achieved through various mechanisms, including enhancement of beneficial bacteria growth, competitive exclusion of pathogenic bacteria (Wu et al., 2011), production of antimicrobial substances (Tran et al., 2022), modification of the immune response (Rooks and Garrett, 2016), and improvement of intestinal barrier integrity (Bilal et al., 2021). Studies have shown that *B. subtilis* and *B. amyloliquefaciens* can maintain commensal bacteria and inhibit the growth of enteric pathogens in the gut of broilers (Wang et al., 2021a; Xu et al., 2021; Rodrigues et al., 2020b). *Bacillus* spp. also produce several metabolites, such as bacteriocins (subtilin and barnase) that exert antimicrobial effects on proteolytic bacteria resulting in a decrease in toxic metabolites such as ammonia, indole and biogenic amines in the caecum and excreta of broilers (Wang et al., 2021b; Teng et al., 2017; Lisboa et al., 2006). However, the impact of these *Bacillus* spp. on gut microbiota of broilers has been shown to be inconsistent, with some studies reporting no effect (Rodrigues et al., 2020a), while others showed positive effects on gut health, including increased growth of beneficial bacteria like *Lactobacillus* and *Bifidobacterium* (Wang et al., 2021b; Wu et al., 2011) and enhanced production of bacterial metabolites like SCFA and lactate (Rodrigues et al., 2020b).

Bacillus-based probiotics have been shown to exhibit immunomodulatory properties. Addition of *B. subtilis* and *B. amyloliquefaciens* increased cytokine production and immune cell proliferation in the ileum and caecum of broilers, resulting in the maturation of immune system (Bilal et al., 2021; Hong et al., 2021). Moreover, *B. subtilis* has been shown to enhance the production of anti-inflammatory cytokines such as IL-10 and IFN-γ in the ileum, thereby maintaining gut homeostasis (Bilal et al., 2021). The immune-modulating properties of *Bacillus* spp. are attributed to their surface components such as peptidoglycans. These components interact with specific receptors on the epithelial cells and immune cells in the intestine, regulating stimulatory and inhibitory signals, which contribute to the immune responses of the host (Rooks and Garrett, 2016). Moreover, antimicrobial substances produced by *Bacillus* spp. can alter the immune responses against certain enteric pathogens. For example, sublancin, an antimicrobial peptide isolated from *B. subtilis*, decreased the expression of pro-inflammatory cytokines such as IL-1β and IL-6 in the ileum of broilers challenged with *Clostridium perfringens*, resulting in a reduction in intestinal lesion severity (Wang et al., 2015).

In conclusion, *Bacillus*-based probiotics have potential to regulate gut microbiota and immune system, however the mechanisms of action of *Bacillus* spp. on these features in the gut need to be studied further.

2.4. Effect of grape polyphenols on gut health of broilers

Polyphenols are bioactive compounds found in plants including legumes, fruits, vegetables, grains, and essential oils. Polyphenols have been shown to possess potent antioxidant properties and ability that modulate gut microbial populations in chickens (Iqbal et al., 2020). Grapes are one of the fruits that are particularly rich in polyphenols, mainly flavan-3-ol monomers and polymers, also called procyanidins (González-Quilen et al., 2020). An abundant source of procyanidins in grapes have been linked to reduced oxidative stress, and intestinal inflammation in broilers (Wu et al., 2016). Moreover, these phenolic compounds can modify the microbiota composition by selectively inhibiting the growth of some pathogenic bacteria while enhancing the growth of beneficial bacteria (Iqbal et al., 2020).

Grape procyanidins and their polyphenolic compounds present antimicrobial effects through various mechanisms, including inhibition of extracellular enzymes, deprivation of essential microbial substrates, disintegration of bacterial outer membrane, and direct action on microbial metabolism (Dasiman et al., 2022). An *in vitro* study has shown that grape seed extracts (0.5%, 1%, and 2.5%) exhibited bacteriostatic and bactericidal effects against pathogenic bacteria such as *Escherichia coli* O157:H7, *Aeromonas hydrophila*, and *Staphylococcus aureus* as determined by the agar diffusion method (Baydar et al., 2006). Moreover, the inhibitory effects of grape seed extracts on these bacteria increased with higher concentration (Baydar et al., 2006). In broilers, Chamorro et al. (2019) showed that higher level of procyanidin-rich grape extract (5,000 ppm) in the diet decreased the populations of *E. coli, Enterobacteriaceae,* and lactic acid bacteria in the ileum. However, a lower dose (2,500 ppm) of the grape extract did not alter these bacteria compared to the control diet. In contrast to their antimicrobial properties, procyanidins showed prebiotic effects by serving as nutrient sources for selected bacteria (Mena et al., 2015). Grandhaye et al. (2020) found that addition of grape seed extract (1,000 ppm) enhanced the growth of beneficial bacteria including *Bifidobacteriaceae*, *Lactobacilliaceae*, and *Lachnospiraceae* in the caecum of broilers. Similarly, procyanidin-rich grape pomace concentrate (60,000 ppm) and grape seed extract (7,200 ppm) increased the populations of beneficial bacteria including *Enterococcus* in both ileum and caecum of broilers (Viveros et al., 2011).

Procyanidins have been found to play a role in the modulation of host immune functions by regulating enzyme activity and transcription factors involved in the production of cytokines, chemokines, and other inflammatory mediators in epithelial and immune cells (González-

Quilen et al., 2020). Cao et al. (2020) found that grape seed extract supplementation (200-400 ppm) reduced gut inflammation by decreasing pro-inflammatory cytokines such as IL-1β in the jejunum and ileum of broilers. Similarly, Huerta et al. (2022) found that grape seed extract (1,000-4,000 ppm) increased the number of intraepithelial leukocytes in the jejunum, indicating immune system activation in the gut mucosa. Furthermore, procyanidins could be indirectly modulate immune responses by regulating gut microbiota and metabolites (Cao et al., 2020; Williams et al., 2020). Addition of grape seed extract (7,200 ppm) increased beneficial bacteria including *Lactobacillus* in the ileum and caecum, while decreased potential pathogenic bacteria such as *Clostridium* in the ileum of broilers, which may subsequently reduce intestinal inflammation and damage (Viveros et al., 2011). Besides the microbial and immune-modulating effects, it should be noted that procyanidins are considered as anti-nutritional compounds, as they bind to macronutrients and form complexes, leading to decreased nutrient utilization by the host (Chamorro et al., 2013; Viveros et al., 2011). The degree of polymerization of procyanidins is a critical factor in their anti-nutritional effects, as a larger molecule resulting from a higher degree of polymerization can increase the binding sites for nutrients, further reducing nutrient absorption (Brás et al., 2010). Therefore, when applying procyanidins in broilers, it is important to consider the quality and quantity of procyanidins from the extract to avoid negative impacts on nutrient utilization and growth performance.

2.5. Development and functional properties of intestinal mucus layer in poultry

The mucus layer plays a crucial role in protecting the epithelium from various threats in the gut, such as mechanical forces during digestion, digestive enzymes, and gut microorganisms. This protective layer is produced and maintained by goblet cells, and its production is influenced by factors such as the environment, microbiota, and nutrition. To gain a better understanding of the functions of the mucus layer and the role of goblet cells in maintaining it, an extensive review was conducted. This review discusses the impact of age on development of goblet cells and their mucus production, in relation to the functional properties of the mucus layer and its protective mechanism in the chicken intestine. Moreover, this review highlights dietary factors affecting goblet cell proliferation and differentiation and the consequences of these effects on mucosal integrity and dynamic in poultry.

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REVIEW
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Development and Functional Properties of Intestinal Mucus Layer in Poultry

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Intestinal mucus plays important roles in protecting the epithelial surfaces against pathogens, supporting the colonization with commensal bacteria, maintaining an appropriate environment for digestion, as well as facilitating nutrient transport from the lumen to the underlying epithelium. The mucus layer in the poultry gut is produced and preserved by mucin-secreting goblet cells that rapidly develop and mature after hatch as a response to external stimuli including environmental factors, intestinal microbiota as well as dietary factors. The ontogenetic development of goblet cells affects the mucin composition and secretion, causing an alteration in the physicochemical properties of the mucus layer. The intestinal mucus prevents the invasion of pathogens to the epithelium by its antibacterial properties (e.g. β-defensin, lysozyme, avidin and IgA) and creates a physical barrier with the ability to protect the epithelium from pathogens. Mucosal barrier is the first line of innate defense in the gastrointestinal tract. This barrier has a selective permeability that allows small particles and nutrients passing through. The structural components and functional properties of mucins have been reviewed extensively in humans and rodents, but it seems to be neglected in poultry. This review discusses the impact of age on development of goblet cells and their mucus production with relevance for the functional characteristics of mucus layer and its protective mechanism in the chicken's intestine. Dietary factors directly and indirectly (through modification of the gut bacteria and their metabolic activities) affect goblet cell proliferation and differentiation and can be used to manipulate mucosal integrity and dynamic. However, the mode of action and mechanisms behind these effects need to be studied further. As mucins resist to digestion processes, the sloughed mucins can be utilized by bacteria in the lower part of the gut and are considered as endogenous loss of protein and energy to animal. Hydrothermal processing of poultry feed may reduce this loss by reduction in mucus shedding into the lumen. Given the significance of this loss and the lack of precise data, this matter needs to be carefully investigated in the future and the nutritional strategies reducing this loss have to be defined better.

Keywords: mucin, mucus layer, goblet cell, mucosal integrity, intestine, poultry

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INTRODUCTION

Intestinal mucus layer is the first line of defense protecting epithelium against luminal threats including mechanical forces during digestion process, enzymes and gut bacteria. The intestinal mucus also plays important roles in supporting the colonization with commensal bacteria, maintaining an appropriate environment for digestion and facilitating nutrient transport from the lumen to the underlying epithelium. The mucus layer is produced and preserved by mucin-secreting goblet cells. The present manuscript reviews the current state of knowledge about the ontogenetic development of goblet cells and the interactions between the intestinal mucus and gut microbiota as well as the mode of actions behind intestinal mucus functionality in poultry. Furthermore, it highlights dietary factors affecting goblet cell proliferation and differentiation and the consequences of these effects on mucosal integrity and dynamic in poultry.

GOBLET CELL DEVELOPMENT

Goblet cells (GC) are highly polarized columnar epithelial cells which contain secretory granules in the cytoplasm. GC secrete mucins which provide the mucosal surfaces with a thick mucus layer lining, and separate the intestinal epithelium from the luminal cavity. The mucus layer plays important roles in maintaining the intestinal microbial balance, facilitating nutrient transport, preventing pathogen invasion and regulating the microbial–host immune response (1). GC are differentiated from the transit-amplifying cells which are the transition cells between the stem cells and differentiated cells and are located in the crypts of the small and large intestine (**Figure 1**). The intestinal crypt is a harbor of stem cells and transit-amplifying cells which are committed to produce several cell lineages including GC and enterocytes. Maturation of the GC occur along with migrating toward the villus tip, where they are undergoing the apoptosis process or being damaged and shed into the lumen. Immature GC at the crypt base are large, pyramidal in shape, and contain mucin granules. During maturation, GC become a cup-like shape accumulating more mucin granules at the apical portion, whereas the nucleus and synthetic organelles reside at the basal portion. In chicken, the migration of GC along the villus-crypt axis occurs over a duration of 2-3 days (2).

The morphology of GC in chicken can be distinguished from enterocytes at around 16.5-18 days of embryonic age (2, 3). It has been shown that, the density of GC increased by 3.3 times in the duodenum from 18 to 21 days of embryonic age, whereas this number in the jejunum and ileum increased by 4.5 and 7.1 times respectively (4). However, Uni et al. (2) found no change in GC density of the duodenum during the last 3 days of incubation (2). The GC density is variable in the jejunum and ileum during the first week of age, while it is almost constant in the duodenum (2). A marked, 1.8-fold increase in the GC density was reported in the ileum during the first 4 days of age, while no significant change was observed in the duodenum (5). The GC density in the jejunum and ileum increased by approximately 1.5 and 1.8 times from day 4 to 7 of age (6). At the end of the first week post-hatch, different developmental rates of GC along the small intestine led to an anteroposterior increasing trend in its GC density, with the lowest density for the duodenum and the highest for the ileum (2, 6). The massive increase in the intestinal GC density and activity in the first week of age seems to be due to the emerging needs of newly hatched chickens for mucus secretion and immune response, associated with their immediate expose to the surrounding environment and diet. The host-related responses after hatch seem to provide enough functioning GC to maintain mucus thickness and protect the underlying epithelia from the introduced threats in the gut lumen (6, 7).

While the GC density in the jejunum and ileum are relatively high during the first week of age, it tends to decrease afterward

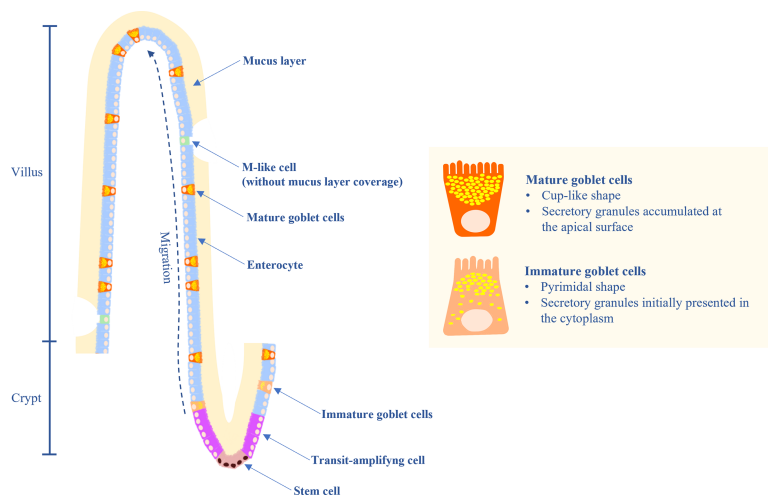

FIGURE 1 | Diagram of the small intestinal epithelium highlighting the characteristics of immature and mature goblet cells (GC), GC migration, and mucus layer.

until the third week of life (**Figure 2**) . The GC density tends to be stable between third and fifth week of age, with an average of 10.4 and 11.3 cells/100 µm of villus length in the jejunum and ileum, respectively (**Figure 2**). Calik and Ergün (8) also reported a stable GC density in the ileum of 21 and 42 days old chickens (14.9 *vs.* 13.9 cells/100 µm villus length). Therefore, although the cell renewal of GC in chickens has not been investigated yet, it can be speculated that the GC population in the small intestine may reach maturity at three weeks of age. This speculation can be supported by the outcome of several poultry studies showing an initial decline in the cell differentiation and migration rate through a decreased mitotic activity at day 21 of age compared with days 7 and 14 (18, 19).

The predominant changes in the GC density of the jejunum and ileum during the first week of age indicates that the age-related GC development rate is region specific. The proximal part of the small intestine including duodenum is very active in digestion and absorption processes. Although GC secretion provides moisturizing and lubricant properties for epithelial cells (20), the proximal part of the intestine may prioritize the proliferation of absorptive cells over secreting cells (21), which can be associated with a lower GC density, lower mucus secretion (22) and larger GC size (23) in the duodenum compared with the jejunum and ileum. The retention time is only a few minutes in the duodenum and up to 2 hours in the ileum (24). Therefore, the lower GC density and mucus secretion in the duodenum may enhance absorptive capacity, making the duodenum accommodate to the short digesta retention time. Furthermore, the number and activity of bacteria, along with the digesta retention time, increase distally in the small intestine. The bacteria and their products are recognized by the sensor system of the intestinal and immune cells leading to an activation of the host innate defense system (25), which in turns stimulate GC differentiation *via* cellular signaling or secreted cytokines such as IL-1β, IL-4, IL-13 and L-22 (1). Thus, the anteroposterior increasing trend in the GC density and mucus secretion (in the small intestine) can be assumed as a host adaptation to enhance protective barrier against the increasing number (and activity) of gut bacteria along the small intestine (25).

MUCIN SECRETION

The primary function of GC is to secrete mucins and create a protective mucus layer. Mucins are the major components in the cytoplasmic secretory granules of GC. Other proteins were also found in the GC secretion including IgA, avidin and lysozyme as well as other secretory components that play major roles in innate immunity of chicken (26). The secretion of GC is suggested to be regulated by two pathways; i) constitutive secretory pathway, and ii) regulated secretory pathway (27). The constitutive secretory pathway is a low-level continuous secretion to maintain the renewal of the intestinal mucus layer. In this baseline secretion, mucin glycoproteins are assembled and stored in membrane-bound granules which are stored within a highly organized array of microtubules and intermediate filaments called theca. The theca separates mucin granules from the rest of the cytoplasm and gives GC goblet cells a large cup-like shape (28). The constitutive secretion is dependent on cytoskeletal movement (e.g. the theca) that moves secretory granules toward the cell surface (29). This steady and unstimulated release results in maintaining the

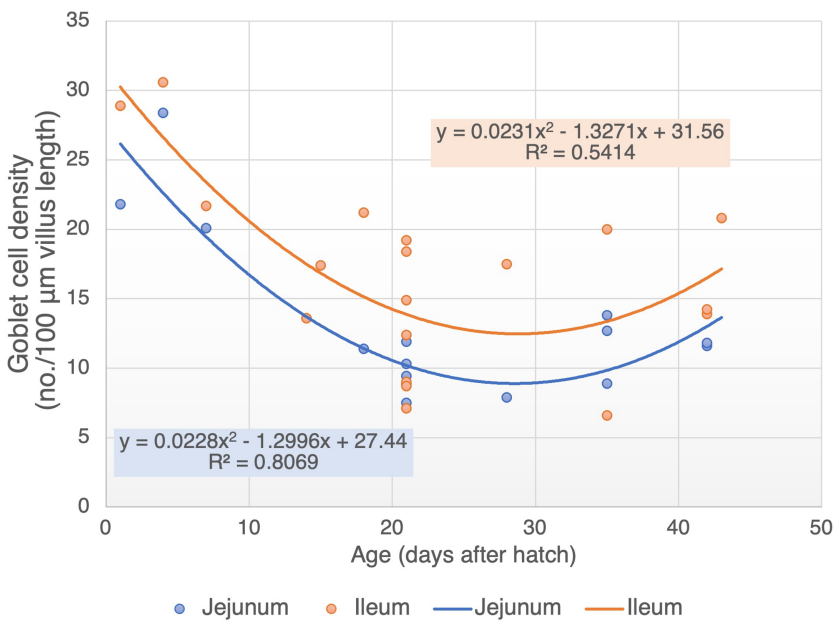

FIGURE 2 | Goblet cell density in jejunum and ileum of broiler during age development (cell number per 100 µm villus length). Adapted data from references (6, 8–17).

mucus layer (1). The regulatory pathway is an exocytosis of GC responding to external stimuli such as neurotransmitters (e.g. acetylcholine), cytokines, bacteria and their products including lipopolysaccharides (27). Acetylcholine, a primary parasympathetic neurotransmitter, plays a role in GC degranulation and can induce mucin secretion (30). Stimulation with acetylcholine or other cholinergic agonists such as carbachol resulted in a rapid transient increase in mucus secretion rates in the small and large intestine of mouse (31). Cytokine secretions by immune cells have been reported to stimulate GC proliferation and mucus production. For example, the secretion of IL-13 by dendritic cells and macrophages, IFN-γ by the activation of Th1 pathway and IL-4, IL-5, IL-9, and IL-13 by T helper 2 have been shown to stimulate GC proliferation and mucus production (1). The presence of bacteria which disrupts mucosal surface, has been also reported to stimulate a rapid release of stored mucin granules (32). The absence of gut bacteria in germ-free chickens led to a reduction in GC number and density as well as MUC2 mRNA expression in the small intestine compared with conventional birds. These observations confirm the stimulating impact of gut bacteria on the mucin development and secretion (33). Immediate bulk release of mucins (triggered by the regulatory pathways) captures the pathogens mechanically and inhibits them chemically with antibacterial peptides/proteins (secreted by GC and other epithelial cells), while the continuous basal secretion maintains the mucus layer during an absence of luminal or physiological stimuli.

GC in chicken has some functionalities similar to Paneth cells in mammals. In mammals, Paneth cells are restricted to crypts of the small intestine and secrete substances like lysozyme, IgA, and defensins which protect host from enteric pathogens. Among these substances, lysozyme is wildly considered as a marker for Paneth cells (34). To date, presence and location of Paneth cells in the small intestine of chickens have remained controversial. An *in situ* hybridization analysis showed that lysozyme-positive cells were specifically located at the bottom of crypts in the small intestinal of 6-month-old chickens. These detected cells also showed morphological similarities to Paneth cells in mammal (34). However, in the small intestine of 17 days old chickens, lysozyme-positive cells were only observed in the villi epithelium and were absent in the crypts (35). In a study on the duodenum of chickens, it has been shown that lysozyme-positive cells are not only found in crypts, but can also be detected along villi. It was suggested that lysozyme-containing cells located in the small intestine villi can be either GC, goblet cells, Paneth cells, or lysozyme-positive enterocytes (26).

Mucins are synthesized and readily secreted by GC at the crypt, while their compositions and secretion rate change along with cell migration. During migration of GC from the crypt, the mucin secretion and renewal rate increase (22) and the oligosaccharide chains in the mucin glycans are elongated by the addition of monosaccharides (36). The elongation of mucin glycans and higher secretion of mucin may indicate the maturation of GC along the migration toward the villus tip. In a mice study, it has been shown that, the duration of mucin synthesis is around 3-4 hours in the crypts and less than 3 hours in the villi (22). A faster mucin production and secretion by GC at the villi compared with crypts may be a physiological response to facilitate luminal mobility and digestion as well as protect the villi surface against mechanical erosion and microbial invasion (22), while the preserved mucins at the crypt can provide a further protection by a massive release in case of gut inflammation and infection (37).

A well-developed mucus layer in the gut is important for an active immunity system. Beside mucin secretion, the gut GC also participate in the immune responses by secretion of various substances acting as antibacterial agents. GC in the duodenum and cecum of broilers were shown to store avidin, lysozyme and other secretory components (26). Avidin was found to be an acute phase protein which is expressed in the intestine during gut injury and inflammation (38) and involves in restoration of a damaged intestinal tissue (39). Secretory components like cleaved fragments of pIgR, have neutralizing properties against pathogen-associated molecules and act as antibacterial substances (26). Lysozyme plays an important role in activating innate immunity and recruiting of leukocytes (26). Immune protection of the gut in early life stage depends on provision of maternal antibodies including IgA, IgG and IgM which can be delivered *via* colostrum and milk in most of mammals (40). However, industrial avian species have no direct contact with parents after the egg is laid; hence, the only source to supply maternal antibody is the egg itself. Maternally derived IgA was found in the GC and epithelial apical surface of newly hatched chickens. Maternally derived IgY (the avian counterpart to mammalian IgG) was observed in the intestinal vessels at the day of hatch. Both Ig appeared later (7-28 days of age) in the plasma cells located in the lamina propria of the small intestine (41). The GC seems to act as a reservoir for maternal IgA antibodies prior to hatch which are slowly secreted along with mucin, thus extend the protection until maturation of the endogenous IgA response (41). During the first week after hatch the maternal IgA in chickens decreases gradually and the maturing antibody secreting cells subsequently take over the immunological protection (7).

MUCIN CHARACTERISTICS AND GOBLET CELLS CATEGORIZATION

Structurally, mucins are large glycoproteins characterized by heavily *O*-glycosylated polypeptides that usually composed of tandem repeats rich in proline, threonine and/or serine sequence (PTS domain). The hydroxyl group of threonine and serine is necessary for ester linkages between the amino acid backbone and carbohydrate groups (42). A dense array of *O*-linked carbohydrates in mucins confers charge to them, giving them the ability to interact with other surrounding molecules such as nutrients and regulate the diffusion of nutrients within the mucus layer. Mucin *O*-glycans account for over 80% of the total mucin molecule by mass and support rigidity of the mucin structure, contributing to specific physical and biological properties essential for their protective functions

(43). For instance, the chicken MUC2 mucin comprises 3,697 amino acids (44) and >100 different *O*glycan structures linked to the mucin protein core (45). Mucin *O*-glycans typically contain several types of sugars (**Figure 3**) including N-acetylglucosamine (GluNac), N- acetylgalactosamine (GalNac), galactose, sialic acid, fucose and mannose (46–48). The glycan biosynthesis begins by the transfer of one sugar to the protein, followed by the addition of more monosaccharides one by one (32). In chicken, *O*-glycans are predominantly composed of GlcNAc, galactose and GalNAc at approximately 37.0, 27.4 and 13.4%, respectively, while the remaining includes sialic acid, fucose and mannose (46). Further structural diversity is obtained through modifications of the saccharides e.g. phosphorylation, sulfation, and acetylation (32). The most common terminal end of glycans in the small and large intestine of chicken contains sulfated and sialylated groups which account for the polyanionic or negative charges of mucins (49). The complexity of mucin carbohydrate structures is regulated by glycosylation within the Golgi apparatus of the GC. The pattern of monosaccharide sequence in mucins varies between individual GC, intestinal location and species (32).

Depending on the ionic charge of the mucin molecule, mucins can be differentiated (**Table 1**) as neutral or acidic (50). The *O*-glycans containing terminal residues of sialic acid and sulfated group represent anionic charge, while other terminal groups such as fucose, hexose and galactose result in neutral charge. The negatively charged mucins are likely to resist mucin degradation by bacterial enzymes therefore; mucin charge is an important factor in determining host defense with particular regard to interactions with microorganisms present in the gut. It is noteworthy that the *O*-glycosylation occurring in the GC is usually very heterogeneous and not only a single type of mucin is produced in an individual GC (50). Based on the sum of ionic charge of secreted mucins, GC can also be categorized into two types; 1) neutral GC predominated by neutral charged mucins and 2) acidic GC predominated by anionic charged mucins (**Table 1**). By using common microscopic observations in histological studies, staining of secretory granules in GC represents a color as the result of a chemical reaction between dyes and the terminal groups of mucins. The anionic mucins can be detected by cationic dye including Alcian blue (AB) and mucicarmine resulting in blue and red in color, respectively (51). In contrast, the neutral mucins can be recognized by the reaction between Periodic acid -Schiff (PAS) reagent and aldehyde groups of the monosaccharide units, resulting in magenta color (51). GC containing almost similar amount of acidic and neutral mucins appear in bluish purple color and are called mixed GC (52). In chicken, the mixed GC are commonly observed along the small intestine (53, 54). Most of the studies conducted on chicken have used both AB and PAS solutions for mucin determination in the gut tissue, individually or together (combined staining method). The individual staining method uses only one solution at the time for each tissue sample (determining acidic and neutral GC in separate tissue samples), while the combined staining method

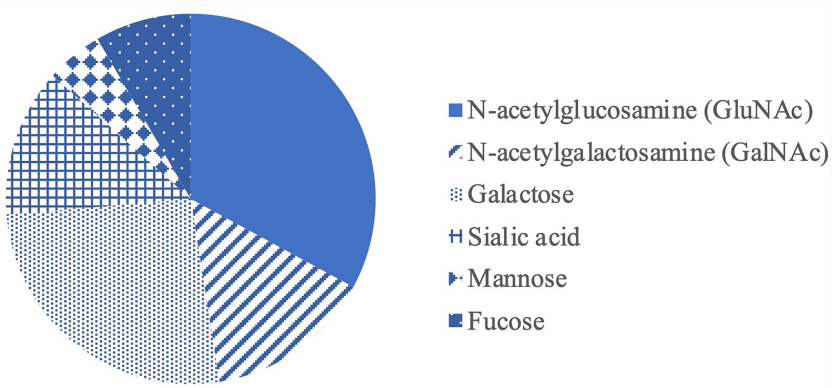

FIGURE 3 | Proportion of mucin monosaccharides in the small intestine of broiler (46, 47).

TABLE 1 | Characteristics of neutral and acidic goblet cells [a].

	Neutral goblet cells	Acidic goblet cells
Mucin structure	Large glycoproteins containing extensive amounts of oligosaccharide chains attached to protein core	
Mucin *O*-glycan composition	Heterogenous arrangement of monosaccharides including GluNAc, GalNAc, galactose, fucose, sialic acid and mannose	
Terminal end of *O*-glycan	Predominant in neutral charged monosaccharides e.g. GluNAc, GalNAc, galactose, fucose and mannose	Predominant in negatively charged monosaccharides e.g. sialic acid and/or sulfated groups
The overall charge of mucins	Neutral charge	Negative charge

[a]*references (49–52).*

uses both solutions in one process (determining acidic and neutral GC in one tissue sample). Given the fact that one GC can produce more than one type of mucin (2), overestimation of acidic and neutral GC number may be more likely to happen by using the individual staining method. Therefore, the histochemical technique of "combined staining method" may be more accurate to define the GC types.

Mucins are also broadly grouped into transmembrane and secretory mucins based on their biosynthesis and secretion. The secretory mucins are initially formed by mucin polymers and packed into secretory granules prior secretion (55). Upon mucin granule exocytosis, the secretory mucins are hydrated and expanded massively, forming a gel-like structure and creating a mucus layer over the intestinal epithelium. The transmembrane mucins are characterized by a single transmembrane protein incorporated into the plasma membrane, a cytoplasmic tail (signaling domain) and the extracellular part of a highly glycosylated mucin domain. These mucins are retained at the apical cell surface of GC (55). The transmembrane mucins do not form gel-like structure but rather serve as anchors for the secretory mucins. However, the strict classification of both mucin types is complicated by their dual occurrence of membrane attachment and detachment; the transmembrane mucins can be forced to detach from the cell surface by mechanical stimuli (56), while the secretory mucins temporarily attached to the apical surface of GC prior the cleavage by proteolytic enzymes (1). Proteolytic enzymes such as Meprin β are likely to cleavage the specific site at the anchor point between secretory mucins and cell membrane. This cleavage is found to be activated through bacterial contact or microbial signaling to the enterocytes, indicating that the presence of gut bacteria may be a key mechanism for secretory mucin detachment and release into the intestinal lumen (57). It has been observed that Meprin β was deficient in the small intestine of germ free mice resulting in less viscous mucus layer, thus more adhesive and attached mucus to the cell membrane (57).

Mucins are encoded by mucin genes, represented as MUC followed by a number that reflects the order in which the particular mucin gene was discovered. A mucin gene is translated into a protein core which is further decorated with extensive glycosylation. The discovered MUC genes in chicken are still limited compared with human (**Table 2**). Different types of mucin are present throughout the gastrointestinal tract in specific locations. Each type plays a different role in maintaining homeostasis and protecting intestinal epithelium in different parts of the gut. In chicken, the MUC2 is the major component of mucus in the small and large intestine but it is weakly expressed in the crop (44), whereas MUC5ac is specifically expressed in the proventriculus and only weakly expressed in the small intestine (6, 45). The composition and sequence of amino acids derived by specific MUC genes have a role in dimerization through covalent and noncovalent cross-links and their subsequent polymerization to form multimers (61, 62). The structure conformation of different MUC type mucins (such as MUC2 *vs.* MUC5ac) was found to differ in cross-linking between mucin dimers, which may determine their functionality in regard to mucus permeability (61) and viscosity (62) in different parts of the gastrointestinal tract. The tissue specific expression of MUC genes seems to be depending on the physicochemical properties of the mucins and their required functionality in maintaining mucosal integrity in that specific part of the gut.

DEVELOPMENT OF ACIDIC AND NEUTRAL MUCINS

The physiological relevance of distinct mucin types is not yet well understood but it has been suggested to be associated with their protective properties. The distribution of mucin types in the GC is regulated by glycosylation process, which can be affected by host (e.g. inflammatory markers, hormones and neurotransmitters) and external (e.g. commensal bacteria, pathogens, pre/probiotics and nutrients in the diet) factors (20). It is known that many bacteria in the gut produce glycosidases or proteases to degrade mucus. The terminal ends of *O*-glycan with *O*-acetylated sialic acid (sialomucins) or

TABLE 2 | Mucin genes expressed in the small and large intestine of human and chicken.

Mucin types	Human [a]		Chicken [b]	
	Small intestine	Large intestine	Small intestine	Large intestine
Transmembrane mucins	MUC1	MUC1	MUC4	n/a
	MUC3	MUC3	MUC13	
	MUC4	MUC4		
	MUC12	MUC12		
	MUC13	MUC13		
	MUC15	MUC15		
	MUC16	MUC17		
	MUC17			
Secretory mucins	MUC2	MUC2	MUC2	MUC2
	MUC5ac	MUC5b	MUC5ac	MUC6
	MUC6			

n/a, no available data.
[a]*references (56, 58).*
[b]*references (59, 60).*

sulfated group (sulfomucins) have been found to play a key role to protect mucin chains from degradation by bacterial enzymes (like proteases and glycosidases) and proteolytic host enzymes (52, 63). Previous studies on the chicken's intestinal mucosal layer and mucin subtypes indicate that during late embryonic development, the produced mucins are more of the acidic subtype than neutral subtype (2, 4, 64). It seems that acidic mucins production prevails before hatch and then neutral mucins are more produced after hatch (6). In newly hatched chicks, the density of acidic and neutral GC was increased during the first week of age in the small intestine, especially in the jejunum and ileum. This increase was suggested to be due to the bacterial colonization in the gut and presence of dietary components (6, 7). The dominance of acidic mucin production by GC right before hatch could be a host adaptation to proteolytic host enzymes (to use yolk sac) and prepare the chicken for exposure to bacterial proteases and glycosidases right after hatch (2).

The development of sialomucins and sulfomucins from late embryonic stage to post-hatch is shown in the **Table 3**. Sulfomucins appear initially as early as 18 days of embryonic age (4), while mucins containing sialic acids appear later at 21 days of embryonic age and considerably increase after hatch, especially during the first week of age (4, 6). The high amount of sulfomucins presented at embryonic stage may be due to the immaturity of GC (because of the low number and activity of gut bacteria) (6). No exposure of hatching eggs to the caecal content of the layers caused a reduction in the number of both neutral and acidic GC with the absence of sialomucins during the first 7 days of age, compared with the chickens hatched from conventional eggs (33). The presence of intestinal bacteria could induce GC maturation and increase mucus production which could associate with increasing amount of sialomucins after hatch (6). It was found that GC initially contain higher amount of sulfomucins and lower amount sialomucins, but as they mature and migrate toward the villus tip, the mucins are increasingly sialylated (63). This can indicate the importance of bacterial exposure for GC differentiation and maturation (33).

MUCUS LAYER AND ITS THICKNESS

The thickness of mucus layer is a result of dynamic balance between secretion rate by GC and destruction rate through mechanical shear and enzymatic degradation (66). During a normal physiological condition, the mucus thickness is determined by the basal secretion which involves the continuous production and release of mucins into the gut lumen as previously discussed. Two methods have been applied to measure mucus thickness from the basal secretion. One is by using cryostat-sectioned tissue stained with histochemical staining (CRHS) and the other is by using anesthetized animal (*in vivo)*. The CRHS method is a simple method that can be used for a wide range of tissue types and can show the characterization of normal mucus layer. The *in vivo* method provides information about the dynamic of mucus secretion under a real physiological regulation (67).

By microscopic observation, it has been shown that the intestinal mucus consists of 2 layers; i) a thin inner layer which is strictly attached to the epithelial membrane ii) a thick outer layer which is loose, non-attached and forming viscous gel between the lumen and the thin inner layer (68). It can be expected that the inner layer contains transmembrane mucins because it was hardly removed by mucolytic agents (69), while the outer layer may contain secretory mucins which form a viscous mucus layer (1). In human and rodent, mucus in the small and large intestine have both layers, while the inner layer of the small intestine is thinner than the large intestine. However, several studies in both species showed that the inner layer was absent in the small intestine, especially in the duodenum and jejunum (56, 70). It seems that generally, the thickness of mucus is higher in the distal part (caecum > ileum > duodenum and

TABLE 3 | Percentage of acidic goblet cell number in the jejunum and ileum of broiler at different ages.

Intestinal part	Age [a]	Percentage of acidic goblet cell number [b]			Reference
		Sialomucin	Sulfomucin	Intermediate	
Jejunum	E18	–	100%	–	(4)
	E21	20%	56%	24%	(4)
	D1	–	100%	–	(6)
	D4	31%	38%	38%	(6)
	D7	18%	41%	41%	(6)
	D18	34%	49%	17%	(65)
	D18	30%	54%	16%	(10)
Ileum	E18	–	100%	–	(4)
	E21	37%	18%	45%	(4)
	D1	–	100%	–	(6)
	D4	28%	39%	39%	(6)
	D7	28%	39%	33%	(6)
	D18	33%	47%	20%	(10)

[a]*Age was reported as days of embryo age (E) or chicken age (D).*

[b]*Acidic goblet cell was determined by the combined staining Alcian Blue/High Iron Diamine (AB/HID) method. AB-positive goblet cells (blue) are categorized as sialomucin, HID-positive goblet cells (brown) are categorized as sulfomucin, and goblet cells that are positive to both AB and HID stains (brown-black color) are called intermediate. The percentage of each type is relative to the total number of acidic goblet cells (the sum of sialomucin, sulfomucin and intermediate).*

jejunum) of the intestine (68), which may be explained by the digestive and protective functions of mucus and the fact that, gut bacterial number and activity increase from the proximal part of the small intestine to the distal part of the gut. Few studies measured the mucus thickness in chicken by the histological method. The average of mucus thickness in 42 days old broilers ranged from 14.9 μm in the duodenum to 18.6 μm in the ileum (46, 47). In a CRHS study using anesthetized rats, the basal mucus secretion rate was the highest in the colon (3.9-5.2 μm/min), while the secretion in the small intestine ranged from 1.9 to 4.7 μm/min, with the highest rate in the ileum compared with duodenum and jejunum (68). Furthermore, it has been shown that by removing mucus layer with a suction probe, the inner mucus layer remained attached to the mucosal surface, while the outer layer in all parts of the gut was easily removed by suction collection. The mucus secretion was immediately stimulated after the mucus suction, with a lower secretion rate in the small intestine and a higher rate in the colon compared with those prior mucus removal (68). Thus, the mucus layer at the lower part of the gut seems to be better maintained resulting in a thicker mucus layer covering the epithelial surface of the colon.

NUTRIENT TRANSPORT THROUGH MUCUS LAYER

The intestinal mucus must provide a robust barrier that traps and immobilizes potentially hazardous compounds such as pathogens, while allowing the passage of nutrients to the epithelial surfaces. These properties are particularly important in the small intestine, where the mucus layer is the thinnest in the gut and the nutrients absorption needs to be highest. A thinner mucus layer in the small intestine could facilitate nutrient absorption, whereas a thicker mucus layer in the colon must be a barrier to the dense bacterial population (71). Since more than 90% of the total nutrients absorption including carbohydrates, proteins and lipids occur in the small intestine (72), the mucus properties involved in nutrient diffusion are of interest. It has been reviewed by Leal et al. (72) that physiochemical properties of mucus like pore size, viscoelasticity, pH, ionic strength, and net charge of mucus layer and mucin polymers can alter the transportation of molecules (**Table 4**) (72). They suggested that these factors regulate permeability of mucus layer which not only restrict the diffusion of bacteria and macromolecules but selectively, allow absorption of nutrients (72).

The net-like structure of mucus layer creates pores which allow only small molecules from the lumen pass through the mucus layer and restrict the flow of large molecules including polysaccharides and polypeptides (1). Limited studies evaluated the pore size of the intestinal mucus but it is known that the mucin network expands 2-3 times in volume when moving from the inner layer to the outer one (1). Several studies reported that particle size ranged from 0.5-2 μm in diameter could diffuse through the outer mucus layer of the jejunum (73) and ileum (70), while the inner mucus layer are sufficiently small (< 0.5 μm diameter) to hinder penetration of bacteria or beads (1).

The viscosity of mucus layer is attributed to the capacity of mucin monomers to form polymeric structures. Only the secretory mucins are properly assembled into a disulfide-bridged covalent network, giving mucus its viscous properties, while transmembrane mucins are monomers that are integrated into membranes and do not form viscous gels. The viscosity of the mucus ranged from 1 to 30,000 millipascal second along the villus surface of the pig's small intestine, with a numerically higher mean viscosity at the inter-villus space (the space between the villi) compared with the villus tip (73). In general, a low viscous mucus provides a higher permeability for diffusing molecules (74), thus the diverse viscosity at different part of the villi may indicate the preferential area for nutrient diffusion. However, diffusion of particles through mucus layer was greater at the inter-villus space compared with villus tip due to a hindrance of the apoptotic cells that shed into the mucus (73).

Different components in the mucus such as water, mucins, globular proteins, salts, DNA, lipids, cells and cellular debris are stabilized by covalent and noncovalent interactions including hydrophobic, electrostatic and hydrogen bonds (72). These binding interactions are the main factors that contribute to viscoelasticity and permeability of a mucus layer (72). Generally, charged groups of the mucins can interact with charged particles and immobilize them through the mucus (74). Peptides which contain both basic and acidic amino acids have both positive and negative charges simultaneously. It was found that when positive and negative charges were both present on a peptide, the diffusion through gastric mucins was higher than the isolated charged peptides (75). The interaction between charged particles and mucins also rely upon the intestine's pH as well as ionic strength of the intestinal mucus (74). Lowering the mucus pH altered mucus conformation by promoting the exposure of hydrophobic domains of the mucins, decreasing repulsive forces between mucins and increasing mucus viscosity (72). Thus, a reduced electrostatic interaction between mucins

TABLE 4 | Physiochemical characteristics of mucus layer affect nutrient transportation [a].

Characteristics	Impact on nutrient transportation
Pore size	Size-filtering property
Viscoelasticity	Lower mucus viscosity provides a higher permeability for diffusing molecules
pH	Higher pH increases electrostatic interaction between mucins which enhances selective permeability of charged particles
Ionic strength	Higher ion concentration enhances permeability of positively charged molecules
Net charge	Attractive or repulsive forces between diffusing molecules and the mucus

[a]*references (72–76).*

enhances selective permeability against charged particles compared with neutral particles (72). In porcine gastric mucins with pH adjustment to 3, the positive and negative charged polyethylene glycols (PEG) were less mobile leading to low diffusion, while both charged and neutral particles diffused almost freely in mucus at pH 7 (74). The pH limitation for nutrient diffusion through mucus layer may be lesser in the distal part of the small intestine compared with the proximal part because of the fact that in chicken, pH increases from the duodenum (5.0-6.0) to the jejunum (6.5-7.0) and ileum (7.0-7.5) (76).

Changes in ionic strength cause shrinkage or swelling of mucus and, thus, significantly alter mucus viscoelasticity (77). The strength of the attractive or repulsive forces between mucin molecules depends on the ion content in the mucus layer including sodium, chloride, potassium and calcium ions (74, 78). In general, increases in ion concentration correlate with a decrease in the viscosity of mucus (77). The investigation of ionic strength in the intestinal mucus layer of chicken has not been yet explored. Using porcine gastric mucus (*ex vivo*), it was demonstrated that the mobility of positively charged particles was considerably increased at high ionic strength (500 mM NaCl, pH 3) compared with low ionic strength conditions (20 mM NaCl, pH 3), while neutral particles diffusivity remained unaffected by changes in the ionic strength (74). Similarly, increasing the ionic strength (5-200 mM NaCl, pH 7) of porcine gastric mucus accelerated the transport rate of cationic peptides (lysine residue), while the anionic peptides (glutamic acid residue) maintained a high diffusion at various ionic strengths (75). Therefore, it can be speculated that that high ionic strength of mucus layer in a neutral pH condition, which usually occurs in the lower part of the small intestine (compared with the proximal part of the gut), may lead to a higher permeability of positively charged molecules in this part of the gut.

PROTECTIVE MUCUS LAYER AGAINST GUT BACTERIA

The intestinal mucus layer provides a protective shield for epithelium against gut microbiota which begins to colonize within an hour after hatch (79). The bacterial colonization was initially observed in the cecum, possibly because of yolk sac utilization and absorption effect, which was dominated by facultative aerobes such as *Enterobacteriaceae* and *Streptococcus* spp. and then spread throughout the gastrointestinal tract within 24 hours (79, 80). It has been reported that the bacterial concentration increases distally along the small intestine due to increasing luminal pH and retention time in the distal ileum compared with the duodenum (79). Approximately one third of the commensal bacteria is comprised of genes involved in carbohydrate digestion and many bacteria have specialized genes for degrading different type of complex carbohydrates such as non-starch polysaccharides (81). In a normal condition, gut bacteria locate only in the outer mucus layer where they can degrade mucin glycans or proteins and utilize them as energy source for colonization, while the inner layer is relatively impermeable for bacteria (82). However, when the mucosal barrier function is disrupted, the mucus becomes more permeable and a higher number of bacteria can be found in the inner layers (82). A more invasive bacteria including pathogens may extensively degrade mucins and compete with the gut microflora for mucin-derived nutrients, establishing their colonization and epithelial attack.

The homeostasis of gut bacteria in chicken can be affected by mucin (MUC) types, *O*-glycan composition (extent of glycosylation and oligomerization of mucin), and the mucus layers characteristics (inner and outer mucus layer thickness) (50, 83). Mucin types and *O*-glycan composition affect physicochemical properties of mucins and the effectiveness of bacteria for reaching epithelial cells by degradation of mucins (50). Several mechanisms for the intestinal mucus were reported which prevent the invasion of pathogens, while maintaining a homeostatic microbial population. The continuous secretion of mucus pushes the pathogens away from the enterocytes and flushes them out distally with peristaltic moves (73). Moreover, antibacterial peptides and proteins within the mucus prevent a direct access of bacteria to the epithelial surface. In chickens, antibacterial compounds in GC secretion including β-defensin, lysozyme, avidin, IgA, and free secretory component (a glycoprotein that binds and transports the secretory immunoglobulins) were found as responses to both gram positive and negative bacteria (26). The continuous secretion of these peptides and proteins creates an antibacterial gradient within the mucus layer with an increasing antibacterial activity from the lumen to the cell surface, creating stricter protection at a closer area to the epithelium (56). Due to a shielding or charge repulsive effect of the anionic glycans, the interaction between mucins and pathogens could also slow down their penetration. A high abundance of sulfated and sialylated mucins could reduce the adhesion and penetration ability of *Campylobacter jejuni* through intestinal mucus of chicken and protects the mucins from degradation by bacterial glycosidases (84). The protective properties of mucus may also rely on mucin subtypes (e.g. MUC2 and MUC5ac) through the interaction between protein domains which as discussed, can determine the permeability of the mucus layer (25, 61).

Although the small intestine contains lower bacterial population than the lower gut, it may be a better target for pathogenic bacteria due to its thin and patchy distributed mucus layer. Furthermore, a particular area in the small intestine lacks of mucus coverage (**Figure 1**). This area composes of M-like cells overlying on the lymphoid tissues of the digestive tract and bursa of Fabricius (32). The M-like cells act as sentinel cells which transport endocytosed microorganisms and other antigenic substances into the underlying lymphoid structures and initiates immune response. However, some bacteria including *Salmonella* Typhimurium, *Shigella flexneri, Yersinia enterocolitica* and *Vibrio cholerae* take advantage of the low protective barrier of this area and invade the epithelial cells (32).

Intestinal mucus not only serves as a protective layer but also accommodates the colonization of bacteria by providing i) ligands for bacterial adhesion, and ii) nutrient sources for selective bacterial community that contains mucin degrading enzymes or receives degraded mucin saccharides from the others (83). The colonization ability of bacteria depends on the bacterial attachment, bacterial enzymes for mucin degradation and utilization capacity of them for mucin-derived carbohydrate. These mechanisms were extensively reviewed by Sicard et al. (83). The bacterial adhesion to mucins is believed to initiate the colonization process which involves one or more mechanisms including Van der Waals forces, electrostatic interaction, and hydrophobic forces (85). Bacteria frequently use different strategies like cell-surface proteins, pili, fimbriae and flagella to bind to mucins (83). Some commensal bacteria including lactic acid bacteria occupy mucus binding proteins and pili to adhere to mucin oligosaccharide (e.g. mannose), while other adhesion strategies were observed for pathogenic bacteria. For example, *C. jejuni* uses the carbohydrate-lectin, flagella subunit proteins and major outer membrane proteins to adhere to mucins (83).

Mucolytic bacteria possess specific enzymatic activity necessary to degrade glycan chains and facilitate their colonization. Their enzymes include neuraminidases/sialidases, fucosidases, exo- and endo-β-N-acetylglucosaminidases, β-galactosidases, α-N-acetylglucosaminidase, and α-N-acetylgalactosaminidases (86). The members of mucolytic bacteria are groups from both commensal (such as *Bifidobacterium bifidum, Bacteroides fragilis, Akkermansia muciniphila)* and pathogenic bacteria (such as *Clostridium perfringens, Salmonella* Typhimurium*, Vibrio cholerae, Enterococcus faecalis).* These bacteria compete for mucin-derived nutrients (86). Some of the intestinal pathogens have developed strategies to win this competition with commensal microflora for nutrients. It has been found that *Clostridium perfringens* have developed an ability to secrete a wide range of glycosidases with broader substrate specificity (87). *Clostridium perfringens* has shown a wide range of enzymatic activity in cleaving the terminal residues (exoglycosidases) and inner parts of sugar chains (endoglycosidases) including α-L-fucosidases, endo-α-GalNAcase and sulfatases, which promote adherence to the mucin carbohydrate receptors and mucin degradation (43). Therefore, a broader range of substrates is available for *Clostridium perfringens* which increases their chance to win the competition and cause necrotic enteritis in poultry. Another set of gut bacteria without mucolytic activity can also take advantage of released saccharides by scavenging the liberated sugars, leading to an increased in their colonization in the gut. For example, the presence of *B. thetaiotaomicron* or *B. fragilis* that produce sialidases enabled the expansion of sialic acid-scavenging bacteria including *E.coli* (88).

It has been shown that the common modification of terminal glycans of human mucins including sulfation and sialylation, increases resistance to microbial glycosidases and thus, the protective barrier is thought to remain more intact (6, 86). The 5increase in anionic (sialytated, sulfated and sialytated-sulfated) glycans was also reported in chicken and other avian species as a strategy to provide a charge-repulsion effect between mucins and maintain relatively low pH of mucins, aiming to create strong protection for mucins against bacterial degradation (84). In chicken, the *O*-glycans were abundantly sulfated and sialylated, with 33% and 23% in the jejunum and 34% and 29% in the cecum, respectively, while the remaining was neutral glycans (40% and 26%) and sulfo-sialylated intermediate (4% and 11%) in the respective locations (84). However, some bacteria possess strong sialidase and/or sulfatase activity. For example, *Ruminococcus gnavus* and *Akkermansia muciniphila* have sialidase activity, while *Prevotella* spp., *Bifidobacterium* spp. and *Helicobacter pylori* have sulfatase activity (86). Some bacteria produce both enzymes such as *Bacteroides fragilis, B. thetaiotaomicron* and *Bifidobacterium bifidum* (86, 88). Among bacteria with sialidase activity, the released sialic acid can be only utilized by some groups like *R. gnavus* and *B. fragilis* since they are the ones encoding specific genes responsible for sialic acid metabolism. On the other hand, *Salmonella* Typhimurium and *Clostridioides difficile* are able to utilize sialic acid but lack the sialidase enzyme, and thus, they rely on other sialidase-producing organisms to acquire this potential nutrient source (86, 88).

ENDOGENOUS LOSS OF MUCUS LAYER

Mucus degradation generally occurs due to physical disruption by mechanical shear forces of peristalsis and enzymatic cleavage by bacteria, after which the mucus is transported with the intestinal content and excreted (20). Broilers have a short intestinal retention time to support a high feed intake despite the limitations of the digestive tract volume. The average retention time in broilers is 2.9 and 5.7 hours for the small intestine and total tract, respectively (24). Furthermore, chicken has a unique mechanism of intestinal reflux that propel liquid material from the proximal ileum or cloaca ascendingly to as far as the duodenum and gizzard in order to enhance digestibility of major nutrients such as starch (89). The high passage rate and frequent intestinal movement in chicken may contribute more to loss of the mucus layer compared with other species. It is noteworthy that the outer mucus layer is more loose and prone to be propelled with digesta transportation compared with the inner layer (68). Hydrothermally processed diets such as pelleted, extruded and expanded, feed may provide low mechanical shear force due to the rapid disintegration once moistened (90). Moreover, these forms of feed have higher starch digestibility compared with the non-processed one (91), reducing the need for frequent intestinal reflux to enhance digestibility of starch. It has been shown that pelleted feed increased villus height, decreased crypt depth and GC density in the small intestine of broilers compared with mash feed (92). It can be assumed that higher GC density in broilers fed mash feed is an adaptive response to increase mucin secretion and replace the part of the mucins which has been lost due to enzymatic hydrolysis, mechanical shear forces or intestinal refluxes. Thus, hydrothermal processing of poultry feed may reduce mucus shedding into the lumen.

To our best knowledge, the direct effect of particle size (fine/coarse) on mucins secretion in poultry has not been investigated yet. The coarse and fine feed are usually characterized by discrete mean particle size (dMEAN) based on dry sieving analysis; dMEAN above and below 1.8 mm are defined as coarse and fine particle sizes, respectively (93). It is generally believed that fine particles increased the accessibility of digestive enzymes to the substrate and enhance nutrient digestibility (90). However, some studies have shown that reduction in particle size of cereals (e.g. corn) did not affect ileal digestibility of amino acids that are accounted for more than 50% of the amino acid content of each type of mucin i.e. glutamic acid, aspartic acid, proline, threonine and serine (93) as well as crude protein (94, 95). Other studies showed a lower ileal digestibility of crude protein in fine corn compared with coarse corn (96, 97). It was suggested that finely ground particles may cause gut functional impairment due to a faster passage rate (98), while coarse particles reduce passage rate and enhance gizzard activity which may subsequently stimulate more bile acid and pancreatic secretion and also improve digestibility of nutrients like starch (91, 99). The dietary replacement of fine corn (2.4 mm) with coarse corn (7.16 mm) by 25% or 50% in pelleted feed increased digesta retention time, gizzard weight, and apparent ileal digestibility of energy and nitrogen, while the digesta pH seemed to be decreased in the proventriculus (100). The lower pH of the digesta entering duodenum may enhance mucus degradation by enzymatic hydrolysis (101) and may increase mucus secretion in response to prevent epithelial damage (102). In terms of nutrients absorption, the lower pH of the digesta entering duodenum can increase mucosal viscosity which as discussed, subsequently may cause lower permeability for certain nutrients. Furthermore, replacing fine particles with coarse ones could increase intestinal muscle (tensile strength) activity (100) which on one hand, may increase mechanical shear force between luminal materials and intestinal mucosa, enhancing mucus loss into the lumen and on the other hand, increases retention time of digesta leading to a higher nutrient digestibility and subsequently less intestinal reflux and loss of the mucus layer.

Mucins are high molecular mass (2 x 10^6 Da) of heavily *O*-glycosylated polypeptides which resist to digestive processes resulting in a significant fraction of endogenous losses (103). The endogenous loss of mucin carbohydrates and amino acids are commonly determined at the end of ileum instead of total tract because of the variable and modifying effects of the hindgut microbiome on nutrient utilization (104). The quantification of mucins in ileal digesta is therefore often undertaken using mucin carbohydrate as markers. The measurement of sialic acid in ileal digesta or excreta is used to estimate the total mucins secreted into the lumen of chicken (105), while other components including fucose, galactose, glucosamine and galactose are more commonly used in mammals (106). In chicken, sialic acid concentration in the ileal digesta was reported to be between 31.7 to 171 mg/100 g dry ileal digesta (107, 108). The observed variation in the studies may be due to the different methods in sample preparation. The mucin extraction from digesta may give a lower sialic acid concentration compared with measuring it in an intact digesta (as-is). The mucin extraction method may provide only mucin-bound sialic acids and exclude bacteria-derived sialic acids including peptidoglycans and lipopolysaccharides (109). Approximately 74% of sialic acid content in the ileal digesta of pig did not bind to the mucin subunits, while the remaining (26%) was found as a mucin-bound form (110), thus the considerable amount of non-mucin-derived sialic acids may affect the overall sialic acid concentration in the ileal digesta. The variation in sialic acid concentration was also observed in the total tract excreta ranging from 76.5 to 148 mg/100 g dry excreta (111–113). Therefore, sialic acid content of the intact digesta cannot be a reliable representative for mucus loss since a major part of the measured sialic acid might be originated from bacterial cell surface (104). To gain more realistic data on the mucin loss, a further step to achieve purified crude mucin prior to carbohydrate quantification may be needed.

The endogenous proteins entering the digestive tract are predominantly originated from various digestive enzymes, mucoprotein and desquamated enterocytes. These proteins are mainly reabsorbed in the small intestine. The basal loss of endogenous amino acids in broilers (measured at the end of the ileum) was ranged from 3.08 g/kg dry matter intake (DMI) for a protein-free diet to 8.81 g/kg DMI for a casein diet (104). It is assumed that highly purified and digestible proteins such as casein are completely digested and absorbed in the small intestine of broilers (114) therefore, the detected amino acids at the end of the ileum of broilers fed by casein diet should be of endogenous origin. The remaining of unabsorbed endogenous proteins that passes beyond the ileum is considered as a loss of protein and energy to animal. The mucin polypeptides are relatively resistant to endogenous protease since the central mucin domains are protected by high density of *O*-glycosylation and animals do not secrete enzymes that can degrade the *O*-glycans (115). Therefore, increasing mucin secretion into the gastrointestinal tract spontaneously increases endogenous protein loss. In chicken, the amino acids of mucins are mainly composed of glutamic acid, proline, aspartic acid, threonine and serine (101). It has been reported that the mucin domains in the small intestinal of chicken are composed of proline, threonine and serine at approximately 22, 29 and 30% of the total amino acid sequences, respectively (44). A study conducted on broilers, laying hens and roosters has speculated that relative comparison of proline, glutamic acid, aspartic acid, serine and threonine concentration (as the main amino acids of mucoproteins) in the ileal digesta can be used for quantitative estimation of mucoproteins contribution (mucus loss) to the ileal endogenous amino acids loss of poultry (116). To our best knowledge, a specific method for measuring the contribution of mucoproteins to ileal endogenous amino acids in poultry has not been established yet, while it has been done for other species. In pig, the contribution of mucins in digesta was determined by the regression equation of the GalNAc : GluNAc ratio of purified mucin. The amount of mucins secreted into ileal digesta accounted for 13% by weight of which, 64% was gastric mucin and 36% was intestinal mucins (117, 118). Although endogenous

loss of proteins and amino acids in chickens has been widely studied, the contribution of intestinal mucins in it has not been clearly explained. In the poultry studies that have considered this matter, the contribution of intestinal mucins in the endogenous amino acids' loss has been attributed to the endogenous flow of the predominant mucin-derived amino acids.

ALTERATIONS IN MUCUS PRODUCTION BY DIET AND FEEDING

Mucus production is associated closely with the digesta and gut movements as well as bacterial enzymatic digestion. Therefore, alterations in feeding strategies and diet e.g. feed restriction, protein level, carbohydrate sources, feed form, etc., affecting nutrient digestibility and gut bacterial status (e.g. symbiosis and dysbiosis) could potentially influence the intestinal GC as well as mucins production and dynamic.

Feed restriction in chicken has been shown to alter the number and secretion of GC in the small intestine. Feed restriction after hatch reduced cell proliferation and migration rate in the small intestine resulting in decreased number of enterocytes per villus, increased GC density and reduced villus surface area (2, 119). Moreover, during feed restriction (24-36 hours) in newly hatched chicks, a reduction in number of GC migrating from the crypt to the villus base has been reported, causing a lesser GC number in the lower half of villus in the jejunum and ileum compared with the upper part of villus (120). On the other hand, the expression of MUC2 mRNA in the small intestine was reduced in newly hatched chickens with delayed feed access up to 72 hours, which led a decrease in mucus production (7, 120). This may also be considered as an indication for immature GC or/and lower total GC number in the small intestine. It has been shown that feed restriction up to 36 hours suppressed proliferation and differentiation of stem cells therefore, the number of GC in the crypt did not change (increase) and cell migration out of the crypt decreased (120). The reason for the observed increase in GC density in the villi by feed restriction could be the reduction in villus surface area (not increase in GC number *per se*) or boosted host defense mechanism during the delayed development of gut barrier (121). The gut barrier and immune system are generally immature in newly hatched chicks therefore, the presence of mucus is of high importance for gut protection against the invasion of pathogenic bacteria and toxins (7). Immediate feeding of hatchlings is essential and required to support the development of intestinal epithelium including enterocytes and GC, in order to strengthen mucosal barrier to prevent damages by pathogens and toxins.

A few reports showed that delayed feeding in newly hatched chicks influences mucin composition in the GC. The proportion of acidic mucins increased in 48 hours fasted newly hatched chicks compared with fed chicks (7, 122). The presence of acidic mucins can have a protective role against bacterial invasion because of the fact that they are less prone to degradation by bacterial enzymes (63). Thus, the reported increase in acidic mucins may be associated with reported mucosal injury and bacterial overgrowth triggered by stress and decreased intestinal movement in fasted chickens (123). However, after having access to feed, the fasted chickens showed an ability to restore cell proliferation and their GC density. The cell proliferation and migration rate can be recovered within 3-4 days after refeeding (119). The GC density in these refed chicks was similar to immediately fed chicks during the first week of age (2, 120).

The effects of fasting on intestinal mucus layer has been investigated in older broiler chickens. Interestingly, the amount of mucus secreted per area of intestinal tissue of growing chickens (>4 weeks of age) was decreased with 72 hours feed restriction (45, 124), while the mucin concentrations (measured by the intensity of the bands using Western blot analysis) was increased (45). The decreased amount of mucus secreted per area might be resulted from an alteration in mucus composition especially reduction in water content, which also leads to a higher mucus concentration (125). It was proposed that the reduction in mucus secretion due to feed withdrawal in chicken may be associated with physiological regulation *via* cholinesterase activity (45). Mucus secretion is stimulated by acetylcholine. Feed restriction could increase acetylcholinesterase activity and subsequently decreases the stimulating signal for mucin secretion (30). In conclusion, the consequences of delayed feeding of newly hatched chicks and feed withdrawal for growing chickens is a reduction in differentiation and secretion of GC, leading to a thinner protective mucus layer and increased risk of exposure of the epithelium to luminal harmful agents. The restoration of GC population after feed introduction has been found at young age but the secretion ability of GC after feed introduction still needs to be studied further.

Dietary proteins and specific amino acids have been shown to alter mucin secretion through increasing renewal of mucus layer (126) or through providing amino acids essential for the mucin synthesis (127). Certain amino acids including threonine, proline and serine are of particular interest because of the role they play in the mucin amino acids backbone. Threonine is one of the essential amino acids which cannot be synthesized by poultry and must be provided by the diet. It was found that a reduction in dietary threonine by 60% and 30% of broiler requirement (8.2 g/kg diet) decreased crude mucin concentration in excreta by 50% and 20%. It also reduced sialic acid concentration by 49% and 9%, respectively (105). Similarly, feeding a low protein diet (19% CP) to broilers between 21 and 42 days of age, with a decreased level of threonine, proline and serine by 4, 18 and 17% respectively, caused a reduction in crude mucin excretion by 6.4-8.8% in compare with those received the standard (21% CP) diet (128). Therefore, mucin secretion may be disturbed by deficiency of some the amino acids like threonine.

Indigestible carbohydrates have an effect on luminal components including gut microbiota, gut epithelium and mucin secretion which was discussed by Montagne et al. (106). The indigestible carbohydrates including dietary fiber (DF) play a role in regulating mucus secretion due to their properties including water-holding capacity, viscosity and abrasive surface,

which could potentially alter the quantity, physicochemical properties and protective function of the intestinal mucus layer (105). The secretion of intestinal mucins in rat was found to be proportionally increased with increasing the volume of insoluble DF attained in water (bulk-forming properties) (129) and with the viscosity of soluble DF (130). The stimulatory effects of DF on mucin secretion could be because of an increased luminal pressure and flow resistance of bulky and viscous digesta which enhance mucus loss and GC differentiation and subsequently, increase mucin secretion (130). In chicken, several studies showed that the addition of soluble or insoluble DF stimulated GC population and mucin secretion in the small intestine (121, 131). As an example, adding either insoluble (2-4% cellulose) or soluble fiber (2-4% carboxymethyl cellulose) to chicken diets increased ileal GC number compared with the control group (131). Similarly, the addition of insoluble DF compound (rice hull, 100 g/kg) enhanced MUC2 expression, increased number of GC per villus and increased mucin secretion in the jejunum and ileum compared with the control (cornstarch) diet (121). Different types of DF sources have also been shown to increase the excretion of mucins at the terminal ileum in pigs (e.g. peas, wheat, straw, corn cobs and cellulose), rats (psyllium seed husk) and human (soya fiber) (106).

Different cereal types provide varied composition and amount of DF which could modify bacterial fermentation and their metabolic activities (132). Non-starch polysaccharides (NSP) are of high importance since they are largely indigestible in the small intestine of poultry and are mainly fermented in the hindgut by bacteria (106, 133). Cereals with high soluble NSP content like wheat, barley, rye and oat, can lead to a high viscous conditions in the small intestine and may alter the intestinal bacterial composition and activities compared with cereals like corn which contains lower soluble NSP (79, 134). The main fermentation by-products of NSP are short chain fatty acids (SCFA), predominantly acetate, propionate and butyrate. Approximately 95 to 99% of SCFA that are produced in the hindgut of non-ruminants, are absorbed and have specific roles in the body (135). As for example, acetate which is the most abundant SCFA, acts as an energy substrate for muscle tissue and can be utilized by bacteria as a precursor for butyrate synthesis. Propionate regulates glucose synthesis in liver and butyrate is used as a major source of energy for cellular metabolic activities (132). It has been shown *in vitro* and rat studies that SCFA, in particular butyrate, also involve in supplying energy for intestinal GC proliferation and differentiation and subsequently increase mucus production and MUC2 gene expression in the gut (136, 137). Feeding broilers with wheat/rye-based diet instead of corn-based diet increased bacterial number in the small intestine, most notably enterobacteria, and increased SCFA concentration, especially acetate and n-butyrate in the cecum (133). It also increased GC size and their number in the ileum and the cecum (134). Although soluble NSP provide the energy for bacteria which allow them to use other nutrients such as nitrogen as substrates for metabolite production, it should be concerned that the presence of these viscous-forming fibers have adverse effects on nutrient absorption. The viscous NSP can physically complex with intestinal enzymes reducing the interaction with substrates, thus decrease nutrient digestibility (133). Using SCFA as feed additives for poultry has been shown to promote intestinal development and modulate gut bacteria (138). Adding sodium butyrate (0.8 g/kg) to broiler diets caused a distinct impact on the bacterial community and increased the number of bacteria related to the fermentation of undigested carbohydrate including *Firmicutes, Bacteroidetes* and *Proteobacteria* in the cecum and thus, further increased microbial-derived SCFA compared with the control group (138). Addition of sodium butyrate (0.2-1 g/kg) in broiler diets increased villus length and GC density in the jejunum and ileum, and also increased mucus secretion compared with the control diet (138). Supplementing sodium butyrate (0.5-1 g/kg) in broiler diets also increased acidic GC number per villi of the small intestine, suggesting a stimulating effect for butyrate to promote protective mechanism against mucin degradation by gut bacteria (9). In conclusion, the recent obtained data shows a potential role for dietary and bacterial SCFA specially butyrate, in regulating GC differentiation and modulating mucus production and dynamic.

The oxidative stress of the intestinal cells (e.g. colonic GC) induced by high fat diet, led to upregulation of intestinal inflammatory cytokines (e.g. IL-1b, TNF-α and IL-17a), along with a decrease in GC differentiation and MUC2 expression in mice (139). There is a limited information available regarding the impact of dietary fat on mucus properties in poultry. However, the intracellular fatty acid-binding proteins (FABPs) have been subject of several studies. In poultry, FABPs modulate lipid metabolism via regulation in the fatty acid uptake (in line with the concentration gradient) into the cell (140). Several FABPs including FABP1, FABP2, and FABP6 have been identified to be predominantly expressed in the digestive tract of chickens (140, 141). It has been shown that, enhancing the dietary fat level in poultry feed could increase the concentration of FABPs in the intestine (142). A downregulation in mRNA expression of FABP2 occurred in compromised gut barrier chickens (challenged with coccidiosis vaccine) along with decreased MUC2 and occludin expression, which may indicate an association between FABP2 and gut integrity in chickens (141). It was suggested that necrotic enteritis infection in broilers caused downregulation of FABP1 and FABP2 in the small intestine. These downregulations were assumed to be attributed to structural damage and intestinal epithelium loss in the small intestine and lead to reduction in fatty acid utilization (140). However, to the best of our knowledge, no direct interaction between FABPs and mucus production and/or quality has been reported so far.A downregulation in mRNA expression of FABP2 occurred in compromised gut barrier chickens (challenged with coccidiosis vaccine) along with decreased MUC2 and occludin expression, which indicate the role of FABP2 in maintaining intestinal integrity in chicken (141). It was suggested that the downregulation of FABP1, FABP2 and other genes that are related to reduced fatty acid utilization may be also associated with intestinal inflammation and structural damage of the epithelium (140). The concentration of lipids in poultry feed is considerably lower than carbohydrates and

proteins, but they still influence gut microbiota (143, 144). Feeding isoenergetic diets with different fat sources to broilers affected the pH and fermentation products in the ileum and cecum, which shows differences in activity and composition of the gut microbiota between these groups (144). For example, diet with palm kernel fatty acids distillers (4.3%) increased concentration of total SCFA and lactate in the ileum and cecum and decreased digesta pH in the ileum compared with soybean oil (4.0%) diet (144). As mentioned above, higher SCFA production in the gut can lead to higher GC proliferation and differentiation as well as higher mucus production (136, 137). Therefore, dietary fat level and type in poultry feed can have an indirect impact on intestinal integrity and mucus production. However, the extent of this impact needs further investigation.

CONCLUSION AND SUGGESTIONS

It can be concluded that the intestinal mucus layer plays an important role in maintaining the intestinal microbial balance, facilitating nutrient transport, preventing pathogen invasion, and regulating the microbial–host immune response. The intestinal mucus layer made by mucins secreted by goblet cells possesses a particular structure and molecular glycan composition for each part of the gut which contributes to its main functions including protecting itself against sheer force of dietary materials, transporting nutrient, maintaining the colonization of commensal bacteria and protecting the epithelial surfaces against pathogenic bacteria. In chicken, the considerable increase in the intestinal goblet cells density and activity in the first week of age is a response to emerging needs of newly hatched chickens for mucus secretion and immune response associated with their immediate expose to the surrounding environment and diet. The goblet cell population in the small intestine of chickens reaches maturity at 3 weeks of age. There is an anteroposterior increasing trend in the goblet cells density and mucus secretion in the small intestine of chickens which is a host adaptation to enhance protective barrier against the increasing number (and activity) of gut bacteria. Furthermore, the proximal part of the small intestine including duodenum is very active in digestion and absorption and may prioritize the proliferation of absorptive cells over goblet cells, which is associated with a lower goblet cell density, lower mucus secretion and a larger goblet cell size in the duodenum compared with the jejunum and ileum. The continuous production of mucins by goblet cells mainly renews the outer mucus layer which is easily lost into the lumen by mechanical erosion and bacterial degradation. However, the regulated secretion of mucus is a rapid response to external stimuli and acts as the first defensive mechanism of the gut. The distribution of mucin types in the goblet cells is regulated by glycosylation of *O*-glycan, which can be affected by the host (e.g. inflammatory markers, hormones and neurotransmitters) and external (e.g. commensal bacteria, pathogens, pre/probiotics and nutrients in the diet) factors. Increasing in acidic mucins is known as an adaptation strategy to protect mucin from degradation by bacteria. However, several bacteria have an ability to still degrade this barrier. Any factor affecting nutrient digestibility, gut motility and digesta flow, gut bacterial status and their metabolic activity e.g. dietary factors (physical and chemical properties of feed) could potentially influence the intestinal goblet cells as well as mucin production and dynamic. The mode of action and mechanisms behind these effects need to be studied further. Mucins resist to digestive processes; therefore, a significant fraction of endogenous losses in chicken is mucins which can be considered as a loss of protein and energy to animal. Hydrothermal processing of poultry feed may reduce this loss by reduction in mucus shedding into the lumen. Given the significance of this loss and the lack of precise data about it, this matter needs to be carefully investigated in the future and the nutritional strategies reducing this loss have to be defined better.

AUTHOR CONTRIBUTIONS

Conceptualization, YD, JZ, and FGB. Writing-Original draft, YD. Writing-Review and Editing, YD, JZ, and FGB. All authors contributed to the article and approved the submitted version.

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3. Chapter 3: Main part of the thesis (part I)

Impact of feed additives and host-related factors on bacterial metabolites, mucosal integrity and immune response in the ileum of broilers

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RESEARCH

Impact of feed additives and host-related factors on bacterial metabolites, mucosal integrity and immune response in the ileum of broilers

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Abstract

The present study aimed to investigate the effect of age, breed, and sex of broilers, as well as a probiotic or phytobiotic product on mucosal morphology, bacterial metabolites, and immune traits in the ileum of broilers. A total of 2,880 one-day-old male and female broiler chicks from two breeds (Ross308® and Cobb500®) were randomly assigned to 72 pens. Broilers were offered a wheat-soybean diet without (CO), or with either a probiotic (PO; 2.4×10^9 CFU/kg of *Bacillus subtilis* DSM32324 and DSM32325 and *B. amyloliquefaciens* DSM25840) or a phytobiotic (PY; grape extract, 165 ppm procyanidin and 585 ppm polyphenols of the diet) product. The trial was conducted with a $3 \times 2 \times 2$ factorial arrangement of diet, breed, and sex in a completely randomized design (6 replicate-pens per treatment). At day 7, 21, and 35, one chicken per pen was slaughtered for collecting ileal tissue to evaluate of histomorphology and mRNA expression, as well as ileal digesta to measure bacterial metabolites. Data were subjected to ANOVA (the main factors; age, diet, breed, and sex) and Four-Way ANOVA (interactions) using GLM procedure. Overall, the concentration of acetate and total short chain fatty acids reached the peak and lactate decreased to its lowest on day 21, but their concentrations at day 7 and 35 were similar ($p > 0.05$). Spermine, spermidine, and ammonia decreased after day 7, while putrescine and cadaverine increased after day 21 ($p < 0.05$). mRNA expression of cytokines, mucin 2 (*MUC2*) and claudin 5 (*CLDN5*) was similar; increased from day 7 to 21 and decreased afterward ($p < 0.05$). Villus height, crypt depth and villus surface area increased with age ($p < 0.05$). Acidic goblet cells (GC) number and density increased after day 21 ($p < 0.05$). Ross broilers showed higher D-lactate concentration and *IFN-γ* expression, while Cobb broilers had greater *IL-4*, *IL-6* and *TNF-α* expression and higher total GC number ($p < 0.05$). Female displayed higher villus height and GC number and density (mixed and total GC) than male ($p < 0.05$). The effect of dietary treatment was not found on any investigated variables ($p > 0.05$). In conclusion, aging of broilers affected ileal histomorphology, cytokine expression, and barrier integrity, as well as bacterial activity. These observed impacts could be attributed to host-microbiota interaction and the direct effects of bacterial metabolites on intestinal cells and immune system.

Keywords Goblet cell · Host-microbiota interaction · Immune response · Phytobiotic · Probiotic

Introduction

The prohibition of antibiotic growth promotors (AGP) has put tremendous pressure on the poultry industry to look for reliable alternatives. As a result, probiotics and phytobiotics have been widely used to reduce the use of AGP in poultry production. Probiotics are used to regulate intestinal microbiota and can directly influence gut immune system through the pattern-recognition receptors (PRRs) present in both epithelial and immune cells of the host (Tarradas et al. 2020). Spore forming bacteria are resistant to

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environmental stresses like heat, disinfectants and low pH. *Bacillus* based probiotics have demonstrated superior stability and viability in both feed processing and the gut of broilers compared with non-spore-forming probiotics, making them one of the best probiotic candidates for poultry nutrition (Goodarzi Boroojeni et al. 2016; Zentek and Goodarzi Boroojeni 2020). Feeding *B. subtilis* and *B. amyloliquefaciens* to broilers have been shown to reduce proliferation of pathogens, modify gut microflora, minimize gut inflammation and modify mucosal morphology which finally led to an improved growth performance (Park et al. 2020; (Wang et al. 2021a).

Phytobiotics are plant-based, naturally occurring substances which promote health-related benefits (Chamorro et al. 2019). The beneficial impacts of phytobiotics are attributed to their bioactive compounds including polyphenols which exhibit antioxidant, anti-inflammatory, and antibacterial effects (Viveros et al. 2011). Procyanidins are the main polyphenols found in grape extract and known to reduce pro-inflammatory responses and epithelial damages caused by oxidative stress in the small intestine of broilers (Yang et al. 2017a; Cao et al. 2020). Grape procyanidins can be metabolized by the intestinal microbiota into phenolic acids and other metabolites that help reducing oxidative stress and inflammation in gut of broilers (Chamorro et al. 2019; Cao et al. 2020). Furthermore, addition of procyanidin-rich grape extract to broiler diet has been shown to increase populations of some beneficial bacteria such as *Enterococcus*, whereas it decreased the number of *Clostridium* in the ileum of broilers (Viveros et al. 2011).

The ileum is the terminal part of the small intestine that plays a key role in nutrient absorption. However, in comparison to the proximal part of the small intestine, it appears to be an important site for microbial fermentation as evidenced by an increase in bacterial density and metabolite production distally along the small intestine (Rehman et al. 2007). Gut microbiota directly interacts with intestinal epithelial cells, communicates with immune cells and modulates cell proliferation and barrier function (Mahapatro et al. 2021). Intestinal microbiota of newly hatched broiler chicks is known to have a limited diversity. However, it undergoes successional changes over time and tends to become more diversified and stabilized as the host ages (Glendinning et al. 2019). Dramatic changes in bacterial community composition and activity have been shown to occur naturally as broilers mature (Oakley et al. 2014; Duangnumsawang et al. 2022). However, the direction of these changes seems to be affected by different factors such as intestinal morphology and environment condition provided by the host (Bindari and Gerber 2022).

It has been reported that ileal microbial composition as well as its physiological functions were affected by broiler genotype (Emami et al. 2022) and sex (Lumpkins et al. 2008). Modern broilers are genetically selected for performance and immunocompetence, yet the immunological responses to certain challenges greatly vary between breeds (Jang et al. 2013). On the other hand, distinct gut morphology, such as villus height and crypt depth as well as the mucin composition of the intestinal mucus layer may provide a specific niche for intestinal microbiota, contributing to a breed-specific bacterial community (Mabelebele et al. 2017; Richards-Rios et al. 2020). As a result, different breeds of broilers may exhibit various intestinal bacterial communities and immunological status, even when grown in the same environment and fed the same diet. Considering that male broilers generally have higher growth rates than female broilers, the sex-related physiological growth may selectively influence bacterial colonization (Kers et al. 2018). Therefore, host-related biological elements (e.g. age, breed and sex) can affect gut microbial composition and activity as well as immune responses. Dietary treatments including probiotics and phytobiotics may interact with the host-related biological elements and boost or discount their impacts on gut microbial community and immune responses (Kers et al. 2018).

The present study aimed to bridge the gap between diet, host, gut microbial activity, physiology and immune responses. In order to achieve that, this study investigated the effect of age, breed, and sex of broilers (host-related factors) as well as inclusion of a probiotic or phytobiotic product (nutritional treatment) on mucosal morphology, goblet cell count, bacterial metabolites, as well as mRNA expression of the cytokines and the proteins involved in mucus production and epithelial tight junction in the ileum.

Materials and methods

Animals and experimental diets

A total of 2,880 one-day-old male and female broiler chicks consisting of 1,440 Ross308® and 1,440 Cobb500® were randomly allocated into 72 pens (2.25 m^2) with a softwood shaving floor. The allocation of chicks to pens was based on breed and sex, with individuals of the same breed and sex housed together in each pen. The sex of day-old chicks was determined through vent sexing. All birds were vaccinated against Avian Infectious Bronchitis and Gumboro diseases according to the vaccination program at the hatchery and examined upon arrival (e.g., general behavior, physical appearance, and feathers). The housing system used in this study was described in Tous et al. (2022). In brief, the barn was equipped with an automatic environment control system. The light program consisted of 24 h of light for the

first 2 days, followed by 18 h of light until day 7, and 14 h of light per day thereafter. The temperature program was initially set at 32–34 °C for the first 2 days, then reduced to 29–31 °C from day 3 to 7, and subsequently decreased by 3 °C per week until it reached 21 °C.

Table 1 Dietary ingredients and nutrient composition of the experimental diets (as-fed basis)

Ingredients (g/kg)	Starter (0–7 days old)	Grower (8–21 days old)	Finisher (22–37 days old)
Wheat	528	612	620
Soybean meal (48% CP)	394	305	159
Soybean oil	41.6	48	0
Animal Fat (5 SYSFEED)[1]	-	-	40.1
Extruded soybean	-	-	150
Dicalcium phosphate	18.5	16.6	15
Calcium carbonate	5.3	4.8	4.4
Vitamin-mineral premix[2]	4	4	4
Sodium chloride	3.7	3.7	3.5
dl-methionine	2.7	2.3	1.9
L-lysine HCl	1.6	1.9	1.5
L-threonine	0.5	0.5	0.4
Choline chloride	0.3	0.5	0.5
Antioxidant (Noxyfeed 56P)[3]	0.2	0.2	0.2
Sodium bicarbonate	-	0.1	0.02
Calculated nutrients and energy (g/kg, unless noted)			
AME, kcal/kg	2900	3000	3100
Lysine	14.2	12.1	10.8
Methionine + cysteine	10.1	8.8	8.1
Threonine	9.3	7.9	7.2
Calcium	9.6	8.7	8.1
Total phosphorus	6.9	6.3	6.0
Sodium	1.6	1.6	1.6
Analyzed nutrients (g/kg)			
Dry matter	892	894	901
Crude protein	245	213	201
Ether extract	57	63	84
Ash	58	52	49

[1] Product of Sysfeed SLU (Granollers, Spain, containing 1.5% myristic acid (C14:0), 18% palmitic acid (C16:0), 2% palmitoleic acid (C16:1 n-7), 14% stearic acid (C18:0), 28% oleic acid (C18:1 n-9 cis), 12% linoleic acid (C18:2 n-6 cis) and 6% α-linolenic acid (C18:3 n-3 cis).

[2] One kg of feed contains: Vitamin A: 10 000 IU; Vitamin D3: 4 800 IU; Vitamin E: 45 mg; Vitamin K3: 3 mg; Vitamin B1: 3 mg; Vitamin B2: 9 mg; Vitamin B6: 4.5 mg: Vitamin B12: 40 µg; Folic acid: 1.8 mg; Biotin: 150 µg; Calcium pantothenate: 16.5 mg; Niacin: 65 mg; Mn (as MnSO4.H2O): 90 mg; Zn (as ZnO): 66 mg; I (as KI): 1.2 mg; Fe (as FeSO4.H2O): 54 mg; Cu (as CuSO4.5H20): 12 mg; Se (as NaSeO3): 0.18 mg; BHT: 25 mg; Calcium formiate, 5 mg; Silicicic acid, dry and precipitated, 25 mg; Calcium stearate, 25 mg; Calcium carbonate to 4 g.

[3] Product of Itpsa (Barcelona, Spain), containing 56% of antioxidant substances (butylated hydroxytoluene + propyl gallate), 14% of citric acid and 30% of sepiolite as carrier.

Three experimental diets including a standard wheat-soybean based diet without (CO) or with supplementation of either a probiotic (PO) or a phytobiotic (PY) product were produced and randomly assigned to birds. The trial was conducted with a $3 \times 2 \times 2$ factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen (24 replicate-pens per diet, 36 replicate-pens per sex and 36 replicate-pens per breed). The experiment lasted 37 days. The experimental diets (starter diets for day 0–7, grower diets for day 8–21 and finisher diets for day 22–37) were formulated (Table 1) to meet or exceed recommendations of FEDNA (2018). The diets were offered in crumble form for the starter period and in 3 mm pellets later on. The probiotic product (GalliPro EPB5, Chr. Hansen, Denmark) which consists of *Bacillus subtilis* DSM32324 and DSM32325 and *B. amyloliquefaciens* DSM25840 was added into the PO diets at a dosage of 2.4×10^9 CFU/kg diet. The concentration of probiotics in the diets was measured and was on average 3.7×10^9 CFU/kg. The phytobiotic product (NutriPhy® White Grape 100, Chr. Hansen, Denmark) was included into the PY diets making a final concentration of 165 ppm procyanidin and 585 ppm total polyphenol in the diets. The applied dosages were according to the manufacturer recommendation.

Sample collection

At day 7, 21, and 35 of age, six birds per pen were randomly selected, weighed, slaughtered and used for sample collection. The one which had the closest body weight to the averaged pen-weight was used for the present analysis (6 birds per treatment). The birds selected for the analysis were sacrificed in compliance with the ethical requirement RD 53/2013 (Spain). Following euthanasia, the birds were individually collected for ileal digesta and tissue. The digesta was collected from the distal one-third of the ileum and subsequently were frozen in liquid nitrogen and stored at -80 °C until further analysis. The distal ileal tissue was collected and used for histomorphological analyses and mRNA expression of the proteins related to epithelial barrier and inflammatory markers. For histological measurement, the tissues were fixed in 4% (vol:vol) phosphate-buffered formaldehyde immediately after slaughtering and then transferred to 70% ethanol until further analysis. For mRNA expression analysis, the entire tissues were stored in RNAlater buffer (Qiagen GmbH, Hilden, Germany) at -80 °C until further analysis.

Histomorphological analyses

All tissue samples collected at day 7, 21, and 35 of broiler age were dehydrated, cleared with xylene and embedded with paraffin. Serial of 3 µm sections were prepared, mounted on glass slides and stained with Alcian blue-periodic acid-Schiff (AB-PAS) following manufacture's protocol (AB-8GX, Sigma; Schiff's reagent, Merck, Darmstadt, Germany). Ten villi and ten crypts per sample were randomly selected for morphological analysis. Villus height (*VH*) was measured from the tip to the base of the villus. The villus width (*VW*) was determined at the midpoint of the villus. Crypt depth (*CD*) was defined as its invagination depth. The villus height to crypt depth (*V/C*) was calculated from VH divided by CD. The villus surface area (*VSA*) was calculated by multiplying VH with VW. Acidic (blue), neutral (pink), mixed (purple) and total goblet cell (*GC*) were counted for each villus (GC number) and calculated as the number of GC per 100 µm of VH (GC density). All measurements were performed with an Olympus light microscope (BX 43, Olympus, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). Image analysis was performed by using cellSens Standard software (version 1.14, Olympus, Germany) and ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Metabolite analyses

Analysis of short chain fatty acids (SCFA), including acetate, propionate, i- and n-butyrate, i- and n-valerate was performed by gas chromatography on an Agilent 6890 gas chromatography system with a flame ionization detector and autosampler (Agilent Technologies, Böblingen, Germany). The separation of compounds was achieved by using the column Agilent 19,095 N-123 HP-INNOWAX polyethylene glycol (Agilent Technologies, Böblingen, Germany). D- and L-lactate were analyzed by high-performance liquid chromatography on an Agilent 1100 chromatograph equipped with a Phenomenex C18 (4.0×2.0 mm^2) guard column followed by a Phenomenex Chirex 3126 (D)-penicillamine column (150×4.6 mm^2) and a UV detector at 253 nm. Ammonia was quantified using the Berthelot reaction assay and a photometric measurement was carried out at 620 nm. These methods were described by Goodarzi Boroojeni et al. (2014). Biogenic amines (putrescine, cadaverine, histamine, spermidine and spermine) were analyzed with reversed-phase high pressure liquid chromatography (HPLC) as described earlier (Rehman et al. 2008).

RNA isolation and real time-quantitative PCR

Sample preparations and real-time PCR conditions have been previously described (Duangnumsawang et al. 2022). Briefly, entire tissue samples of the ileum were homogenized in buffer provided in the NucleoSpin® RNA Plus kit and RNA was isolated from the resulting tissue homogenates with the NucleoSpin® RNA clean-up according to the manufacturer's recommendations (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The mRNA quality and quantity were analyzed by a Bioanalyzer (Agilent 2100, Agilent, Waldbronn, Germany). Subsequently, reverse transcription of 100 ng of total RNA into cDNA in a final volume of 20 µL was executed using the Super Script III Reverse Transcriptase First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, California). Primers used for the interleukin (*IL*)-*1β*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-17α*, *IL-18*, Tumor necrosis factor-α (*TNF-α*), interferon γ (*IFN-γ*), transforming growth factor-beta 2 (*TGF-β2*), Mucin 2 (*MUC2*) and Claudin 5 (*CLDN5*) are presented in Table 2. The RT-qPCR was conducted with a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands). The reference mRNA level of β-actin, glycerinaldehyde-3-phosphate-dehydrogenase (*GAPDH*) and β2-microglobulin were used for normalization and times-fold expression was determined based on mean cycle threshold values of the references using the software tool REST© (Pfaffl 2002). The mRNA expression of all cytokines, *MUC2* and *CLDN5* was calculated as copy number per ng of total RNA. Then this value was divided by mean copy number of the references to obtain the expression of targeted mRNA in different treatment groups.

Statistical analysis

Statistical analysis was conducted using SPSS 26 (SPSS Inc. Chicago, IL, United States). Data were analyzed by GLM procedure (using ANOVA) to evaluate the main factors including three ages (day 7, 21 and 35 of age), three dietary treatments (CO, PO and PY), two breeds (Ross and Cobb), and two sexes (male and female). A Four-Way ANOVA was performed to evaluate interactions between the main factors. Means were separated by the Tukey least significant difference post hoc test at $p < 0.05$ statistical level. Means and pooled standard error of the mean (SEM) were reported for all variables measured. Replicate-pen was the experimental unit for all variables measured. Figures were illustrated in GraphPad Prism 9.0.2 for Windows (GraphPad Software, San Diego, California United States).

Table 2 Primer sequences used for RT-qPCR analysis

Targets[1]	Sequences of primers (5′ to 3′)	A_T[2]	Reference
IL-1β	GACATCTTCGACATCAACCAG CCGCTCATCACACACGACAT	60	(Duangnumsawang et al. 2022)
IL-2	TCTGGGACCACTGTATGCTCT ACACCAGTGGGAAACAGTATCA	60	(Hong et al. 2006)
IL-4	AACATGCGTCAGCTCCTGAAT TCTGCTAGGAACTTCTCCATTGAA	60	(Avery et al. 2004)
IL-6	CTGCAGGACGAGATGTGCAA AGGTCTGAAAGGCGAACAGG	60	(Duangnumsawang et al. 2022)
IL-8	GGCTTGCTAGGGGAAATGA AGCTGACTCTGACTAGGAAACTGT	60	(Hong et al. 2006)
IL-10	GGAGGTTTCGGTGGAAGGAG GTTAAGCTGCCATTGAGCCG	60	(Duangnumsawang et al. 2022)
IL-12	AGACTCCAATGGGCAAATGA CTCTTCGGCAAATGGACAGT	60	(Hong et al. 2006)
IL-17α	AAGCGGTTGTGGTCCTCAT CTCCGATCCCTTATTCTCCTC	60	(Hong et al. 2006)
IL-18	GGAATGCGATGCCTTTTG ATTTTCCCATGCTCTTTCTCA	60	(Hong et al. 2006)
TNF-α	CTCGTTGGTGTGGGACGAC CGGCGGCGTATCGAAGTA	60	(Duangnumsawang et al. 2022)
IFN-γ	CTCCCGATGAACGACTTGAG CTGAGACTGGCTCCTTTTCC	60	(Sadeyen et al. 2004)
TGF-β2	TGCACTGCTATCTCCTGA ATTTTGTAAACTTCTTTGGCG	60	(Sundaresan et al. 2008)
MUC2	TGGCTGTGTAACTGCACCAA GTGGGTTTAGGAGGTGGCTC	60	(Duangnumsawang et al. 2022)
CLDN5	CATCACTTCTCCTTCGTCAGC GCACAAAGCTCTCCCAGGTC	60	(Osselaere et al. 2013)
β-actin	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	60	(Li et al. 2005)
GAPDH	GGTGGTGCTAAGCGTGTTA CCCTCCACAATGCCAA	60	(Li et al. 2005)
β2-microglobulin	AAGGAGCCGCAGGTCTAC CTTGCTCTTTGCCGTCATAC	60	(Li et al. 2005)

[1] Three references including β-actin, *GAPDH* (glycerinaldehyde-3-phosphate-dehydrogenase) and β2-microglobulin were used as house-keeping genes. IL, interleukin; *TNF-α*, Tumor necrosis factor alpha; *IFN-γ*, interferon gamma; *TGF-β*, transforming growth factor beta; *CLDN5*, Claudin 5; and *MUC2*, Mucin 2

[2] A_T, annealing temperature (°C)

Results

When there was no significant interaction effect between the main factors, only the results of the main effects will be addressed. No interaction effect between age, dietary treatment, breed and sex on histomorphology of the ileum was observed (supplementary Table 1A), except for GC density (supplementary Table 1B). The effect of the main factors on histomorphology of the ileum is shown in Table 3. Age affected all the morphological variables measured. Overall, the measurement of VH, VW, and CD (expressed as µm), as well as the ratio of V/C and VSA (µm^2) increased between 7 and 21 days of age by 64%, 28%, 51%, 11% and 109%, while only VH, CD and VSA showed a further increase (by 13%, 14% and 22%, respectively) from day 21 to 35 ($p < 0.05$). The effect of dietary treatment, breed and sex was not significant for theses morphological variables ($p > 0.05$), except for VH which was slightly higher for female birds (5.6%) compared with male ones ($p < 0.05$).

Along the villi, approximately 79–88% of the detected GC seemed to be mixed type, while the remaining GC (12–21%) were mainly acidic type (Fig. 1). Neutral type of GC was not present in most of the samples and when present, their number was negligible. The number of acidic, mixed and total GC (per villi) was affected by age ($p < 0.05$). The number of mixed and total GC increased from day 7 and 21 ($p < 0.05$) by approximately 47% and 48%, respectively, however, the 57% increase in number of acidic GC from day 7 to 21 was not statistically significant ($p > 0.05$). The numbers of acidic and total GC further increased from day 21 to 35 ($p < 0.05$) by around 188% and 21%, respectively, while the number of mixed GC remained stable. The density of GC (per 100 µm VH) was also affected by age ($p < 0.05$). The density of acidic GC was similar at day 7 and 21 of age

Table 3 The effect of age, dietary treatment, breed and sex on histomorphology in the ileum of broilers [1]

Parameters*	Age (A)			Treatment (T)			Breed (B)		Sex (S)		SEM	*p*-value			
	7	21	35	CO	PO	PY	Ross	Cobb	Male	Female		A	T	B	S
Morphology[2]															
VH	343 [c]	564 [b]	636 [a]	520	511	516	524	507	502 [b]	530 [a]	10.7	<0.001	0.994	0.124	0.034
VW	109 [b]	139 [a]	147 [a]	133	129	133	131	133	132	132	2.2	<0.001	0.767	0.845	0.871
CD	99 [c]	149 [b]	170 [a]	143	139	138	141	139	138	142	2.9	<0.001	0.746	0.409	0.423
V/C	3.5 [b]	3.9 [a]	3.9 [a]	3.7	3.7	3.8	3.8	3.7	3.7	3.8	0.05	0.009	0.567	0.365	0.164
VSA	37.6 [c]	78.4 [b]	95.3 [a]	71.3	68.7	72.2	71.9	69.5	69.7	71.8	2.31	<0.001	0.818	0.374	0.541
Goblet cell number[3]															
Acidic	7.5 [b]	11.8 [b]	34.0 [a]	17.6	17.2	17.3	17.9	16.8	18.0	16.6	1.39	<0.001	0.942	0.539	0.860
Mixed	75.6 [b]	111.4 [a]	115.3 [a]	100.5	97.7	103.5	103.7	97.3	94.2 [b]	107.2 [a]	2.63	<0.001	0.618	0.135	0.004
Total	83.1 [c]	123.2 [b]	149.3 [a]	118.1	114.8	120.7	121.6 [a]	114.1 [b]	112.2 [b]	123.8 [a]	2.67	<0.001	0.515	0.026	0.001
Goblet cell density[4]															
Acidic	2.1 [b]	2.2 [b]	5.4 [a]	3.3	3.1	3.2	3.2	3.2	3.3	3.1	0.22	<0.001	0.967	0.926	0.820
Mixed	22.3 [a]	19.8 [b]	18.1 [b]	19.5	20.1	20.5	19.7	20.4	19.3 [b]	20.8 [a]	0.37	<0.001	0.510	0.363	0.039
Total	24.5 [a]	21.9 [b]	23.4 [ab]	22.8	23.2	23.8	22.9	23.6	22.6 [b]	23.9 [a]	0.30	0.002	0.362	0.289	0.017

[1] The trial was conducted with a 3×2×2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex.

[2] Villus height (VH), villus width (VW), and crypt depth (CD) are measured in μm, V/C ratio was calculated by dividing villus height with crypt depth, villus epithelial surface area (VSA) was calculated by the multiplication of villus height and villus width, expressed as 10^3 μm^2.

[3] The average number of goblet cells per villus. Acidic represents the cells that are positive to Alcian blue dye. Mixed represents the cells that are positive to both Alcian blue and PAS dye. Total represents the sum of acidic and mixed goblet cells.

[4] The average number of goblet cells per 100 μm villus height. Acidic represents the cells that are positive to Alcian blue dye. Mixed represents the cells that are positive to both Alcian blue and PAS dye. Total represents the sum of acidic and mixed goblet cells.

[a,b,c] Means within a row of each main factors lacking a common superscript differ ($p < 0.05$).

* CO, Control; PO, Probiotic product; PY, Phytobiotic product.

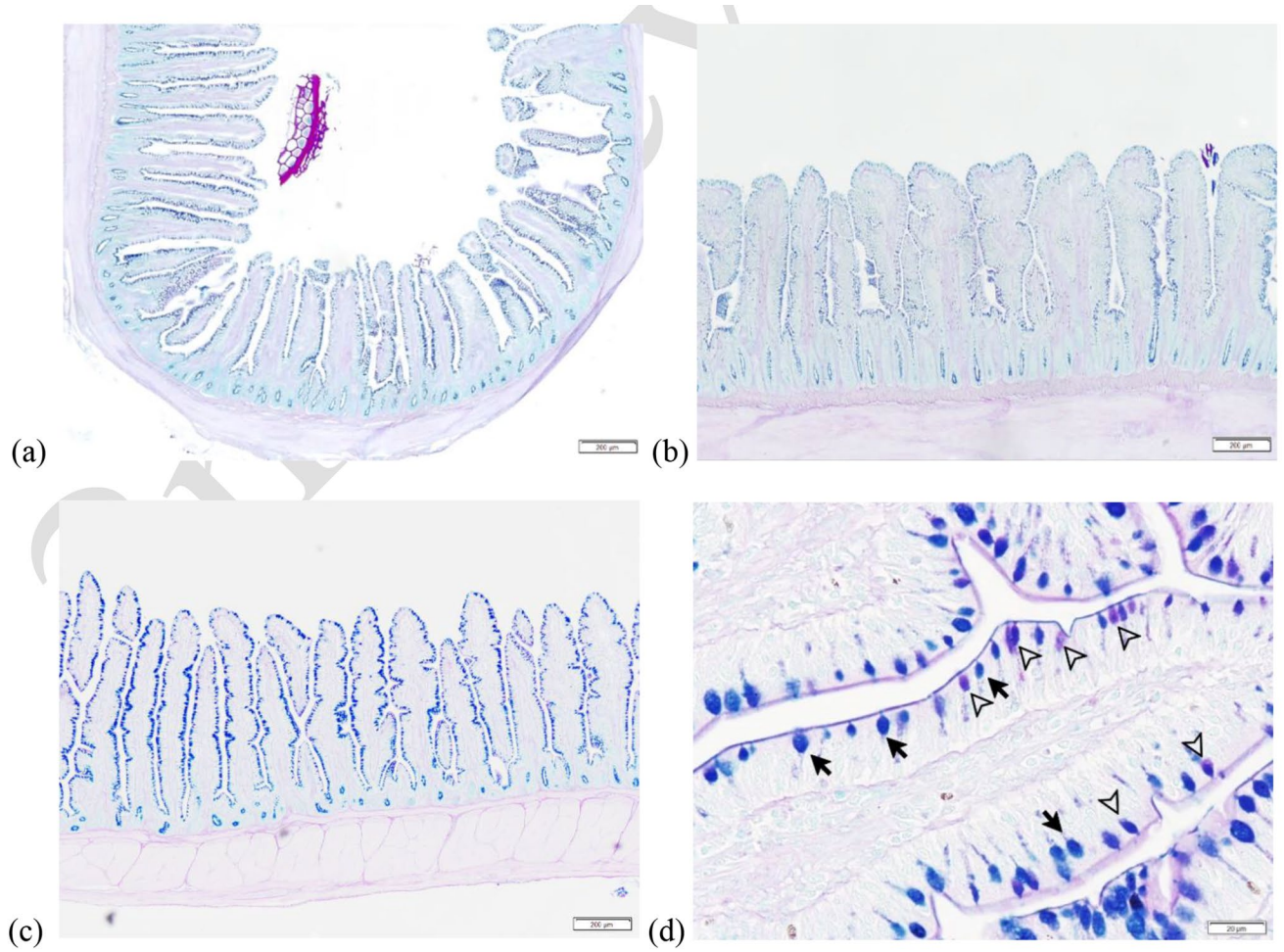

Fig. 1 Alcian blue-periodic acid-Schiff stain on the ileal tissue. Low magnification (10x, a-c) shows the positive stained mucins (blue-purple color) in the goblet cells lining along the villus and crypt of the ileum at day 7 (**a**), day 21 (**b**), and day 35 (**c**). High magnification (100x, **d**) of the villus part of the ileum shows the goblet cells containing blue-stained mucins (acidic goblet cells, solid black arrow) and purple-stained mucins (mixed goblet cells, open arrowhead). The magenta-stained mucins (neutral goblet cells) were not observed in this sample.

($p > 0.05$), while it increased by 145% from day 21 to 35 ($p < 0.05$). In contrast, the density of mixed GC and total GC decreased by 11.2% and 10.6% from day 7 to 21 ($p < 0.05$) and remained constant from day 21 to 35 of age ($p > 0.05$). Breed only affected the number of total GC ($p < 0.05$) with Ross showing 6.2% greater total GC number than Cobb. Sex had an impact on both GC number and density in the ileum and females showed slightly higher number (13.8% and 10.3%, respectively) and density (7.8% and 5.8%, respectively) of mixed and total GC compared with males ($p < 0.05$). However, no differences were observed for acidic GC number and density of males and females ($p > 0.05$). No influence of dietary treatments was found for the GC measurements ($p > 0.05$). The only significant interaction was between age and sex for total GC density ($p < 0.05$, supplementary Table 1B) and females showed higher total GC density at day 35 compared with males at the same age and both females and males at day 21 ($p < 0.05$). Females also showed greater total GC density at day 7 compared with females at day 21 and males at day 35 ($p < 0.05$).

The effects of the main factors on metabolite concentration (µmol/g of fresh sample) of the ileum are presented in Figs. 2 and 3 and supplementary Table 2A and 2B. The significant interaction effects on bacterial metabolites are shown in supplementary Table 2C and 2D. The main effect of dietary treatment, breed and sex had no impact on ileal metabolites concentrations ($p > 0.05$), except for D-lactate and D- to L-lactate ratio which was higher for Ross than Cobb ($p < 0.05$, Fig. 3). Age was the only main factor that altered concentration of all metabolites measured in the ileum ($p < 0.05$, Fig. 2), except for n-butyrate and histamine. Concentration of acetate accounted for approximately 90% of the total SCFA in the ileum, followed by propionate (4%), i-butyrate (3%) and n-valerate (1%). Concentration

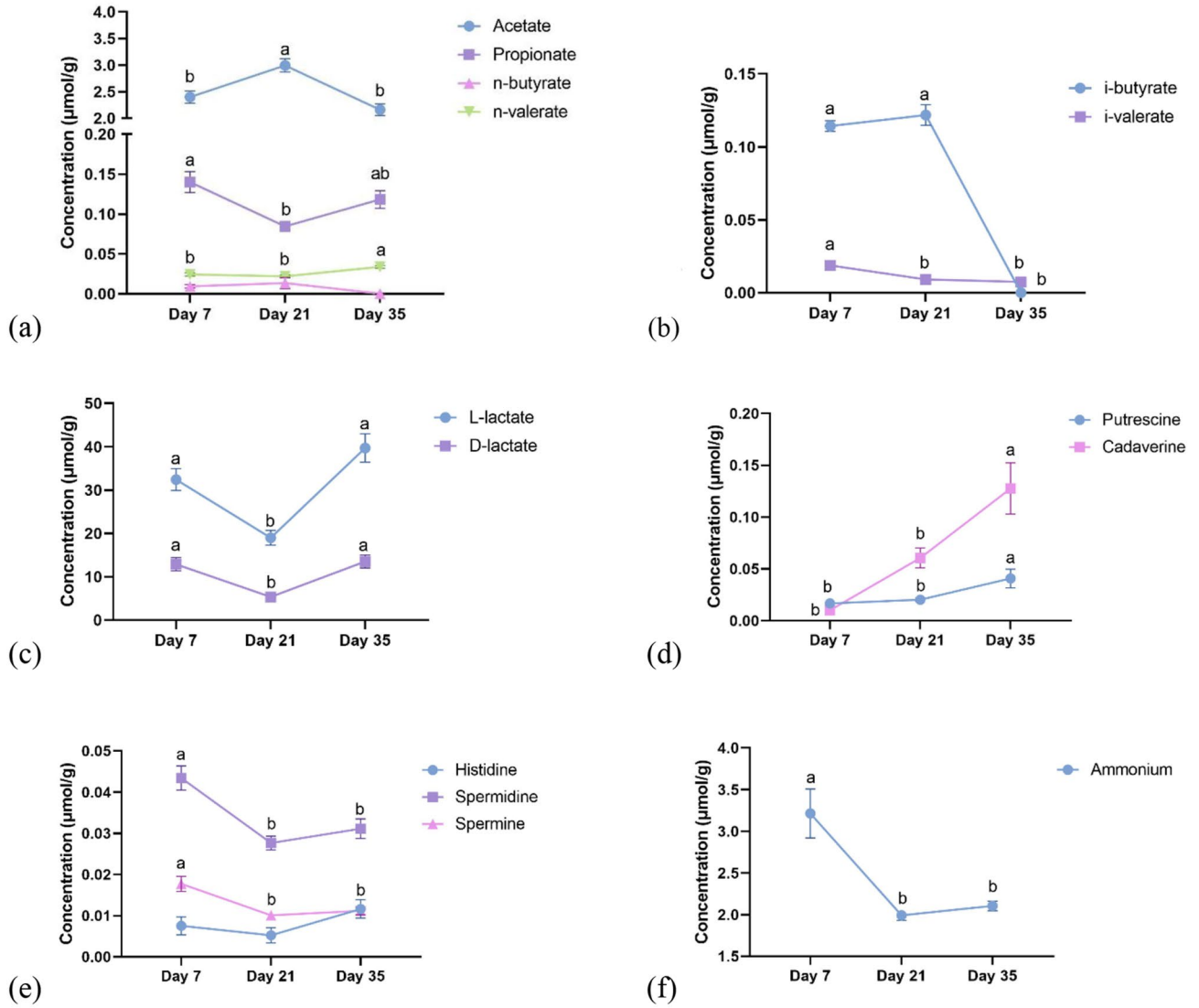

Fig. 2 (**a-f**) The effect of age (day 7, 21, and 35 of age) on metabolite concentration (µmol/g of fresh sample) in the ileum of broilers. The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex. All the data was presented in supplementary Table 2A and 2B.

of n-butyrate and i-valerate in ileal digesta was almost negligible (less than 0.4% of total SCFA). Concentration of acetate increased by 24.6% from day 7 to 21 and then decreased by 27.8% at day 35 ($p < 0.05$, Fig. 2a). Propionate concentration decreased by 42.9% from day 7 to 21 and then increased by 50.0% thereafter ($p < 0.05$, Fig. 2a). Concentration of n-valerate was higher in 35 days old broilers compared with the younger ones ($p < 0.05$), while n-butyrate was not affected by age ($p > 0.05$, Fig. 2a). Both i-butyrate and i-valerate concentrations decreased with age ($p < 0.05$, Fig. 2b) and were almost absent at day 35. Concentration of D- and L-lactate, total lactate and their ratio (D- to L-) was lowest at day 21, while those variables were not different between day 7 and 35 ($p < 0.05$, Fig. 2c). Concentration of D-, L- and total lactate concentration in the ileum decreased by 58%, 41%, and 46% between day 7 and 21, and increased by 152%, 109%, and 119% between day 21 and 35 ($p < 0.05$). Concentrations of all biogenic amines were also influenced by age ($p < 0.05$), except for histamine. Putrescine and cadaverine concentration were not different between day 7 and 21 ($p > 0.05$) but, from day 21 to 35, both metabolites increased their concentration by approximately 102% and 117%, respectively ($p < 0.05$, Fig. 2d). In contrast, spermidine and spermine concentration decreased by 25% and 50%, respectively from day 7 to 21 ($p < 0.05$, Fig. 2e) and remained stable after that. Ammonium concentration

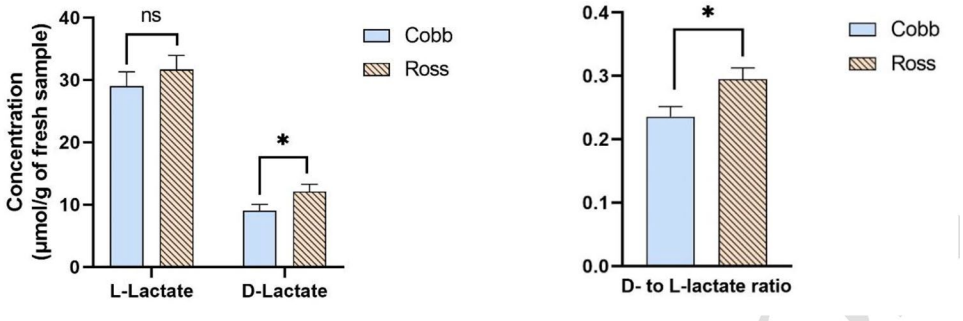

Fig. 3 (**a-e**) The effect of breed on L- and D-lactate concentration (µmol/g of fresh sample) and its ratio in the ileum of broilers. The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex. All the data were presented in supplementary Table 2A and 2B. *, significant difference ($p < 0.05$); ns, no significant difference ($p > 0.05$)

reduced by 38% from day 7 to 21 ($p < 0.05$, Fig. 2f) and remained stable thereafter. An interaction between breed and sex was observed for spermidine (supplementary Table 2C). The concentration of spermidine was lower in female-Ross broilers compared with male-Ross broilers ($p < 0.05$), but both male- and female-Ross broilers showed no difference in spermidine concentration compared with Cobb broilers, regardless of sex ($p > 0.05$). An interaction between age and breed was detected for ammonium, with 7 days old Ross broilers having the highest concentration of ileal ammonium, while other groups were not different from each other (supplementary Table 2D).

The main impacts of age, dietary treatment, breed, and sex on mRNA expression ($\log_{10}$ copy number per ng of RNA) of cytokines and the proteins related to epithelial barrier integrity of the ileum are shown in Figs. 4 and 5, and supplementary Table 3A and 3B. In addition, the significant interaction effect is shown in supplementary Table 3C. Age was the main factor that altered investigated mRNA expression ($p < 0.05$), while no impact of dietary treatment, breed and sex on the variables was observed ($p > 0.05$), except for impacts of breed on *IL-4*, *IL-6*, *TNF-α* and *IFN-γ* ($p < 0.05$). Overall, all mRNA expression of cytokines (*IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-17α* and *IL-18* as well as *IFN-γ* and *TGF-β2*) and epithelial barrier related proteins (*MUC2* and *CLDN5*) increased from day 7 to 21 where they reached the peak, and then decreased at 35 days of age ($p < 0.05$). Among the cytokines investigated, *IL-4* and *TGF-β2* showed a considerable change in their expression during 35 days of life; both were upregulated (537- and 631-fold) from day 7 to 21 and downregulated (117- and 417-fold) from day 21 to 35 ($p < 0.05$, Fig. 4b and c). There was an mRNA upregulation of *IL-1β* and *IL-12* by 295- and 107- fold between day 7 and 21, while a lesser degree of upregulation (between 7- and 81-fold) was found for the remaining cytokines, with the following order *IL-10* > *IL-18* > *IFN-γ* > *IL-2* > *TNF-α* > *IL-8* > *IL-17α* > *IL-6* ($p < 0.05$, Fig. 4a-d). On the other hand, mRNA expression of *IFN-γ* was downregulated by 100-fold between day 21 and 35, while a lesser degree of downregulation (between 13- and 61-fold) was found for *IL-17* > *TNF-α* > *IL-1β* > *IL-8* > *IL-2* > *IL-18* > *IL-12* > *IL-10* > *IL-6* in the following order ($p < 0.05$, Fig. 4a-d). The mRNA expression of barrier integrity related proteins including *MUC2* and *CLDN5* was upregulated by 148- and 214-fold from day 7 to 21 and downregulated by 55- and 60-fold from day 21 to 35 ($p < 0.05$, Fig. 4e).

For Cobb, mRNA expression of *IL-4*, *IL-6* and *TNF-α* was higher than Ross, while *IFN-γ* was higher for in Ross compared with Cobb ($p < 0.05$, Fig. 5). The interaction between age and breed had an impact on *IFN-γ* ($p < 0.05$, supplementary Table 3C). The expression of *IFN-γ* was highest in the ileum of Ross and Cobb at day 21 and was lowest in Ross at day 35 ($p < 0.05$). However, at day 7, *IFN-γ* expression was similar for Ross and Cobb but it was higher than Cobb at day 35 ($p < 0.05$).

Discussion

Before discussing the present findings, it is worth mentioning that the growth performance data of broilers used in the present study as well as the data on their caecal histomorphology, GC density, bacterial metabolites, and mRNA expression of cytokines and proteins related to intestinal integrity have been already published by Tous et al. (2022) and Duangnumsawang et al. (2022).

Over the past decade, it has been revealed that gut microbiota and their metabolites play a vital role in gut health of broilers and alter development and functionality of the gut and its immune system (Tang et al. 2020). Host-related factors including age, breed, and sex have been shown to affect

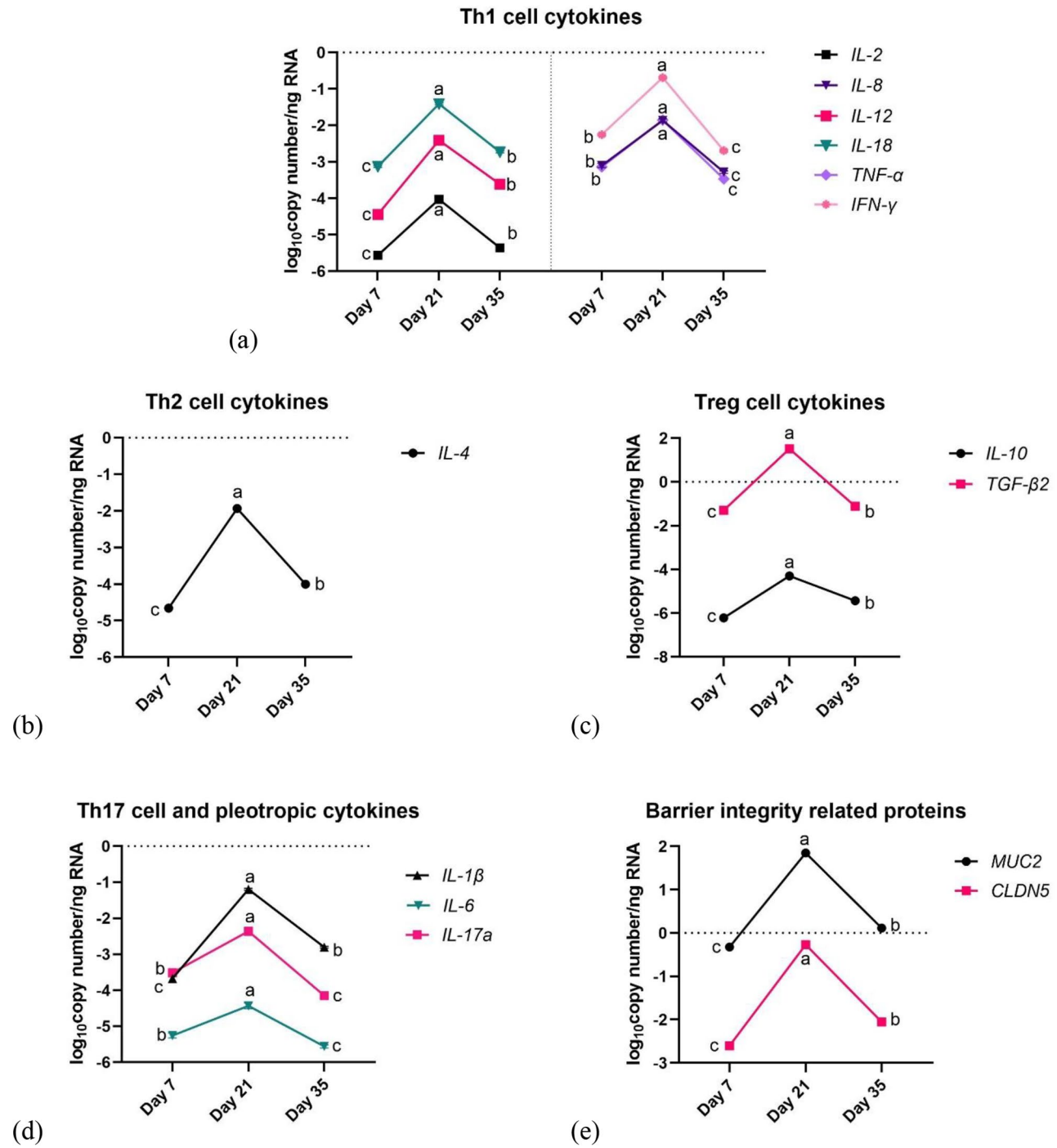

Fig. 4 (**a-e**) The effect of age on mRNA expression in the ileum of broilers. The mRNA expression represents as $\log_{10}$ copy number per ng of RNA (calculated by dividing the copy number of targeted mRNA with the copy number of the housekeeping genes, converting values to the copy number per total RNA, and then transformed to $\log_{10}$ scale). The trial was conducted with a $3 \times 2 \times 2$ factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex. All data were presented in supplementary Table 3A and 3B. [a, b, c] Means with different superscripts in each variable differ significantly ($p < 0.05$). *IL*, interleukin; *TNF-α*, Tumor necrosis factor alpha; *IFN-γ*, interferon gamma; *TGF-β2*, transforming growth factor beta 2; *CLDN5*, Claudin 5; *MUC2*, Mucin 2

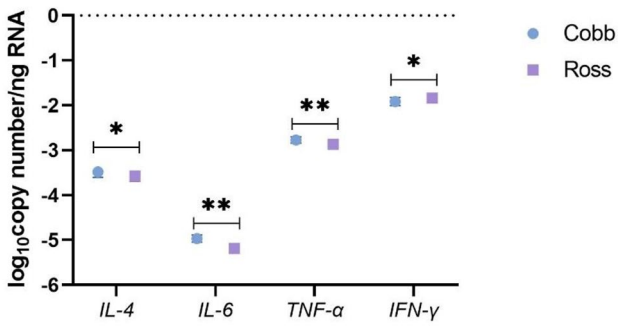

Fig. 5 The effect of breed on mRNA expression in the ileum of broilers. The mRNA expression represents as $\log_{10}$ copy number per ng of RNA (calculated by dividing the copy number of targeted mRNA with the copy number of the housekeeping genes, converting values to the copy number per total RNA, and then transformed to $\log_{10}$ scale). The trial was conducted with a $3 \times 2 \times 2$ factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex. All data were presented in supplementary Table 3A and 3B. *,** Means in each variable differ significantly (*, $p < 0.05$; **, $p < 0.01$). *IL*, interleukin; *TNF-α*, Tumor necrosis factor alpha; *IFN-γ*, interferon gamma

intestinal microbial community and immune responses (Torok et al. 2013; Richards-Rios et al. 2020). Genes are known to contribute to variation in physiological traits. Genotypic variations of broiler breeds and sexes may influence their gut physiology which creates a specific environment for microbial colonization. Differences in microbial composition and activity could result in distinct immune traits in the gut (Kers et al. 2018). In the present study, female broilers showed longer villi and higher number and density of mixed and total GC compared with male broilers, while their VW, CD, V/C, and VSA were similar. Studies reviewed by Heak et al. (2017) suggested that greater VH may be associated with increased surface area for nutrient absorption, while an increased number of GC may be linked to greater production of intestinal mucin resulting in higher endogenous loss (e.g., energy and protein) to the birds (Duangnumsawang et al. 2021). As a result, differences in these morphological variables may have an impact on growth performance between male and female broilers. Male birds showed better growth performance in terms of body weight gain, feed intake, and feed conversion ratio compared with females (Tous et al. 2022). However, it seems difficult to draw a clear conclusion regarding significance of the observed differences in gut morphology of male and female broiler chickens for their growth performance. In terms of the differences between breeds, Ross chickens had a higher total GC number, D-lactate concentration and D- to L-lactate ratio than Cobb broilers. In addition, Ross showed an upregulation of *IFN-γ*, while Cobb had a higher expression of *IL-4*, *IL-6*, and *TNF-α*. However, no significant differences between breeds were found for other variables measured ($p > 0.05$). A similar pattern for cytokine expression has been also reported in the caecum of the current broilers (the same trial, focusing on the caecum) by Duangnumsawang et al. (2022). Alterations in bacterial metabolites such as lactate (which is important energy source for the host) and differences in cytokine expression between breeds have been reported to affect immune responses and growth performance of the birds (Gadde et al. 2017; Lee et al. 2018). Differences in growth performance of these current birds (the same trial focusing on growth performance) were also observed, with Cobb having higher body weight gain, and feed intake, as well as lower feed conversion ratio compared with Ross (Tous et al. 2022). Therefore, differences in growth performance between Ross and Cobb may be attributed to variations in bacterial metabolite concentration and cytokine expression in the gut but the mode of actions behind it is not clear. Nevertheless, a nearly identical gut microbial activity and immune responses for both breeds and sexes in the present study could be attributed to the absence of harmful stimuli which are reportedly responsible for disrupting intestinal microbial populations and resulting in microbial dysbiosis, and induce gut immune responses in order to protect the gut from potential injuries (Mabelebele et al. 2017; Paraskeuas and Mountzouris 2019; (Wang et al. 2021b).

Modifications in gut microbiota of broilers induced by probiotics and phytobiotics have been demonstrated to be advantageous for gut homeostasis with promoting proliferation and metabolic activities of beneficial bacteria and suppressing those of pathogenic species (Krysiak et al. 2021). These modifications have been linked to improved growth performance in broilers (Lee et al. 2015). In this study, adding multi-strain *Bacillus* based probiotic or a procyanidin-rich phytobiotic to broiler diets did not show any impact on bacterial metabolic activity, morphology and mRNA expression of cytokines and proteins associated with mucus production and epithelial integrity in the ileum. In addition, the applied probiotic or phytobiotic product did not affect growth performance of the current birds (Tous et al. 2022). Broilers fed diets with *B. subtilis* showed an unchanged bacterial diversity (e.g. Shannon index) in the caecum (by using 16s rRNA analysis) compared with those fed control diet (Lin et al. 2017; Jacquier et al. 2019). However, these studies displayed that *Bacillus* spp. altered bacterial genera and their functional activities related to activation of immune responses in the ileum of broilers. Several studies showed that modification of gut microbiota by dietary *Bacillus* spp. improved gut barrier integrity and activated immune response in the ileum of broilers, as shown by an upregulation of tight junction proteins (e.g. occludin, *ZO-1* and *JAM-2*) and mucin (e.g. *MUC2*) as well as cytokines

(e.g. *IL-1β*, *IL-12*, *IFN-γ* and *IL-10*) (Rajput et al. 2013; Lee et al. 2015; Bilal et al. 2021). *Bacillus* based probiotics used in previous studies showed positive effects on gut barrier integrity and modulate host immune system, but the extent of these effects varied from strain to strain and also seemed to be dependent on dietary inclusion level, diets composition, environmental condition and age of animals (Yaqoob et al. 2022). Grape extract was found to affect bacterial metabolic activity in the gut through modulating phenolic metabolism of bacteria (Chamorro et al. 2019). An abundant source of polyphenolic compounds in grapes, mainly procyanidins, have been linked to reduced oxidative stress and intestinal inflammation in broilers (Chamorro et al. 2019). In the current study, beneficial impacts of grape extract on ileal bacterial activity, morphology, and immune responses of broilers have been scarce. Previous reports showed that polyphenol rich grape extracts could suppress pro-inflammatory cytokines (e.g. *IL-1β*) in the gut of broilers (Cao et al. 2020), while they stimulated anti-inflammatory cytokines including *IL-10* and *TGF-β1* in Caco-2 human colon cells (Nallathambi et al. 2020) and caecum of broilers (Duangnumsawang et al. 2022). Increasing grape procyanidin level in broiler diets has been shown to reduce concentration of sialic acid in ileal digesta, which may reflect the direct or indirect (through microbial alterations) effect of procyanidins in modifying mucin composition (Chamorro et al. 2019). However, adding procyanidin rich phytobiotic to broiler diets in the present study did not alter GC count and expression of *MUC2* (related to mucin production) in the ileum, which is in line with our previous study evaluating the same variables in the caecum of broilers (Duangnumsawang et al. 2022). The observed inconsistency in the outcome of different studies testing grape extracts could be because of environmental condition, experimental diet composition, molecular structure (e.g. degree of polymerization of procyanidins) and concentration of the active substances in the final diets (González-Quilen et al. 2020), as well as host-related factors, for instance, age, breed, and sex, which differed between trials and could have impacted gut microbiota development (Kers et al. 2018).

In the current study, ileal microbial metabolites, gut morphology and immune traits changed during the growth period. During co-development of the host and gut microbiota, products of bacterial metabolic activity including SCFA and lactate, could be the main factors modulating the host immune system (Yang et al. 2017b). At 35 days of age, lactate presented the highest concentration in the ileum with an average of 40.9 ± 2.29 mol/g, followed by acetate with an average of 2.5 ± 0.07 mol/g of fresh digesta. However, the age-related changes in concentration of acetate and lactate were in opposite directions; acetate concentration were highest on day 21, while lactate concentration was at its lowest point. In another study, the same pattern was observed for SCFA (increase) and lactate (decrease) concentration in the caecum of broilers after 2 weeks of age and it was attributed to the direct effect of lactate-utilizing bacteria or indirect effect of bacterial groups that playing a role in metabolic cross-feeding of fermentation products (Meimandipour et al. 2011). In this study, concentration of propionate, i- and n-valerate, and i-butyrate were present at low levels (up to 4% of total SCFA), while n-butyrate was nearly undetectable in the ileum, which seems to be in line with previous reports (Goodarzi Boroojeni et al. 2014; Liao et al. 2020). Biogenic amines, primarily putrescine and cadaverine, were found at a relatively low concentration during the first 21 days of life in this study, while their concentration increased by around 2-fold at day 35. Another study also found increased cadaverine levels (around 2-fold) in the ileum of older broilers (day 41) compared with younger ones (day 20), but putrescine levels slightly decreased as broilers aged (Tiihonen et al. 2010). The derivatives of putrescine including spermine and spermidine were present at low level (up to 0.05 µmol/g fresh digesta) in the ileum and their concentration was higher in young birds (day 7) compared with the older ones (day 21 and 35). Biogenic amines, especially putrescine, spermine and spermidine, have been found to enhance homeostasis of the intestinal mucosa and increase the rates of epithelial cell division and apoptosis through modulating the expression of various growth-related genes (Timmons et al. 2013). Thus, increasing the concentration of biogenic amines, especially putrescine with age in the present study may be associated with enterocytes and GC proliferation, altering villus and crypt structure as well as mucus production. This could be supported by previous study showing that putrescine *in ovo* injection enhanced cell proliferation as shown by increased VH and GC number in the ileum of broilers (Goes et al. 2021). In this study, ammonium concentration decreased and remained low after the first week of age. The putrefactive metabolites including branched chain fatty acids, biogenic amines and ammonium are products of protein fermentation, while SCFA and lactate are mainly derived from saccharolytic (carbohydrate) fermentation (Qaisrani et al. 2015). Thus, the concentration of these metabolites could be altered by availability of nutrients for the gut microbiota as well as the number and metabolic activity of protein- and carbohydrate-fermenting microorganisms. Using 16s rRNA sequencing has been shown that the relative abundance in bacterial composition and genome (gene based clone libraries) in the ileum and caecum of broilers were concurrently changed with age, during the first 7 weeks of life (Lu et al. 2003). Therefore, age-related changes in the gut bacterial metabolites may reflect alterations in microbial composition and metabolism which could be caused by different factors such as feeding

transition (e.g. feed form, structure, quality and composition), nutrient digestibility of feed, environmental factors (e.g. microbial load and hygiene status) and stress (e.g. environmental and physiological stresses). However, most metabolites, such as SCFA, are quickly absorbed by intestinal cells or transformed into other types of metabolites by gut bacteria (Gomez-Osorio et al. 2021). Thus, it should be noted that measuring concentration of bacterial metabolites in fresh digesta (per weight of digesta) provides only a snapshot of bacterial activity at that particular time and may not accurately represent the actual amount of metabolites produced over time.

The interaction between the intestinal immune system and commensal microbiota in chickens begins at hatching and the host immune system simultaneously responds to changes in the luminal environment as broilers grow. Cytokines act as intercellular immunological messengers promoting intestinal mucosal homeostasis, and they can also be significant drivers of intestinal inflammation and damage (Siddiqui et al. 2020). In general, the investigated cytokines in the present study were selected according to their immune regulatory function and production by T helper (Th) cells (Lee et al. 2019): Th1 cytokines (*IL-2*, *IL-8*, *IL-12*, *IL-18*, *TNF-α*, and *IFN-γ*), Th2 cytokines (*IL-4*), Th17 (*IL-17α*) and regulatory T (Treg) cytokines (*IL-10* and *TGF-β2*) as well as pleiotropic cytokines (IL-1β and *IL-6*). As reviewed by Rescigno and Di Sabatino (2009), Th2 cells are primarily related to the secretion of B cell growth factors including *IL-4*, while Th1 cells are inflammatory cells that direct immune reactions against intracellular pathogens and Th17 cells play a critical role in host defense against a variety of bacteria and fungi. In contrast, Treg cells suppress the functions of effector T cells and are essential to counteract inflammatory responses. The activation of multiple cell types by *IL-1β* and *IL-6* was previously reported; *IL-1β* is a pro-inflammatory cytokine that stimulates Th1, Th2 and Th17 cell proliferation (Muñoz-Wolf and Lavelle 2018) and *IL-6* has both pro- and anti-inflammatory actions that activates Th17 and inhibits Treg cell proliferation (Murakami et al. 2019). In the current study, mRNA expression of all the cytokines was highest at 3 weeks of age and then decreased. In another study, expression of *TGF-β1* and *IFN-γ* in the ileum of broilers was upregulated form day 20 to 27 and then downregulated at day 34, which was associated to an increase in T and B cell proliferation activity (Song et al. 2021). In general, epithelial cells can recognize luminal antigen and transmit this information to the immune cells in the lamina propria to secrete cytokines and restore the balance in the intestine (Mahapatro et al. 2021). During the first week after hatching, antigens from diet and environment construct an immune response in the gut of broilers via recruiting granulocyte and T-lymphocyte and generating cytokines, which could trigger immunological adaptation to luminal antigens and microbiota (Van Immerseel et al. 2002; Bar-Shira et al. 2003; Crhanova et al. 2011). When immunological stimulations (dietary and environmental stimuli) in the lumen reduce, the restoration of immunological balance can take place (Broom and Kogut 2018). An immune stabilization process following a shift of bacterial composition in the gut was suggested to be a mechanism that prevent the body from entering a state of excessive immune activity and to maintain the body's immune balance (Song et al. 2021). The temporary upregulation of all cytokines in the present study could be indicative of an overall immunological response to the physiological changes, microbial establishment/maturation and environmental stress during growth. Downregulation of all cytokines after day 21 may imply adaptation of the gut immune system to luminal antigens and microbiota after 3 weeks, leading to a lesser degree of immune stimulation in the gut. In this study, age-related changes in expression of the pro-and anti-inflammatory cytokines followed the same pattern. During the activation of pro-inflammatory pathway, the presence of anti-inflammatory cytokines may play a role in negative feedback mechanism of the inflammatory activity (Park et al. 2014). In this study, the observed age-related fluctuation of lactate and SCFA which are the main bacterial metabolites in the ileum, seemed to trigger both pro- and anti-inflammatory cytokines, which could be advantageous for immunological maturation and adaptation. Variations in ileal cytokine expression and acetate concentration appeared to be parallel and aligned, while variations in cytokines and lactate concentration were parallel but pointing in opposite directions. It has been shown that microbial metabolites such as SCFA and lactate regulate T cells differentiation and cytokine secretion (Park et al. 2014; Manoharan et al. 2021). In vitro addition of acetate, propionate, and butyrate promoted the differentiation of naïve $CD4^+$ T cells to effector (Th1 and Th17) and Treg cells, resulting in an upregulation of cytokines e.g. *IL-10*, *IFN-γ*, and *IL-17* (Park et al. 2014). It was also found that in vivo regulation of the host immune system by SCFA has been attributed to the direct effect of SCFA on the immune cells or their indirect impact through the cellular signals of the intestinal epithelial cells (Park et al. 2014). Lactate could also modulate the cellular signaling of immune cells such as dendritic cells and macrophages and regulated the development of Treg/Th1/Th17 cells, resulting in an induction of immune regulatory factors and inhibition of pro-inflammatory cytokines (Ranganathan et al. 2018).

MUC2 is a major constituent of mucins, forming a net-like structure of the intestinal mucus layer (Zhang and Wu 2020). In this study, expression of *MUC2* was upregulated during the first 21 days of age and then downregulated

until day 35. A previous study showed an increased *MUC2* expression in the ileum during the first week of life and then become steady until day 14 of age (Proszkowiec-Weglarz et al. 2020). However, Zhang et al. (2015) reported a steady expression of *MUC2* in the ileum and caecum of broilers after hatching until 3 weeks of age. The pattern of *MUC2* expression could be influenced by bacterial colonization and subsequent host response that increases mucin secretion to limit the epithelial contact with intestinal bacteria (Zhang et al. 2015). The claudin family is a key component that forms epithelial tight junctions that regulate paracellular permeability, epithelial polarization, and conservation of transepithelial resistance, as well as the selective passage of molecules and ions in the chicken intestine (Turner 2009; von Buchholz et al. 2021). *CLDN5* is the main barrier-forming claudins between adjacent epithelial cells in the gut of chicken which involves in paracellular permeability and intestinal homeostasis (Ozden et al. 2010). The immunostaining of *CLDN5* has been shown to be stronger in the crypt and lower villus regions of the small intestine of newly hatched broilers compared with other *CLDN* family such as *CLDN3* (Ozden et al. 2010). Therefore, expression of *CLDN5* in ileal mucosa may be a good marker for evaluating tight junction in the ileum. Like *MUC2* and other cytokines, *CLDN5* expression reached a peak at day 21 in this study. In contrast to this study, expression of tight junction proteins including *CLDN1* and *CLDN5* in the jejunum and ileum of broilers decreased after hatch and became stable during the first 2 weeks of life. It has been discussed that expression of tight junction proteins could be a result of a compensatory mechanism responding to alterations in microbial composition and restoring intestinal permeability (Proszkowiec-Weglarz et al. 2020). Similarities in the expression patterns of *MUC2*, *CLDN5* and all the investigated cytokines may imply that alterations in immune system (e.g. cytokines) may subsequently influence mucus production and epithelial integrity of the gut. This speculation is supported by Mahapatro et al. (2021) study which demonstrated the regulatory mechanisms of pro-inflammatory cytokines (e.g. *IL-4*, *IL-18*, *IFN-γ* and *TNF*) that induced the differentiation of progenitor cells to cells of the secretory lineage such as GC and increased mucus production, thereby restoring the intestinal epithelial barrier.

The crypt-villus morphology in the ileum provides the environment for digestion and absorption, while its structure could be simultaneously affected by commensal or pathogenic microorganisms residing in the gut. In this study, all morphological variables including VH, VW, CD, V/C and VSA increased with age, with VH and VSA showing 85% and 153% increase from day 7 to 35. Longer intestinal villi are associated with an increase in the absorptive surface of the intestines which support increase in nutrients requirement during broiler growth (Awad et al. 2009). Bacterial metabolites such as SCFA and lactate are known to affect villus and crypt morphology (Lee et al. 2018). As a source of energy, butyrate plays a vital role in promoting intestinal development and maintaining the integrity of the intestinal epithelial cells (Zou et al., 2019). Acetate has been shown to alter intestinal cell apoptosis and mucus production (Liu et al., 2017). Propionate is also a potent fatty acid that modulate intestinal cell activity including differentiation and apoptosis (Hosseini et al., 2011). Lactate possesses diverse metabolic and regulatory properties, such as being an energy source and a signaling molecule for intestinal stem cell and goblet cell regeneration (Lee et al. 2018). Besides these main metabolites, as mentioned earlier, some biogenic amines also alter regeneration of the epithelial cells, while high concentration of ammonia may cause cell damage (Rehman et al. 2007). Therefore, age-related alterations in the investigated metabolites in this study may influence cell differentiation and proliferation in the ileum.

In the present study, the number of total GC (per villus) increased from day 7 to 35 of age, while the density of total GC decreased during the first 3 weeks of age and then it became stable. Other studies have also shown an increase in GC number per villus in the ileum of broilers during 3–5 weeks of age (Sikandar et al. 2017; Thiam et al. 2021). In accordance with the present data, Duangnumsawang et al. (2021) demonstrated that the GC density in the ileum is relatively high during the first week of age, but it tends to decrease afterward until the third week of life and then becomes stable between the third and fifth week of age. Mucin-secreting GC are the first line of defense in the mucosa and mucins secreted by GC can protect epithelial cells from pathogens, chemical and mechanical damages. Therefore, mucin-secreting GC develop and mature after hatch as a response to external stimuli including intestinal microbiota, dietary factors and antigens from diet and environment (Duangnumsawang et al. 2021). It has been also reported that changes in GC number of the gut could be due to biological mechanisms such as cell proliferation and apoptosis regulated by direct and/or interaction effect of gut microbiota (dysbiosis and symbiosis) and host immune response (Deplancke and Gaskins 2001). The observed pattern for age-related changes in GC density in the current study, might be also caused by immunological adaptation of the gut immune system to the luminal substances (e.g. feed, microbiota, antigens, etc.).

Mucins are the major components of the intestinal mucus layer and can be classified into neutral and acidic subtypes based on their net molecular charge. Acidic type expresses a net negative charge and neutral type exhibits a net neutral charge of the mucin molecule (Derrien et al. 2010). The distinct pattern of mucins in the gut may reflect

differential host responsiveness to specific bacterial communities or metabolites (Deplancke and Gaskins 2001). In this study, the majority of GC population was presented as a mixed type (containing relatively similar proportion of acidic and neutral mucins) and the remaining GC can be categorized as acidic type, suggesting that the proportion of secreted acidic mucins in the mucus layer of the ileum may be greater than neutral mucins. High prevalence of acidic mucins was also reported in villi of the duodenum, jejunum, and ileum (Sikandar et al. 2017) as well as crypts in the caecum of broilers (Duangnumsawang et al. 2022). In the current study, the number of acidic GC increased by 4.5 times from day 7 to 35, whereas mixed GC number increased only by 1.5 times during 21 days of age and remained stable afterward. The density of acidic GC was also increased by around 2.6 times during the whole period of this study, while mixed GC density decreased with age. A greater number of GC, particularly acidic GC, may result in the production of more acidic mucins which appear to be less degradable by bacterial and host enzymes, thereby increasing resistance to pathogens and mechanical irritation (Montagne et al. 2004). Indeed, increasing the proportion of negatively charged (acidic) mucins alters physiochemical interactions between mucin molecules causing an increase in viscosity of the mucus layer, which may be associated with an age-related increase in gut bacterial diversity and bacterial-derived compounds (Liao et al. 2020). Modification of mucin molecules such as sialylation and sulfation, converts neutral mucins into acidic mucins and is reportedly promoted along with GC maturation (Hino et al. 2012). Thus, increased acidic mucins in the villus of ileum of current broilers may reflect GC maturation with age, which also enhance the protective property of intestinal mucus layer. According to Duangnumsawang et al. (2022), GC density, especially the acidic type was lower in the caecum (crypts) of broilers (used in the present study) compared with their ileum (villi). The observed variation in GC density in the ileum and caecum could be attributed to their morphology, physiological function and absorption capacity as well as bacterial number, composition and activity. In the ileum, GC population along villi secretes protective mucus layer to cover the epithelial surface while facilitating nutrients transportation from lumen to the underlying epithelium. In contrast, caecum acts as a fermentation chamber for microflora with higher bacterial number and activity than the ileum (Goodarzi Boroojeni et al. 2014), while nutrient absorption is not its main physiological function. Moreover, the mucus layer in the small intestine is usually thinner than the hindgut due to gut motility which propel digesta and mucus to the distal part of the intestine (Herath et al. 2020), thus may increase mucus renewal and stimulate GC proliferation in the ileum compared with the caecum.

Conclusion

The present data demonstrated that age of broilers had a significant impact on microbial activity and immunological responses in the ileum, while the effect of probiotic or phytobiotic supplementation was totally absent. The genetic background of broilers, particularly their breed (Ross and Cobb), was found to have an effect on goblet cell count, certain bacterial metabolites, and cytokines expression in the ileum, while sex had almost no impact on these variables. A few interaction effects between the main factors were found on some of the investigated variables but they did not show meaningful biological patterns. This study was able to capture the alterations in microbial metabolites in the ileum of broilers at different ages which could potentially affect the development of gut morphology, goblet cell density, as well as expression of the cytokines and the proteins involved in epithelial barrier integrity. The observed age-related effects could be explained by the interaction between the gut microbiota and immune system and the direct effect of microbial metabolites on the gut morphology and cytokine response profile. Gut microbiota could affect maturation of the host immune system through its bioactive substances. However, further research is required to understand better the mechanisms behind the interaction between the host and its gut microbiota.

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Author contributions All authors designed the experiments, JT and FGB performed the experiment. WV provided mRNA abundance data. YD analyzed the histomorphology, performed data analysis, and wrote the manuscript. JZ and FGB revised and finalized the manuscript. All authors approved the final version of the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval The animal study was reviewed and approved by Ethical Committees of Generalitat de Catalunya, Spain (Proceeding number 10,226).

Conflict of Interest Authors declare no conflict of interest.

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4. Chapter 4: Main part of the thesis (part II)

Alterations in bacterial metabolites, cytokines, and mucosal integrity in the caecum of broilers caused by feed additives and host-related factors

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Alterations in bacterial metabolites, cytokines, and mucosal integrity in the caecum of broilers caused by feed additives and host-related factors

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A total of 2,880 one-day-old male and female broiler chicks from two breeds, Ross308 and Cobb500 were randomly assigned to 72 pens. Broilers were offered three diets: a wheat-soybean diet without (CO), or with either a probiotic (probiotic; 2.4 x 10^9 CFU/kg diet of *Bacillus subtilis* DSM32324 and DSM32325 and *B. amyloliquefaciens* DSM25840) or a phytobiotic (phytobiotic; grape extract with 165 ppm procyanidin and 585 ppm polyphenol) product. The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment (40 birds per pen). At day 7, 21, and 35, one chicken per pen was slaughtered for caecal sampling to quantify bacterial metabolites (digesta) as well as evaluate mRNA abundance and histomorphology (tissue). Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex and their interactions. Spearman's correlation (r) was analyzed between metabolite concentration and mRNA abundance. Overall, the concentration of short chain fatty acids increased with age, while lactate decreased from day 7 to 21 ($p < 0.05$). The mRNA abundance of IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IL-18, IFN-γ and TGF-β2 increased with age but IL-1β and TNF-α increased in abundance from day 7 to 21 and then decreased ($p < 0.05$). Abundance of MUC2 and CLDN5 increased after day 21 ($p < 0.05$). Caecal crypt depth increased with age ($p < 0.05$). Acidic goblet cell (GC) number peaked at day 21 ($p < 0.05$), while mixed GC number was not affected by age. A few impacts of breed, diet and interactions on the investigated variables showed no meaningful biological pattern. Propionate positively correlated with all cytokines investigated (r = 0.150–0.548), except TNF-α. Lactate negatively correlated with pro-inflammatory cytokines like IL-1β (r = –0.324). Aging affected caecal histomorphology, bacterial activity and genes responsible for barrier integrity and inflammatory response. This effect could be attributed to the interaction between gut microbiota and immune system as well as the direct effect of metabolites on gut histomorphology and cytokine mRNA abundance.

KEYWORDS

short chain fatty acids, goblet cells, mucosal immunity, cytokines, host-microbe interactions, commercial broilers

1 Introduction

Probiotics and phytobiotics have been used as potential substitutes for antibiotic growth promoters, with the goal to improve animal health and performance. The health advantages of probiotics are suggested to be related to their ability to modify the gut microbiota and its metabolic activity, as well as their subsequent role in modulation of the immune system (Lee et al., 2015; Park et al., 2020). Probiotics from genus *Bacillus* spp. have been receiving a great interest lately, because of their spore-forming abilities, which give them a number of advantages in terms of viability and stability during feed processing and also in the gut (Goodarzi Boroojeni et al., 2016; Zentek and Goodarzi Boroojeni, 2020). Adding *Bacillus* spp. to broiler feed inhibit intestinal pathogens, modify the bacterial community and their metabolic activity, diminish gut inflammation, modify mucosal morphology, and finally improve growth performance (Song et al., 2014; Park et al., 2020). Phytobiotics are a diverse group of plant-based products (including essential oils, herbs, and fruit extracts) with demonstrated health effects on gut health through their antioxidant, anti-inflammatory, and antibacterial properties (Viveros et al., 2011). Plant polyphenols such as procyanidins improve gut immunity through modifying intestinal microbiota, reducing oxidative stress, and modulating the expression of cytokines in the gut (Gessner et al., 2017). Procyanidins, the primary polyphenols in grape extract, can be catabolized by the intestinal microbiota into phenolic acids and other metabolites that help reducing oxidative stress and inflammation in the broilers gut (Chamorro et al., 2019; Cao et al., 2020). Grape polyphenols also found to increase short chain fatty acids (SCFA), and regulate the immune response and gut barrier integrity in broilers (Yang J. Y. et al., 2017; Cao et al., 2020).

The interactions of gut microbiota with their host affect immune responses, gut morphology and integrity (Apajalahti and Vienola, 2016). Adaptations in the intestinal microbial population occur concurrently with broiler growth. In newly hatched chicks, the gut bacterial community was already present but could only be characterized by limited bacterial diversity. However, the bacterial community composition, diversity and richness evolved over time (Glendinning et al., 2019). There are also some evidences showing that broiler's intestinal microbiota can be affected by host genotype (Emami et al., 2022) and sex (Lumpkins et al., 2008). Despite the fact that commercial broilers are co-selected for performance and immunocompetence, their genetic make-up still affects their immune response to certain challenges (Cheema et al., 2003; Mayahi et al., 2016). For instance, different immunological developments and inflammatory responses (e.g. expression of pro-inflammatory cytokines) in Ross308 and Cobb500 have been attributed to their differences in gut microbial composition and activity (Hong et al., 2012; Richards et al., 2019). On the other hand, different characteristics in the gut morphology, such as villus height and crypt depth (Mabelebele et al., 2017), as well as distinct immunological traits and response to pathological challenges (Hong et al., 2012) contribute to a breed-specific bacterial community. Therefore, bacterial community and immunological status of different commercial breeds that are reared under the same environmental and nutritional conditions, may still differ. Generally, male broilers are known to have a higher growth rate and final body weight than females. This difference on growth rate between sexes may have an effect on composition of gut bacteria (Lumpkins et al., 2008). Since host-related factors can modulate the composition of gut microbes and gut immune responses, the environmental factors such as dietary treatment may interact with them and boost or discount their impacts. Hence, this study was conducted to evaluate the effect of feed additives (probiotics and phytobiotics), host-related parameters (age, breed, and sex) and their interactions on mucosal morphology, goblet cell number, bacterial metabolites, and mRNA abundance of cytokines, Mucin 2 (MUC2) and Claudin 5 (CLDN5) in the caecum of broilers. Additionally, the relationship between bacterial metabolites and cytokine responses in the gut was investigated, which may describe the interactions between gut microbiota and immune system.

2 Materials and methods

2.1 Animals and experimental diets

A total of 2,880 one-day-old male and female broiler chicks consisting of 1,440 Ross308® (RS) and 1,440 Cobb500® (CB) were randomly allocated into 72 pens (2.25 m^2) with a softwood shaving floor. Three experimental diets including a standard wheat-soybean based diet without (CO) or with supplementation of either a probiotic (PO) or a phytobiotic (PY) product were produced and randomly assigned to birds. The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen (24 replicate-pens per diet, 36 replicate-pens per sex and 36 replicate-pens per breed). The experiment lasted 37 days. The experimental diets (starter

TABLE 1 Dietary ingredients and nutrient composition.

Ingredients (%)	Starter (0–7 days old)	Grower (8–21 days old)	Finisher (22–37 days old)
Wheat	52.8	61.2	62.0
Soybean meal (48 % CP)	39.4	30.5	15.9
Soybean oil	4.16	4.80	0.00
Animal Fat (5 SYSFEED) [a]	-	-	4.01
Extruded soybean	-	-	15.00
Dicalcium phosphate	1.85	1.66	1.50
Calcium carbonate	0.53	0.48	0.44
Vitamin-mineral premix [b]	0.40	0.40	0.40
Sodium chloride	0.37	0.37	0.35
DL-methionine	0.27	0.23	0.19
L-lysine HCl	0.16	0.19	0.15
L-threonine	0.05	0.05	0.04
Choline chloride	0.03	0.05	0.05
Antioxidant (Noxyfeed 56P) [c]	0.02	0.02	0.02
Sodium bicarbonate	-	0.010	0.002
Calculated nutrients			
AME, kcal/kg	2900	3000	3100
Lysine, g/kg	14.2	12.1	10.8
Methionine + cysteine, g/kg	10.1	8.8	8.1
Threonine, g/kg	9.3	7.9	7.2
Calcium, g/kg	9.6	8.7	8.1
Total phosphorus, g/kg	6.9	6.3	6.0
Sodium, g/kg	1.6	1.6	1.6
Analyzed nutrients			
Dry matter, g/kg	892	894	901
Crude protein, g/kg	245	213	201
Ether extract, g/kg	57	63	84
Ash, g/kg	58	52	49

[a]Product of Sysfeed SLU (Granollers, Spain), containing 1.5% myristic acid (C14:0), 18% palmitic acid (C16:0), 2% palmitoleic acid (C16:1 n-7), 14% stearic acid (C18:0), 28% oleic acid (C18:1 n-9 cis), 12% linoleic acid (C18:2 n-6 cis) and 6% α-linolenic acid (C18:3 n-3 cis).
[b]One kg of feed contains: Vitamin A: 10,000 IU; Vitamin D3: 4 800 IU; Vitamin E: 45 mg; Vitamin K3: 3 mg; Vitamin B1: 3 mg; Vitamin B2: 9 mg; Vitamin B6: 4.5 mg: Vitamin B12: 40 μg; Folic acid: 1.8 mg; Biotin: 150 μg; Calcium pantothenate: 16.5 mg; Niacin: 65 mg; Mn (as MnSO4.H2O): 90 mg; Zn (as ZnO): 66 mg; I (as KI): 1.2 mg; Fe (as FeSO4.H2O): 54 mg; Cu (as CuSO4.5H20): 12 mg; Se (as NaSeO3): 0.18 mg; BHT: 25 mg; Calcium formiate, 5 mg; Silicic acid, dry and precipitated, 25 mg; Calcium stearate, 25 mg; Calcium carbonate to 4 g
[c]Product of Itpsa (Barcelona, Spain), containing 56% of antioxidant substances (butylated hydroxytoluene + propyl gallate), 14% of citric acid and 30% of sepiolite as carrier.

diets for day 0–7, grower diets for day 8–21 and finisher diets for day 22–37) were formulated (Table 1) to meet or exceed recommendations of FEDNA (2018). The diets were offered in crumble form for the starter period and in 3 mm pellets later on. The probiotic product (GalliPro EPB5, Chr. Hansen, Denmark) which consists of *Bacillus subtilis* DSM32324 and DSM32325 and *B. amyloliquefacens* DSM25840 was added into the PO diets at a dosage of 2.4 x 10^9 CFU/kg diet. The phytobiotic product (NutriPhy® White Grape 100, Chr. Hansen, Denmark) was included into the PY diets making a final concentration of 165 ppm procyanidin and 585 ppm total polyphenol in the diets. The applied dosages were according to the manufacturer recommendation.

2.2 Sample collection

Six birds per pen were randomly selected at 7, 21 and 35 days of age and the one with the closest body weight to the averaged

pen-weight was used for the intended analysis. The birds selected for the analysis were sacrificed to dissect the caecum. The digesta was collected from proximal part of the right caecum and subsequently were frozen in liquid nitrogen and stored at -80°C until further analysis to quantify metabolite concentration. The caecal tissue collected from distal part of the right caecum was used for histomorphological analyzes. Tissues were fixed in 4 % (vol:vol) phosphate-buffered formaldehyde immediately after slaughtering and then transferred to 70 % ethanol until further analysis. Distal part of the left caecal tissue was collected for measuring mRNA abundance related to epithelial barrier proteins and inflammatory markers. Then, the tissue was stored in RNAlater buffer at -80°C until further analysis.

2.3 Histomorphological analyzes

The tissue samples were dehydrated, cleared with xylene and embedded with paraffin. Serial of 3 µm sections were prepared, mounted on glass slides and stained with Alcian blue-periodic acid-Schiff (AB-PAS) following manufacture's protocol (AB-8GX, Sigma; Schiff's reagent, Merck, Darmstadt, Germany).

Ten crypts from each caecal sample were selected for histomorphological analysis. Crypt depth (CD) was defined as its invagination depth. The number of acidic (blue), neutral (pink), mixed (purple), and total goblet cells (GC) in each crypt was counted. The density of GC was calculated from the number of GC per crypt divided by 100 µm of CD. All measurements were performed with an Olympus light microscope (BX 43, Olympus, Germany), which was equipped with a digital camera (DP72, Olympus, Germany). Image analysis was performed by using cellSens Standard software (version 1.14, Olympus, Germany) and ImageJ software (Rasband, W.S. ImageJ, United States National Institutes of Health, Bethesda, Maryland, United States).

2.4 Metabolite analyzes

Analysis of SCFA was performed by gas chromatography on an Agilent 6890 gas chromatography system with flame ionization detector and autosampler (Agilent Technologies, Böblingen, Germany), using the method described by Goodarzi Boroojeni et al. (2014). D- and L-lactate were analyzed by high-performance liquid chromatography on an Agilent 1100 chromatograph equipped with a Phenomenex C18 (4.0 × 2.0 mm^2) guard column followed by a Phenomenex Chirex 3126 (D)-penicillamine column (150 × 4.6 mm^2) and a UV detector at 253 nm, using the method described by Goodarzi Boroojeni et al. (2014).

2.5 RNA isolation and real time-quantitative PCR

The total RNA of caecal tissue was extracted by using NucleoSpin® RNA Plus kit and NucleoSpin® RNA clean-up (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The mRNA quality and quantity were analyzed by a Bioanalyzer (Agilent 2100, Agilent, Waldbronn, Germany). Subsequently, reverse transcription of total RNA into cDNA in a final volume of 40 µl was executed using the Super Script III Reverse Transcriptase First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, California). Primers used for the interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IL-18, tumor necrosis factor-α (TNF-α), interferon γ (IFN-γ), transforming growth factor-beta 2 (TGF-β2), MUC2 and CLDN5 are presented in Table 2. The RT-qPCR was conducted with a Stratagene MX3000p (Stratagene, Amsterdam, Netherlands). The reference genes β-actin, glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) and β2-microglobulin were used for normalization and times-fold abundance was determined based on mean cycle threshold values of the housekeeping genes using the relative abundance software tool REST© (Pfaffl, 2002). The mRNA abundance was calculated as copy number per ng of total RNA. Then this value was divided by mean copy number of house-keeping genes to compare the abundance of targeted genes in different treatment groups.

2.6 Statistical analyzes

Statistical analyzes were conducted using SPSS 26 (SPSS Inc. Chicago, IL, United States). Data were subjected to ANOVA using GLM procedure to evaluate the main factors including three ages (day 7, 21 and 35 of age), three dietary treatments (CO, PO and PY), two breeds (RS and CB), and two sexes (male and female) and their interactions. Means were separated by the Tukey least significant difference post hoc test at $p < 0.05$ statistical level. Means and pooled standard error of the mean (SEM) were reported for all variables measured. Replicate-pen was the experimental unit for all variables measured.

Spearman's rank correlation coefficients (expressed as r) were used to assess associations between bacterial metabolites and mRNA abundance of the investigated genes, using the Spearman's test in SPSS 26 and illustrated in GraphPad Prism 9.0.2 for Windows (GraphPad Software, San Diego, California United States). A *p*-value below 0.05 was considered as statistically significant.

TABLE 2 Primer sequences used for RT-PCR analysis.

Targets[a]	Sequences of primers (5′–3′)	A_T[b]	References
IL-1β	GACATCTTCGACATCAACCAG CCGCTCATCACACACGACAT	60	Institute of Animal Nutrition, Freie Universität Berlin
IL-2	TCTGGGACCACTGTATGCTCT ACACCAGTGGGAAACAGTATCA	60	Hong et al. (2006)
IL-4	AACATGCGTCAGCTCCTGAAT TCTGCTAGGAACTTCTCCATTGAA	60	Avery et al. (2004)
IL-6	CTGCAGGACGAGATGTGCAA AGGTCTGAAAGGCGAACAGG	60	Institute of Animal Nutrition, Freie Universität Berlin
IL-8	GGCTTGCTAGGGGAAATGA AGCTGACTCTGACTAGGAAACTGT	60	Hong et al. (2006)
IL-10	GGAGGTTTCGGTGGAAGGAG GTTAAGCTGCCATTGAGCCG	60	Institute of Animal Nutrition, Freie Universität Berlin
IL-12	AGACTCCAATGGGCAAATGA CTCTTCGGCAAATGGACAGT	60	Hong et al. (2006)
IL-17α	AAGCGGTTGTGGTCCTCAT CTCCGATCCCTTATTCTCCTC	60	Hong et al. (2006)
IL-18	GGAATGCGATGCCTTTTG ATTTTCCCATGCTCTTTCTCA	60	Hong et al. (2006)
TNF-α	CTCGTTGGTGTGGGACGAC CGGCGGCGTATCGAAGTA	60	Institute of Animal Nutrition, Freie Universität Berlin
IFN-γ	CTCCCGATGAACGACTTGAG CTGAGACTGGCTCCTTTTCC	60	Sadeyen et al. (2004)
TGF-β2	TGCACTGCTATCTCCTGA ATTTTGTAAACTTCTTTGGCG	60	Sundaresan et al. (2008)
MUC2	TGGCTGTGTAACTGCACCAA GTGGGTTTAGGAGGTGGCTC	60	Institute of Animal Nutrition, Freie Universität Berlin
CLDN5	CATCACTTCTCCTTCGTCAGC GCACAAAGCTCTCCCAGGTC	60	Institute of Animal Nutrition, Freie Universität Berlin
β-actin	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	60	Li et al. (2005)
GAPDH	GGTGGTGCTAAGCGTGTTA CCCTCCACAATGCCAA	60	Li et al. (2005)
β2-microglobulin	AAGGAGCCGCAGGTCTAC CTTGCTCTTTGCCGTCATAC	60	Li et al. (2005)

[a]Three reference genes including β-actin, GAPDH and β2-microglobulin were used as house-keeping genes.
[b]A_T, annealing temperature (°C)
IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β2, transforming growth factor beta 2; CLDN5, Claudin 5; MUC2, Mucin 2; GAPDH, glycerinaldehyde-3-phosphate-dehydrogenase.

3 Results

The results of histomorphological analysis in the caecum is shown in Table 3. Overall, the averaged CD in the caecum increased with age ($p < 0.05$) by 105 % from day 7 to 35. No effect of dietary treatment, breed and sex was observed on the caecal CD ($p > 0.05$). The majority of GC presented in the caecum was mixed GC (61.2 %–85.2 %) and the remaining was acidic. Neutral GC was not present in most of the samples and when present, their number was negligible. The number of acidic GC per crypt was increased by 39 % from day 7 to 21 of age and then decreased by 38 % at 35 days of age ($p < 0.05$). The number of mixed and total GC per crypt was not affected by age ($p > 0.05$), but numerically increased from 7 to 35 days of age. For the GC density (no. of cells/100 um CD), the mixed and total GC density decreased by 39.4 % and 34.3 % from day 7 to 21 of age, respectively ($p < 0.05$) and no further changes were found at day 35 of age ($p > 0.05$). The acidic GC density decreased by 20.0 % from day 7 to 21 and by 42.9 % from day 21 to 35 ($p < 0.05$). Breed effect was found for mixed and total GC density, with a greater density in RS by 11.3 % and 7.37 %, respectively compared with CB ($p < 0.05$). The number of mixed GC per crypt was also 13.5 % higher in RS than CB ($p < 0.05$). Dietary treatment and sex had no impact on GC number and density in the caecal crypt ($p > 0.05$). There was interaction between age and dietary treatment for mixed and total GC density ($p = 0.032$ and 0.049, Supplementary Table S3A,B) and between age, breed, sex and dietary treatment for mixed GC density ($p = 0.038$,

TABLE 3 The effect of age, dietary treatment, breed and sex on histomorphology in the caecum of broilers[a].

Parameters*	Age (A)			Dietary treatment (T)			Breed (B)		Sex (S)		SEM	*p*-value			
	7	21	35	CO	PO	PY	RS	CB	M	F		A	T	B	S
Caecal morphology															
CD[b]	150[c]	257[b]	308[a]	263	257	251	254	260	261	253	5.5	<0.001	0.929	0.652	0.325
Goblet cell number[c]															
Acidic	5.2[b]	7.2[a]	4.5[b]	6.0	5.9	5.3	5.6	5.8	5.8	5.7	0.24	<0.001	0.477	0.835	0.643
Mixed	14.2	15.2	17.2	15.6	15.6	16.1	16.8[a]	14.8[b]	15.6	16.0	0.45	0.052	0.921	0.032	0.741
Total	19.4	22.4	21.6	21.6	21.5	21.4	22.4	20.6	21.4	21.6	0.51	0.157	0.975	0.072	0.627
Goblet cell density[d]															
Acidic	3.5[a]	2.8[b]	1.6[c]	2.5	2.6	2.3	2.4	2.5	2.4	2.5	0.11	<0.001	0.355	0.604	0.339
Mixed	9.9[a]	6.0[b]	6.0[b]	6.5	6.9	6.8	7.1[a]	6.3[b]	6.6	6.9	0.22	<0.001	0.400	0.009	0.448
Total	13.4[a]	8.8[b]	7.5[b]	9.0	9.5	9.0	9.5[a]	8.8[b]	9.0	9.4	0.27	<0.001	0.376	0.047	0.279

[a]The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex and their interactions.
[b]Crypt depth are measured in μm.
[c]The average number of goblet cells per caceal crypt. Acidic represents the cells that are positive to Alcian blue dye. Mixed represents the cells that are positive to both Alcian blue and PAS dye. Total represents the sum of acidic and mixed goblet cells.
[d]The average number of goblet cells per 100 μm length of the crypt depth. Acidic represents the cells that are positive to Alcian blue dye. Mixed represents the cells that are positive to both Alcian blue and PAS dye. Total represents the sum of acidic and mixed goblet cells.
[a,b,c]Means with different superscripts in a row within the main factor differ significantly ($p < 0.05$).
*CO, control; PO, probiotic product; PY, phytobiotic product; RS, Ross; CB, Cobb; M, male; F, female.

TABLE 4 The effect of age, dietary treatment, breed and sex on metabolite concentration (μmol/g of fresh sample) in the caecum[a].

Parameters*	Age (A)			Dietary treatment (T)			Breed (B)		Sex (S)		SEM	*p*-value			
	7	21	35	CO	PO	PY	RS	CB	M	F		A	T	B	S
Short chain fatty acids															
Acetate	57.67[b]	66.39[a]	68.90[a]	66.21	65.14	63.82	65.99	64.15	65.04	65.07	1.423	0.009	0.776	0.605	0.886
Propionate	2.95[b]	5.71[b]	10.98[a]	6.64	8.32	5.96	6.59	7.30	6.47	7.42	0.666	<0.001	0.562	0.607	0.764
i-butyrate	0.64[b]	0.68 [ab]	0.88[a]	0.68	0.84	0.72	0.81	0.69	0.72	0.77	0.038	0.008	0.265	0.152	0.517
n-butyrate	10.13[b]	12.20[b]	15.47[a]	13.02	13.26	12.37	12.43	13.32	12.64	13.12	0.464	<0.001	0.556	0.461	0.602
i-valerate	0.37[b]	0.33[b]	0.59[a]	0.40	0.50	0.43	0.45	0.43	0.46	0.42	0.024	<0.001	0.283	0.774	0.339
n-valerate	0.30[c]	0.64[b]	0.81[a]	0.64	0.63	0.57	0.60	0.63	0.61	0.62	0.026	<0.001	0.226	0.400	0.816
Total SCFA[b]	72.06[c]	85.96[b]	97.64[a]	87.59	88.68	83.86	86.85	86.51	85.94	87.41	1.943	<0.001	0.567	0.953	0.651
Total BCFA[c]	1.01[b]	1.01[b]	1.48[a]	1.07	1.34	1.14	1.25	1.12	1.18	1.18	0.055	<0.001	0.172	0.272	0.996
Lactate															
L-lactate	1.09	0.42	0.85	0.87	0.66	0.73	0.84	0.66	0.69	0.81	0.107	0.075	0.774	0.465	0.641
D-lactate	1.64[a]	0.46[b]	0.56[b]	0.69	0.83	0.89	0.68	0.94	0.82	0.79	0.135	<0.001	0.857	0.121	0.701
Total lactate	2.68[a]	0.86[b]	1.41 [ab]	1.53	1.49	1.61	1.50	1.59	1.48	1.60	0.223	0.006	0.995	0.600	0.959
D- to L-lactate ratio	1.49	1.30	0.95	0.87	1.71	1.01	0.92	1.50	1.04	1.33	0.204	0.550	0.334	0.223	0.556

[a]The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 6 replicate-pens per treatment and 40 birds per pen. Data were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex and their interactions.
[b]Total short chain fatty acid is the sum of acetate, propionate, i-butyrate, n-butyrate, i-valerate and n-valerate concentration.
[c]Total branched chain fatty acid is the sum of i-butyrate and i-valerate concentration.
[a,b,c]Means with different superscripts in a row within the main factor differ significantly ($p < 0.05$).
*CO, control; PO, probiotic product; PY, phytobiotic product; RS, Ross; CB, Cobb; M, male; F, female.

TABLE 5 The impact of age, dietary treatment, sex and breed on expression of the genes (log_{10} copy number per ng of RNA[a]) related to epithelial barrier function and inflammatory markers of the caecum*

Parameters**	Age (A)			Dietary treatment (T)			Breed (B)		Sex (S)		SEM	*p*-value			
	7	21	35	CO	PO	PY	RS	CB	M	F		A	T	B	S
Cytokines															
IL-1β	−3.30[c]	−2.55[a]	−2.99[b]	−2.98	−2.92	-2.94	−3.00[b]	−2.90[a]	−2.97	−2.92	0.029	<0.001	0.360	0.020	0.293
IL-2	−5.00[b]	−5.10[b]	−4.06[a]	−4.71	−4.76	-4.68	−4.70	−4.74	−4.70	−4.74	0.037	<0.001	0.317	0.314	0.269
IL-4	−4.35[b]	−3.50[a]	−3.54[a]	−3.81	−3.83	-3.76	−3.82	−3.78	−3.81	−3.79	0.029	<0.001	0.209	0.255	0.847
IL-6	−5.29[b]	−5.36[b]	−4.95[a]	−5.24	−5.09	-5.27	−5.29[b]	−5.11[a]	−5.18	−5.22	0.040	<0.001	0.148	0.028	0.565
IL-8	−2.66[b]	−2.65[b]	−2.23[a]	−2.53	−2.46	-2.54	−2.53	−2.50	−2.51	−2.51	0.027	<0.001	0.344	0.569	0.897
IL-10	−6.23[b]	−6.24[b]	−4.87[a]	−5.87[b]	−5.74[ab]	-5.71[a]	−5.80	−5.74	−5.80	−5.74	0.050	<0.001	0.004	0.163	0.429
IL-12	−4.60[c]	−4.45[b]	−3.39[a]	−4.16	−4.14	-4.14	−4.15	−4.14	−4.16	−4.13	0.040	<0.001	0.774	0.985	0.595
IL-17α	−3.81[b]	−3.90[b]	−3.07[a]	−3.63	−3.52	-3.63	−3.57	−3.62	−3.65	−3.54	0.045	<0.001	0.398	0.536	0.158
IL-18	−3.09[c]	−2.75[b]	−2.24[a]	−2.74	−2.67	-2.67	−2.70	−2.68	−2.69	−2.70	0.028	<0.001	0.072	0.645	0.691
TNF-α	−3.22[b]	−2.82[a]	−3.41[c]	−3.19	−3.14	-3.12	−3.21[b]	−3.09[a]	−3.15	−3.15	0.022	<0.001	0.098	<0.001	0.906
IFN-γ	−3.21[b]	−2.61[a]	−2.50[a]	−2.81	−2.77	-2.74	−2.75	−2.80	−2.76	−2.78	0.032	<0.001	0.505	0.215	0.550
TGF-β2	−1.43[c]	−0.96[b]	−0.73[a]	−1.08	−1.04	-0.99	−1.06	−1.02	−1.03	−1.05	0.025	<0.001	0.102	0.302	0.514
Gut barrier related proteins															
MUC2	−0.83[b]	−1.21[c]	−0.70[a]	−0.96	−0.88	−0.93	−0.93	−0.91	−0.90	−0.94	0.027	<0.001	0.311	0.712	0.364
CLDN5	−2.19[b]	−2.17[b]	−1.48[a]	−1.97	−1.95	−1.91	−1.98[b]	−1.91[a]	−1.94	−1.95	0.026	<0.001	0.220	0.017	0.703

[a]Log_{10} copy number per ng of RNA was calculated by dividing the amount of mRNA (copy number per ng of RNA) of targeted genes with the amount of mRNA of house keeper genes and then the obtained value was transformed to log_{10} scale.

[a,b,c]Means with different superscripts in a row within the main factor differ significantly ($p < 0.05$).

*Results are reported as means of 6 replicate-pens. The trial was conducted with a 3 × 2 × 2 factorial arrangement of diet, breed and sex in a completely randomized design and consisted of 40 birds per pen. One bird per pen for each group were subjected to ANOVA using GLM procedure to evaluate age, diet, breed and sex and their interactions.

**CO, control; PO, probiotic product; PY, phytobiotic product; RS, Ross; CB, Cobb; M, male; F, female; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β2,transforming growth factor beta 2; CLDN5, Claudin 5; MUC2, Mucin 2

Supplementary Table S3C). Broilers showed a greater mixed GC density at 7 days of age than those at 21 and 35 days of age regardless of dietary treatment ($p > 0.05$), except those 7 days old birds receiving the CO and PY diets were not different from 35 days old birds feeding the PY diet ($p > 0.05$). Similarly, the density of total GC in 7 days old broilers was also greater than in older birds, regardless of dietary treatment ($p < 0.05$), but 7 days old birds fed PY diet showed a similar total GC density to 21 days old birds receiving CO diet ($p > 0.05$). RS-male broilers at day 7 receiving PO diets showed highest mixed GC density and CB-male birds at day 35 receiving PY and PO diets displayed lowest mixed GC density CB ($p < 0.05$).

The effect of age, breed, sex and dietary treatment on metabolite content in the caecum is shown in Table 4. During the whole period of the study, acetate represented approximately 71–80 % of total SCFA concentration, followed by n-butyrate and propionate which represented 14–15 % and 4–11 % of total SCFA concentration, respectively. Concentration of all SCFA increased with age ($p < 0.05$), with the most drastic change in propionate (3.72-fold from 7 to 35 days of age), followed by n- and i-valerate, n-and i-butyrate, and acetate (2.67-, 1.59-, 1.53-, 1.38- and 1.19-fold, respectively). However, concentration of propionate, n-butyrate, i-valerate and total BCFA was stable in the caecum from 7 to 21 days of age ($p > 0.05$). Acetate concentration increased from day 7 to 21 ($p < 0.05$) and stayed stable from day 21 to 35 ($p > 0.05$). Furthermore, i-butyrate concentration at 21 days of age was not different from 7 or 35 days of age ($p > 0.05$). Dietary treatment, breed and sex had no impact on SCFA concentration ($p > 0.05$). There was no interaction between age, breed, sex, and dietary treatment for SCFA concentrations in the caecum ($p > 0.05$, Supplementary Table S4A).

Concentration of caecal D- and total lactate decreased from day 7 to 21 of age by 72.0 and 67.9 %, respectively ($p < 0.05$). Concentration of caecal D-lactate was identical for 21 and 35 days old broilers, while concentration of total lactate in the caecum of 35 days old broilers was similar to 7 and 21 days old broilers ($p > 0.05$). L-lactate and the ratio of D- to L-lactate were not different among age groups ($p > 0.05$). Dietary treatment, breed and sex as well as the interactions between the main factors showed no effect on lactate concentrations in the caecum ($p > 0.05$).

The impact of age, dietary treatment, sex and breed on mRNA abundance related to epithelial barrier function and inflammatory markers of the caecum is shown in Table 5. The mRNA abundance of all cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α and IL-18 as well as IFN-γ and TGF-

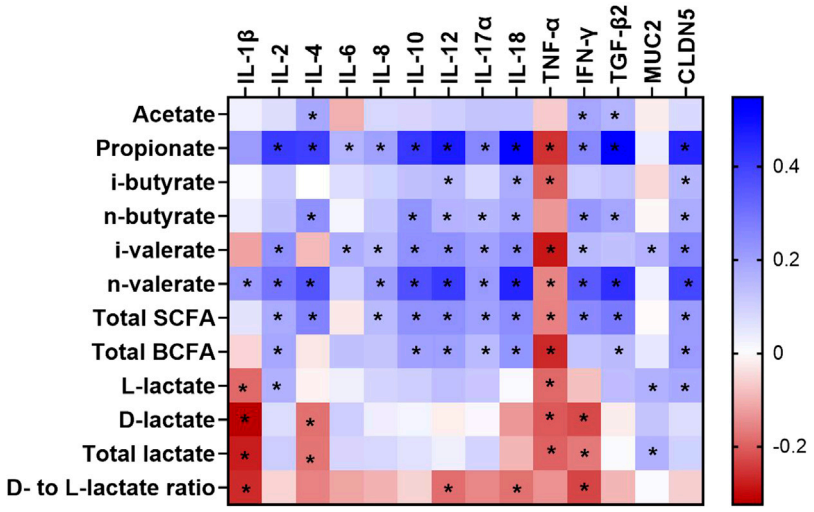

FIGURE 1
A heatmap showing the Spearman's correlation coefficient between metabolites and mRNA abundance in the caecum of broilers between day 7 and 35 of age. The colors represent the correlation, with blue being more positive and red being more negative. Significance is given as * ($p < 0.05$). SCFA, short chain fatty acid; BCFA, branched chain fatty acid; IL, interleukin; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; TGF-β2, transforming growth factor beta 2; CLDN5, Claudin 5; MUC2, Mucin 2.

β2) as well as MUC2 and CLDN5 increased from day 7 to 35 of age, except for TNF-α ($p < 0.05$). However, the abundance of several mRNA including IL-2, IL-6, IL-8, IL-10, IL-17α and CLDN5 was stable from day 7 to 21 of age ($p > 0.05$), while MUC2 decreased by 1.46-fold during this time ($p < 0.05$). Although most of mRNA abundance were increased from day 21 to 35 of age, the abundance of IL-1β and TNF-α were decreased during this period ($p < 0.05$). Dietary treatment only affected IL-10 abundance, with higher level in birds receiving PY diet compared with those fed CO diet ($p < 0.05$). Abundance of IL-1β, IL-6, TNF-α and CLDN5 was higher in CB than RS ($p < 0.05$). Sex had no impact on the mRNA abundance measured ($p > 0.05$). The only significant interaction was between age, treatment and sex on MUC2 abundance ($p < 0.05$, Supplementary Table S5A,B). At day 21, female birds fed CO diets, as well as male and female birds fed PY diets, expressed less MUC2 abundance level than birds aged 7 days (male birds fed PO diets and female birds fed PY diets) and 35 days (irrespective of diet or sex) ($p < 0.05$). In contrast, the abundance of MUC2 of male birds fed CO diet as well as male and female birds fed PO diet at day 21 of age were not different from the other age groups regardless of dietary treatment or sex ($p > 0.05$). The interaction between age, breed and sex was significant for IL-2 ($p < 0.05$). The highest abundance level of IL-2 was observed in 35 days old birds compared with 7 and 21 days old broilers. At day 21, female RS and male CB broilers showed a lesser IL-2 abundance than 7 days old broilers, regardless of their breed or sex (Supplementary Table S5C). The correlations between metabolite concentration and mRNA abundance were also analyzed as shown in Figure 1 (also Supplementary Table S6). The SCFA concentrations in the caecum, predominantly propionate, showed weak to moderate positive correlations (r = 0.150 to r = 0.548) with all the mRNA measured ($p < 0.05$), except for TNF-α, while its correlations with IL-18 (r = 0.518) and TGF-β2 (r = 0.548) were pronounced. The mRNA abundance of TNF-α showed negative correlations (r = −0.154 to r = −0.285, $p < 0.05$) with all the metabolites measured ($p < 0.05$) except for acetate, n-butyrate and D- to L-lactate ratio. Acetate as the predominant SCFA in the caecum, showed weak but positive correlations ($p < 0.05$) with IL-4, IFN-γ and TGF-β2 (r = 0.185, 0.191 and 0.157, respectively). There were only a few significant correlations between lactate and the investigated mRNA ($p < 0.05$) which were mainly weak (r = 0.183 to r = -0.324, respectively). D- and total lactate concentration was negatively correlated ($p < 0.05$) with IL-1β (r = −0.324 and r = −0.279, respectively), IL-4 (r = −0.179 and r = −0.175, respectively), TNF-α (r = −0.209 and r = −0.198, respectively) and IFN-γ (r = −0.227 and r = −0.170, respectively), while total lactate was positively correlated (r = 0.166) with MUC2 ($p < 0.05$). L-lactate concentration was negatively correlated ($p < 0.05$) with IL-1β (r = -0.189) and TNF-α (r = -0.190), but it showed a positive correlation ($p < 0.05$) with IL-2 (r = 0.166), MUC2 (r = 0.167) and CLDN5 (r = 0.183).

4 Discussion

Chicken caecum is inhabited by complex microbial community. These organisms are known to produce

metabolites modulating morphological structure along the gut (Shakouri et al., 2009) and interacting with gut immunity (Willson et al., 2018). Host-related factors including broiler breed, age and sex have been reported to affect intestinal microbiota and immune function (Torok et al., 2013). In this study, the impact of genetic background (breed and sex) was barely observed on caecal bacterial metabolites, histomorphology, integrity and immunological traits. There were no differences between males and females for all the variables measured in the current study. RS showed higher mixed and total GC density as well as higher number of mixed GC per crypt, compared with CB. Abundance of IL-1β, IL-6 and TNF-α (genes related to pro-inflammatory responses) as well as CLDN5 (one of the barrier-forming claudins) was increased in CB compared with RS. These few observed differences in intestinal phenotypes, mainly between breeds, did not show any meaningful biological pattern. The observed similarities between breeds and sexes might be attributed to the optimum rearing condition in the present study and absence of harmful stimuli, causing stress for bacterial population, stimulating the epithelial barrier function and triggering certain gut immune responses in order to protect the gut from additional injury (Mabelebele et al., 2017; Paraskeuas and Mountzouris, 2019; Wang et al., 2021).

Gut microbiota could directly or indirectly (via metabolites) interact with intestinal epithelium and modulate immune responses (Broom and Kogut, 2018). Beneficial impacts of probiotics and phytobiotics on poultry health and performance have been shown to be mainly through gut microbiota by supporting proliferation and metabolic activity of beneficial bacteria and decreasing the number and metabolic activity of those having harmful or pathogenic characteristics (Heak et al., 2018). In this study, probiotic and phytobiotic supplementation did not show any impact on caecal morphology, bacterial metabolic activity, and mRNA abundance, except for IL-10 which was increased in the caecum of birds receiving the phytobiotic product compared with birds in the control group. The cytokine IL-10 plays an essential role in anti-inflammatory response which regulates mucosal immune function (Lu et al., 2014). Grape extract has been reported to modulate cytokine expression through suppressing pro-inflammatory cytokines in the gut (e.g. IL-1β) of broilers (Cao et al., 2020) and increasing anti-inflammatory cytokines including IL-10 and TGF-β1 in human Caco-2 colon cells (Nallathambi et al., 2020). In several broiler studies, it has been shown that *B. subtilis* stimulated the effector and regulatory T cells and increased their cytokine production including IL-1β, IL-12, IFN-γ and IL-10 in the small intestine (Rajput et al., 2013; Lee et al., 2015) and caecum (Bilal et al., 2021). However, adding *B. subtilis* to broiler diets in the present study had no impact on mRNA abundance of all the investigated cytokines and intestinal barrier proteins as well as metabolic activity of bacteria in the caecum. In accordance with the present study, adding *B. subtilis* (Choi et al., 2021; Erinle et al., 2022) and grape seed extract (Cao et al., 2020) to broiler diets had almost no impact on caecal microbiota and their metabolic activity. Other studies, however, have shown the effect of *B. subtilis* on increasing bacterial SCFA concentration in the ileum (Aljumaah et al., 2020) and jejunum (Kan et al., 2021) of broilers. Grape extract was also found to affect bacterial metabolites in the gut of broilers through modulating phenolic metabolism of bacteria (Chamorro et al., 2019). The discrepancy in findings of different studies could be driven by qualitative differences in the extracts used, environmental factors like housing circumstances and diet composition, as well as host-related factors like age, breed, and sex, which differed between trials and could have impacted gut microbiota development (Kers et al., 2018).

In the current study, the observed alterations in the caecum's morphology, mRNA abundance, and bacterial metabolites were mainly age-related. During co-development of the host and gut microbiota, products of bacterial metabolic activity, like SCFA and lactate, could be the main factors triggering the interaction between the host and gut microbiota (Yang L. et al., 2017). In the present study, acetate, propionate, and butyrate concentration increased during 35 days of age, while lactate concentration decreased from day 7 to 21 of age and remained stable afterward. The opposite age-related direction of SCFA (increase) and lactate (decrease) concentrations in the caecum was also previously reported and suggested to be due to a direct stimulation of lactate-utilizing bacteria or indirect action of bacterial groups playing role in metabolic cross-feeding of fermentation products (Meimandipour et al., 2011). Reduction of lactate concentration in the caecum during the first two weeks of age was attributed to replacement of lactic acid bacteria, especially *Lactobacillus*, with other dominant bacterial groups, mainly Clostridiaceae (Ranjitkar et al., 2016). The Clostridiaceae are well-known for conversion of complex polysaccharides to SCFA (Eeckhaut et al., 2011). Most of complex polysaccharides are indigestible in the small intestine of broilers thus, are available as substrates for microorganism in the hind gut. Basically, as chickens become older, the quality of their diets reduces and the concentration of indigestible polysaccharides in their diets increases. Dominance of Clostridiaceae and their increasing access to complex polysaccharides in the caecum can be one of the reasons for increasing SCFA concentration in the caecum of broilers. Furthermore, some bacteria in the Clostridiaceae group, like *Clostridium, Faecalibacterium*, and *Ruminococcus* spp. can utilize lactate as a substrate for butyrate production (Eeckhaut et al., 2011). Thus, the age-related shift from lactic acid bacteria to Clostridiaceae, may increase SCFA and butyrate and reduce lactate concentration, as also have been seen in the present study. Furthermore, it was suggested that the microbial community may exert different metabolic pathways at different ages depending on luminal state, microbial makeup, and host immune response (Wu et al., 2021). In the current study, age had a greater impact on propionate than other SCFA.

Propionate concentration increased by 272 % from day 7 to 35 of age, whereas acetate and butyrate increased only by 19 % and 53 %, respectively. A pronounced increase in propionate over other SCFA was also reported in broilers during 3–6 weeks of age which was suggested to be due to alterations in bacterial composition that increased propionate producers and/or decreased lactobacilli (Meimandipour et al., 2010; Kim et al., 2020; Liao et al., 2020). Several studies have shown that as chickens age, the caecal microbial community becomes more diversified, with a greater number of distinct species (Lu et al., 2003; Oakley et al., 2014; Oakley and Kogut, 2016). The constant (age-related) alterations in metabolites concentration could also reflect that the caecal microbial community of broilers in the present study had not reached its mature and steady state even at 35 days of age. This speculation is in line with prior studies showing (by sequencing 16S rRNA) shifts in the caecal bacterial community (Lu et al., 2003; Oakley and Kogut, 2016) and increases in SCFA concentration (Liao et al., 2020) between 3 and 7 weeks of age. Other studies have also claimed that increasing SCFA concentrations in the caecum of older broilers (Liao et al., 2020) and laying hens (Sun et al., 2021) may reflect maturation process of the microbial community by age. However, there are studies demonstrating a "mature" stage of gut microbiota as early as 3 weeks of age, by comparing phylogenetic diversity in the caecum of 3 weeks old broilers with older (up to 6 weeks of age) birds (Kers et al., 2020) or by a regression model, using the microbiota maturation index for fecal microbiota (Gao et al., 2017). The inconsistency in outcome (the mature stage of gut microbiota) of the studies could be attributed to differences in analytical methodology as well as host genetics, diet, and environmental conditions, which could have affected microbial composition (Pedroso et al., 2005; Richards et al., 2019; Hou et al., 2020).

Intestinal morphology can be considered as a direct measure of intestinal health, as the mucosal epithelium regenerates to replace injured cells and constantly reshapes the mucosal structure in the gut. In the present study, morphology of the caecal crypt was changed with age; CD in the caecum increased by more than 100 % from day 7 to 35 of age. Alterations in the luminal environment by bacterial metabolites may affect villus or crypt structure and mucin production which are important defense structures in the gut (Iacob et al., 2019). As a source of energy, butyrate plays a vital role in promoting intestinal development and maintaining the integrity of the intestinal epithelial cells (Zou et al., 2019). Acetate has been shown to alter intestinal cell apoptosis and mucus production (Liu et al., 2017). Propionate is also a potent fatty acid that modulate intestinal cell activity including differentiation and apoptosis (Hosseini et al., 2011). The concentration of bacterial metabolites such as SCFA is usually higher in the caecum than other areas of the gastrointestinal tract, and their impact on gut histomorphology development should also be highest in the caecum compared with other sections of the gut (Parada Venegas et al., 2019). Therefore, age-related increases in SCFA concentration found in this study may have impacted formation of epithelial cells in the crypt of growing broilers and among other factors, stimulated morphological changes in their caecum. Intestinal GC is the first line of defense for the mucosa. Mucins produced by GC can protect epithelial cells from infections as well as chemical and mechanical damages (Duangnumsawang et al., 2021). In this study, the number of total GC per crypt was not affected by age. Another study found an increased number of caecal GC of 49 days old broilers compared with 28 days old broilers (Jiang et al., 2009). In contrast, Thiam et al. (2021) found that total number of GC per crypt in the ileum and caecum of broilers tended to decrease from day 7 to 21 of age. The changes in GC number of the gut could be due to biological mechanisms such as cell growth and death, which are reported to be influenced by the gut microbial status (dysbiosis and symbiosis) and age (Sovran et al., 2019; Gebert et al., 2020). Therefore, the same number of total GC per crypt during the 5 weeks period of this study, could indicate a stable gut environment that does not require production of additional protective mucins (produced by GC). Mucins are the major components of the intestinal mucus layer and can be categorized as acidic or neutral based on their net molecular charge. Acidic type expresses a net negative charge and neutral type exhibits a net neutral charge of the mucin molecule (Derrien et al., 2010). The diverse forms of mucins found in GC may provide clues to host adaptability to gut microbiota (Sicard et al., 2017). In the current study, the majority (61–85 %) of GC was found as a mixed type (containing relatively similar proportion of acidic and neutral mucins) and around 15–39 % of total GC was an acidic type, suggesting that the proportion of secreted acidic mucins in the mucus layer of the caecum may be greater than neutral mucins. Furthermore, the number of acidic GC per crypt increased with age and peaked at 21 days, whereas the amount of mixed GC was unaffected by age. Increasing the overall negative charge (acidic) of mucins enhances mucus viscosity which may be associated with increased gut bacterial diversity and amount of bacterial-derived compounds as age increases. A greater number of GC, particularly acidic GC, could produce more protective mucins that resist bacterial degradation, thus provides more protection against pathogens and mechanical irritation (Montagne et al., 2004). Mucin modifications (including sialylation and sulfation which result in acidic mucins) are typically promoted along with the maturation process of GC (Hino et al., 2012). The observed increase in the proportion of acidic GC and the number of acidic GC per crypt during the first 3 weeks of age may reflect GC maturation, which is important for the functional protection of the intestinal epithelium. This could be attributed to the increased diversity of the caecal microbial community and the increase in feed consumption (more mechanical irritation) of broilers with growing older. It should be noted that individual sampling from tissues of sacrificed birds provides a snapshot of the intestinal response which may cause a

bias in either direction for time-sensitive variables such as GC due to their rapid, continuous turnover (3–7 days) at the crypt base (Birchenough et al., 2015). Nevertheless, this study was able to capture the variation of GC proliferation during 14-day periods (from day 7 to day 21, and from day 21 to day 35), particularly for acidic GC. In agreement with prior research, the increased number of GCs and the production of acidic mucins in the small intestine of broilers demonstrated gut maturation of broilers due to exposure to intestinal normal flora over time (Forder et al., 2012).

The mRNA abundance of all cytokines, MUC2 and CLDN5 was also mostly regulated by age, with only a few genes being modified by dietary treatment (IL-10) and breed (IL-1β, IL-6, TNF-α and CLDN5). Throughout the whole study period, the mRNA abundance of all cytokines was increased with age, except IL-1β and TNF-α which reached the highest level at 21 days of age. The alterations in cytokine mRNA abundance in healthy unchallenged broilers may only reflect the interaction between the gut immune system and the commensal microflora (Bar-Shira and Friedman, 2006; Crhanova et al., 2011). The mRNA abundance of several cytokines in the caecum like IL-1β, IL-18, IL-22 and TNF-α was found to be fluctuated due to the colonization of normal gut microflora during first 58 days of age, reflecting an adaptation of the gut immune system to microbiota (Crhanova et al., 2011). In another study, it has been shown that mRNA abundance of IL-1β and TNF-α increased after hatch and decreased during the third week of age (Crhanova et al., 2011), which is in accordance with our study. During the first week after hatching, antigens from the diet and environment constructed an immune response in the caecum of broilers via recruiting granulocyte and T-lymphocyte and generating cytokines, which could trigger immunological adaptation to luminal antigens and microbiota (Van Immerseel et al., 2002; Bar-Shira et al., 2003; Crhanova et al., 2011). Immunological adaptation reduce or eliminate the impacts of dietary and environmental challenges and restore the balance in the immune system (Broom and Kogut, 2018). In the current study, the mRNA abundance of IL-1β and TNF-α as pro-inflammatory cytokines was downregulated after 3 weeks of age, which may imply a lesser degree of immune response in the gut, following transitory inflammatory activation as an immunological adaptation process. It has been also shown in another study that, healthy unchallenged broilers had a lower inflammatory response in the caecal tissue after 3 weeks of age, which was associated with a reduction in potential pathogenic bacteria such as *Escherichia* and *Shigella* and an increase in some beneficial bacteria like *Firmicutes* (including *Faecalibacterium)* in the caecum (Oakley and Kogut, 2016).

Previous studies have shown that bacterial metabolites regulate the immunological pathways of intestinal cells (Yang et al., 2018; Parada Venegas et al., 2019). Therefore, the observed correlations between metabolites and mRNA abundance of cytokines and epithelial barrier proteins in the current study may be interpreted as the relative extent of host-microbiota interactions through bacterial metabolites. Several SCFA mainly propionate and n-butyrate showed an association with cytokines investigated in the caecum (Figure 1). This was in line with in several studies indicating the effects of SCFA on intestinal immunity as reviewed by Gasaly et al. (2021). It was proposed that SCFA may regulate cytokine production of the immune or epithelial cells by directly binding to certain receptors such as free fatty acid receptor (or G protein-coupled receptors) and/or by regulating target cell epigenetics after they were taken up into the cells (Liu et al., 2021). Among SCFA, propionate demonstrated a relatively strong positive correlation with most of cytokines analyzed in this study, especially IL-4, IFN-γ and TGF-β2. The balance between pro-inflammatory (e.g. IFN-γ and IL-4) and anti-inflammatory (e.g. TGF-β) cytokines was shown to regulate mucosal inflammation in response to the presence of bacterial antigens (Neurath et al., 2002). Higher mRNA abundance of TGF-β could be associated with inflammatory inhibition through suppressing cytotoxic actions of Th1 lymphocytes and their cytokines such as IFN-γ, while it simultaneously promoted humoral immune mechanisms of Th2 lymphocytes which stimulate IL-4 secretion (Jarosz et al., 2017). In contrast to SCFA, lactate was negatively correlated with several investigated pro-inflammatory cytokines including IL-1β, IL-4, TNF-α and IFN-γ, thus increasing lactate concentration may inhibit inflammatory response in the caecum of broilers. The anti-inflammatory effects of lactate was also observed in the colon of mice through downregulation of pro-inflammatory cytokines such as IL-1β and TNF-α (Iraporda et al., 2016). Although our result illustrated a potential role of bacterial metabolites on the intestinal immunity, their mechanisms of action are difficult to explain due to their complicated interaction with multiple signaling molecules. Sudden changes in microbial community or activity, such as those caused by stress or pathogenic challenges, can rapidly shift the gut's immunological balance toward a pro-inflammatory response, inducing a transient physiological inflammation and immune stimulation (Crhanova et al., 2011). The mRNA related to intestinal barrier integrity including MUC2 (mucus protein) and CLDN5 (one of the barrier-forming claudins) also increased their abundance with age in the current study. Several studies suggested that mRNA abundance of MUC2 and CLDN family genes reflect maturation of the intestinal tract during postnatal development (Holmes et al., 2006; Proszkowiec-Weglarz et al., 2020), as well as shifts in bacterial composition in the caecum during animal's growth (Lu et al., 2003). In the present study, an increased mRNA abundance of MUC2 and CLDN5 occurred

along with more developed CD and certain alterations in bacterial metabolites during 35 days of age, which could reflect an increased protective barrier in response to age-related changes in gut microbiota and their metabolic activity. Correlation between bacterial metabolites and CLDN5 was stronger than their correlation with MUC2, with propionate having the strongest correlation with CLDN5. Propionate was also reported to increase the expression of tight junction proteins including ZO-1, CLDN1, CLDN8 and occludin in rat's colon, hence propionate may enhance intestinal epithelial integrity (Xia et al., 2017).

5 Conclusion

Our result showed that bacterial metabolites could play a significant role in mucosal development and immunological response in the caecum of broilers. Bacterial metabolites, particularly SCFA, seemed to contribute to the formation of crypts in the caecum and modify gut immunity over time. The influence of sex on measured variable was completely obscured in this study, while the other genetic-related factor (breed) and dietary treatments (probiotics and phytobiotics) showed a limited impact on microbial metabolites and immunological responses. However, the few observed impacts of breed and dietary treatment as well as interactions between factors on measured variables did not show any meaningful biological pattern. Age remarkably impacted mucosal morphology, goblet cell proliferation and bacterial metabolic activity as well as mRNA abundance of the genes responsible for inflammatory response in the caecum of broilers. This impact could be attributed to the interaction between the gut microbiota and immune system as well as the direct effect of microbial metabolites on the gut histomorphology and cytokine mRNA abundance.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ethical Committees of Generalitat de Catalunya, Spain (Proceeding number 10226).

Author contributions

All authors designed the experiments. JT and FGB performed the experiment. WV provided mRNA abundance data. YD analyzed the histomorphology, performed data analysis, and wrote the manuscript. JZ and FGB revised and finalized the manuscript. All authors approved the final version of the manuscript.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.935870/full#supplementary-material

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5. Chapter 5: General discussion and conclusion

The use of probiotics and phytobiotics may modulate intestinal microbiota and immune response of modern broilers providing a range of health benefits based on the interactions between host, microbes, and diet. This study aimed to investigate the effect of host-related factors (age, breed, and sex) and feed additives (probiotics and phytobiotics) on mucosal structure, immune response, and intestinal microbial activity. A trial with one-day chicks including two breeds (Ross and Cobb) and two sexes (male and female) was conducted. They were fed three different experimental diets including a standard wheat-soybean based diet without or with supplementation of either a probiotic (*Bacillus subtilis* DSM 32324, *B. subtilis* DSM 32325 and *B. amyloliquefaciens* DSM 25840) or a phytobiotic (white grapes, 165 ppm procyanidin and 585 ppm total polyphenol in the diets) product. The sample collection of broiler's ileum and caecum at three age points (day 7, 21 and 35) were used to analyze mucosal morphology, cytokine expression, and microbial metabolite concentration.

Bacterial metabolites play a crucial role in gut health of broilers through interacting with intestinal epithelium and the immune system. Various microbial metabolites are produced along the gastrointestinal tract of broilers through fermentation of feed derived substrates by microorganisms. In the small intestine, bacterial fermentation is limited due to rapid motility and luminal environment such as the presence of digestive secretions that restrict bacterial growth (Kastl et al., 2020). The ileum is the terminal part of the small intestine that plays a key role in nutrient absorption. However, in comparison to the proximal part of the small intestine, it appears to be an important site for microbial fermentation as evidenced by an increase in bacterial density and metabolite production distally along the small intestine (Rehman et al., 2007). The caeca consist of two blind-ending pouches located between the small and large intestine. The broiler's caeca contain the highest number and diversity of microbiota, making them primary centers for metabolite production in the gut (Oakley et al., 2014b). In addition, longer retention time in the caecum (12 - 20 hours) promotes microbial breakdown of fermentable substrates including nondigestible carbohydrates (e.g., non-starch polysaccharides; NSP) and protein leading to an increase in production of metabolites including SCFA and lactate (Borda-Molina et al., 2018). Regarding different range of fermentation activity in the ileum and caecum, each section's metabolite profile may uniquely interact with epithelial cells and immune system. This could result in various host responses that maintain or promote homeostasis in the different intestinal sections. Besides the regional variation, the impact of microbial metabolites on gut health may be influenced by several intrinsic and extrinsic factors. The former includes genotype, sex, and age, whereas the latter

may involve nutrition and other environmental stimuli (Kers et al., 2018). Feed additives such as PO and PY are commonly used to modulate the intestinal microbiota and immune response of modern broilers, resulting in a range of health benefits (Krysiak et al., 2021). However, these feed additives and host-related factors, independently or in interaction with each other, may show different impacts on metabolite production and immune responses in the broilers gut. Furthermore, given the microbiological, physiological, and functional differences between ileum and caecum, consequences of these impacts may not be necessarily similar for these two sections of the gut. Therefore, the purpose of the current study was to investigate the effect of host-related factors (age, breed, and sex) and feed additives (PO and PY) on intestinal microbial activity, immune response, and mucosal morphology in the ileum and caecum of broilers and comparing consequences of these effects in these gut regions.

5.1. Key findings in the ileum of broilers

Ileal morphology, bacterial metabolites, and mucosal cytokines were only marginally different across breeds, sexes, and diets. However, all the variables substantially changed during growth.

Regarding metabolites concentration during growth, acetate showed the highest concentration during the third week of age, whereas concentration of lactate was the lowest. Biogenic amines, BCFA, and ammonia were found at very low amounts in the ileum and showed various pattern of changes during growth. It was suggested that production of bacterial metabolites in the gut is influenced by the composition and metabolic activity of gut bacteria (Rinttilä and Apajalahti, 2013). The putrefactive metabolites including BCFA, biogenic amines and ammonia are produced by proteolytic bacteria, while SCFA and lactate are mainly but not exclusively derived from saccharolytic bacteria (Qaisrani et al., 2015). Thus, type and amount of nutrients available for bacterial utilization in the distal part of ileum can also be one of the main determinative factors for the observed alterations in ileal metabolites. Nutrient availability at the end of ileum changes over time and depend on different factors like quality of feed and functional maturity of the small intestine in digestion and absorption process. After one week of age, ileal digestibility of starch, fat, and protein becomes relatively stable (Noy and Sklan, 1995). Pancreatic and brush-border enzymes in the duodenum, jejunum, and ileum are responsible for digestion of these nutrients and their activity increases as broilers age, contributing to improved digestion and absorption (Iji et al., 2001). However, highly soluble non-starch polysaccharides (NSP) in the diet can impair digestion and absorption by increasing digesta viscosity which limits accessibility of enzymes to substrates and reduce nutrient absorption (Weurding et al., 2001). Relatively high levels of wheat (53 – 62 %) in this study

can also negatively impact nutrient digestion and absorption due to its high content of soluble NSP (mainly arabinoxylans). Higher amounts of undigested NSP and nutrients lead to higher fermentation activity in the distal part of the gastrointestinal tract. A similar study also showed that increasing soluble NSP content in poultry diet (55 – 60 % of wheat) enhanced ileal digesta viscosity and increased caecal SCFA concentrations (Nguyen et al., 2021).

Number and composition of ileal bacterial communities also play an important role in determining type and concentration of metabolites. As broiler chickens age, gut microbiota undergo a series of changes in species diversity, followed by maturation and stabilization (Barua et al., 2021). Studies have found that the most abundant bacteria in the ileum of newly hatched broilers belong to the family *Enterococcaceae* and *Clostridiaceae* (Richards-Rios et al., 2020; Lu et al., 2003). During the first week of life, the initial bacterial communities are generally replaced by *Lactobacillaceae* and maintained throughout the chicken's life (Schokker et al., 2015). The main genera of *Clostridiaceae* and *Lactobacillaceae* include *Clostridium* and *Lactobacillus* spp. are mainly responsible for producing SCFA and lactate, respectively. Meanwhile, proteolytic bacteria such as *Bacteroides* spp., *Clostridium* spp., and *Streptococcus* spp. also increased as broilers age (Lu et al., 2003), leading to production of putrefactive metabolites (Rehman et al., 2007). Thus, changes in the number and composition of bacteria in the ileum can significantly impact the type and quantity of metabolites produced.

In the present study, gene expression of mucosal cytokines was considerably affected by broiler age. All cytokines were upregulated from day 7 to day 21 and then downregulated at day 35. The upregulation of these cytokines at day 21 could be an indicator for an overall immunological change that could have been driven by physiological maturation, microbial development, or environmental stimuli (Lu et al., 2003). In our study, the increase in cytokine expression at day 21 seemed temporary and could have been triggered by an environmental or immunological stimulus which faded away after a couple of weeks, resulting in downregulation of all cytokines. Expression of mucin and tight junction protein also followed the same pattern as cytokines to maintain gut homeostasis and enhance epithelial barriers during activation of the immune system. Therefore, it can be speculated that cytokines and other epithelial barrier proteins could have an important role in maintaining the gut homeostasis as well as responding to environmental or immunological stimuli in the early stages of life.

In the current study, number and density of GC, especially their acidic type, increased with age. Acidic mucins are predominated by sialic acid and sulfate groups which form a strong net-negative charge of the mucin molecules (Derrien et al., 2010). These negatively charged mucins form numerous cross-links between the mucin molecules, resulting in an increase in mucus viscosity which decrease the permeation of bacteria moving across the mucus layer

(Leal et al., 2017). Furthermore, sialic acid or sulfated groups have been found to play a key role to protect mucin chains from degradation by bacterial enzymes such as proteases and glycosidases as well as proteolytic host enzymes (Hino et al., 2012; Roberton and Wright, 1997). It was suggested that increasing acidic GC could be an adaptive response to age-related increase in diversity and activity of gut microbes (Bergstrom and Xia, 2022). Therefore, the increase in acidic mucins with age is likely to be beneficial in providing epithelial protection from bacterial invasion by increasing the viscosity and reducing degradation of the mucus layer.

5.2. Key findings in the caecum of broilers

Caecal bacterial metabolites, immune responses and morphology were nearly similar across broiler breeds and sex, as well as dietary treatments. The observed alterations in the caecum were mainly driven by age.

Concentration of SCFA in caecal digesta increased by age while its lactate content decreased. It has been shown that lactate concentration in the caecum of older broilers are typically lower than younger ones, which has been explained by transformation of lactate to other metabolites by lactate-utilizing bacteria or by metabolic cross-feeding within communities (Ranjitkar et al., 2016). It has been reported repeatedly that caecal microbiota of broilers becomes more diversified over time, with a greater number of distinct species (Oakley et al., 2014a; Lu et al., 2003). Network analysis of caecal microbiota of broilers showed an increase in microbial complexity with number of microbial taxa increasing between day 7 and 42 of age (Oakley et al., 2014a). As seen by the constant changes in metabolite concentrations over the 35-day period in the present study, it is possible that the caecal bacterial communities may not have reached maturity until, at least, day 21 of age. Beside changes in microbial composition, available indigested-unabsorbed (anti-)nutrients like NSP are known to alter microbial fermentation in the hindgut of broilers. It was suggested that soluble NSP serve as a source of energy for beneficial bacteria through selective fermentation of the oligosaccharides derived from their polymerization (Nguyen et al., 2021). Increasing soluble NSP level in broiler diet enhanced caecal fermentation rates and production of SCFA (Nguyen et al., 2021). In the current study, increasing wheat inclusion from 53 to 62 % between starter and finisher period may play an important role in altering caecal microbiota and their metabolite production, leading to an increase in SCFA concentration with age. It is generally believed that caecal microbes in younger birds are not able to ferment arabinoxylans efficiently, due to limited production of enzymes to break them down (Lu et al., 2003). However, after three weeks, caecal microbiota of broilers seems to develop the ability to hydrolyze and ferment these

compounds, as observed by an increase in arabinoxylan degrading activities and its digestion (Bautil et al., 2019). Therefore, age-related changes in caecal metabolites, especially increase in SCFA concentration, may reflect microbial maturation process and adaptation to ferment indigestible nutrients such as soluble NSP in the caecum. Although soluble NSP could increase production of metabolites that are beneficial for epithelial growth, an excessive amount of soluble NSP should be avoided due to its negative effect on digestion and absorption in the small intestine as well as the bacterial overgrowth in the hindgut (Nguyen et al., 2021).

The levels of cytokines in the caecum of broiler chickens changed with age, with most cytokines showing an upregulation as the chickens grew. Cytokines are important mediators that communicate between innate and adaptive immune systems and regulate them in response to microbiota-derived factors and other luminal stimuli (Mahapatro et al., 2021). This immunological activation seems to be crucial due to the constant changes in the microbial composition of the caecum that is known to occur during broiler growth (Oakley et al., 2014a). Moreover, bacterial metabolites including SCFA and lactate modulate cytokine production by altering cellular signaling of the enterocytes and immune cells (Parada Venegas et al., 2019). As a result, these bacterial metabolites can serve as important regulators of cytokine production and may help to shape the immunological responses to caecal microbiota changes and maturation during broiler growth. With respect to the mucosal barrier in this study, the number of total GC in the caecal crypt did not change as broiler aged. Since GC play a role in producing mucins to protect epithelial cells from bacteria as well as chemical and mechanical damages, an unchanged number of total GC may imply a relative stable luminal environment that does not require production of additional mucins. Mucins are highly glycosylated proteins with varying amounts of negatively and neutrally charged carbohydrate residues, resulting in acidic and neutral mucins (Derrien et al., 2010). The current study showed a constant number of mixed (acidic + neutral) GC, while the number of acidic GC varied, with the highest density at the third week of age. Acidic mucins appeared to dominate the hindgut of broilers and their amount was enhanced by maturation of GC and gut microbiota (Kers et al., 2018; Fernandez et al., 2000). Moreover, as mentioned above, increasing negatively charged mucins has been found to enhance mucus viscosity and preserve mucus from bacterial breakdown (Robinson et al., 2017). The age-related increase in acidic GC in the caecum represent physiological maturation of GC and host adaptability to gut microbiota and metabolites to protect intestinal epithelium from microorganisms.

5.3. Variation in bacterial metabolites and cytokine expression in the ileum and caecum of broilers

The present study demonstrated regional variations (ileum *vs.* caecum) in concentration of bacterial metabolites including SCFA and lactate. In the ileum, the average concentration of SCFA in the digesta ranged from 2.32 to 3.24 μmol/g, while that of lactate varied between 24.4 and 53.3 μmol/g. However, in the caecum, the average concentration of SCFA in the digesta varied from 72.1 to 97.6 μmol/g, whereas lactate concentration was between 0.86 and 2.68 μmol/g. This shows a remarkable difference between bacterial activity in these two regions. As mentioned above, fermentation activity and metabolite products are largely influenced by the microbial composition in the gut (Ranjitkar et al., 2016). By using 16S rRNA sequencing for broilers, it was shown that the relative abundance of *Lactobacillus* ranged from 65 to 74 % in the ileal bacterial community, while *Clostridium* was most abundant in the caecum, ranging from 20 to 48 % (Ranjitkar et al., 2016; Lu et al., 2003). The genus *Lactobacillus* primarily produces lactic acid and *Clostridium* is known to produce a broad spectrum of SCFA (Pessione, 2012). Therefore, variations in bacterial composition in the gut of broilers may account for regional differences in metabolite concentration. As discussed above, another factor that would alter metabolite concentration is attributed to the type and concentration of luminal substrates (Kastl et al., 2020). A range of substrates including monosaccharides, amino acids, peptides, and indigestible dietary materials (e.g., NSP) are presented in the ileum and caecum. The final products of digestion such as monosaccharides and amino acids are mostly absorbed precaecally, while the remaining substrates can be fermented by microorganisms in the distal ileum and caecum. In general, microorganisms would preferably take up easily fermentable nutrients like glucose as an energy source but when these substrates are limited, bacteria would try to harvest energy from complex carbohydrates like NSP or other nutrients such as amino acids (Oliphant and Allen-Vercoe, 2019a). The easily fermentable compounds like unabsorbed monosaccharides could be immediately taken up by ileal microbiota, however utilization (fermentation) of more complex (anti-)nutrients could be limited by the short retention time of digesta in the ileum (Oliphant and Allen-Vercoe, 2019b). In the caecum on the other hand, fermentation of dietary materials by bacteria is relatively high due to longer retention time and favorable luminal conditions for bacterial growth including the absence of digestive secretions and infrequent gut motility (Kastl et al., 2020). Therefore, gut function, microbial composition, as well as the type and amount of substrates in the ileum and caecum of broilers have an impact on production of metabolites, resulting in unique patterns of metabolite concentrations in each intestinal segment.

Age-related variations in cytokine expression displayed distinct patterns in the ileum and caecum. From the first to third week of life, there was a wide range of 7 to 642-fold increases in the expression of all cytokines in the ileum, followed by a range of 13 to 421-fold decreases until the end of the fifth week of life. In contrast, caecal cytokines showed only slight changes in their expression during the first three weeks and most of them reached their peak at the fifth week of life. As discussed earlier, the luminal environments in the ileum and caecum differ with respect to microbiota (e.g., abundance, composition, and activity) and availability of substrates. These factors may interact with intestinal epithelial cells and immune cells to stimulate immunological processes. Given high feed intake of broilers and rapid gut motility, there is a high digesta flow in the ileum which may potentially result in rigorous changes in the luminal environment due to different factors like temporary presence of immunological stimuli. This may trigger an intermittent immune activation in the ileum. However, the luminal conditions in the caecum are more stable with a longer retention time of digesta. Thus, the ileum may have encountered different environmental and immunological situation in this study, particularly at the week three and therefore, displayed different immune responses compared with the caecum.

The number of GC (both acidic and mixed type) was higher in the ileum than caecum, which may imply a greater mucus production in the ileum. The primary physiological functions of the ileum, digestion, and absorption may explain its need for higher mucus production. The mucus layer in the ileum should be maintained by GC secretions to for a barrier on the epithelial surface against luminal materials and digestive enzymes, while it facilitates nutrient transportation across the mucus layer. However, gut motility in the ileum may cause the mucus being removed and transported to the distal intestine constantly. This might have stimulated renewal of the mucus layer by increasing GC proliferation to produce more mucins in the present study. On the other hand, the mucus layer in the caecum seems to be more preserved due to less mechanical and chemical damage by digesta and digestive enzymes, thus the caecal mucosa may require less GC to maintain the mucus layer. As reflected by the prevalence of acidic and mixed GC and a trace amount of neutral GC in both ileum and caecum, modification of mucin molecules (neutral to acidic) was highly occurred in both intestinal sections. As discussed before, a high degree of mucin modification may provide more mucosal protection in the gut of broilers and this molecular modification results in more resistance to enzymatic degradation by bacteria (Robinson et al., 2017).

5.4. Critical evaluation of the study design and implications for future research

The current study demonstrated the temporal and regional development of bacterial activity, immunological traits, and mucin-secreting GC in the broiler gut. The obtained data provide insight into a developmental snapshot of gut microbiota and host immune system interaction. Specifically, this study highlighted the potential actions of bacterial metabolites in modulating immune system in the gut via cytokines and chemokines. Nevertheless, some aspects of the present findings should be viewed with caution or require additional research. This involves interpretation of metabolite data, sample collection, and mucin analysis.

Measuring concentrations of bacterial metabolites in fresh digesta (per weight of digesta) do not represent their actual amount produced by bacteria over time. This is because most metabolites, particularly SCFA, are rapidly absorbed by intestinal cells or can be transformed into other metabolites by bacteria (Gomez-Osorio et al., 2021). As a result, the metabolite concentration measured at a particular time point cannot be equivalent to the overall production of metabolites in the gut and may only provide a snapshot of bacterial activity at the time. Increasing the frequency of sample collection could have provided greater insight into the dynamics of microbial activity. However, it would be demanding to take frequent samples from an individual bird at different time points, especially from young birds due to limited amount of digesta.

The impact of bacterial metabolites on immune responses has been extensively studied, yet the effects of individual metabolites on the immune system remain unclear. The present study suggests that lactate may inhibit the inflammatory response by negatively correlating with pro-inflammatory cytokines. This finding is supported by previous research in mice and in vitro experiments (Manosalva et al., 2022; Iraporda et al., 2014). Conversely, the concentration of SCFA, such as propionate and n-butyrate, showed positive correlations with both pro- and anti-inflammatory cytokines in this study. These metabolites have been considered as potential mediators in the regulation of T cell proliferation, including T helper and regulatory T cells (Vinolo et al., 2011). These immune cells are typically present in the broilers intestine even in the absence of infectious stimuli (Mwangi et al., 2010). T helper cells promote inflammation by secreting cytokines, while regulatory T cells suppress inflammation by producing cytokines. Therefore, it is important to note that the activation of T cells by SCFA may result in various effects on inflammation and immune responses (Vinolo et al., 2011). This highlights the need for further research to fully understand the mechanisms behind the impact of bacterial metabolites on the broiler's immune system.

The evaluation of mucin-related gene expression has been used as an indicator of mucin biosynthesis processes, but it should be noted that this does not accurately reflect the actual amount of mucins secreted into the lumen of the gut. Mucins are synthesized and stored in the cytoplasmic secretory granules of GC, and their secretion can be either constitutive or stimulated by external factors such as bacteria and their metabolites (Duangnumsawang et al., 2021). Once secreted, mucins are usually not degraded by digestive enzymes and remain intact along the small intestine, so the secretion of mucins into the lumen can be measured at the end of the small intestine, specifically the distal ileum. To evaluate mucin secretion in the gut, markers such as N-acetyl-glucosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc) have been used in broiler studies (MacMillan et al., 2019; Tsirtsikos et al., 2012). GlcNAc and GalNAc are abundant carbohydrates found in mucin molecules, but their presence in feed and bacteria is limited (Montagne et al., 2000), making them useful markers to evaluate the secretion of host-derived mucins. Thus, measuring the amount of GlcNAc and GalNAc in the distal ileum could be a method to quantify mucin secretion in broiler's gut.

6. Summary

Title of the PhD thesis:

Phenotyping the Broiler Intestine: Influence of Host-related Factors and Feed Additives on Bacterial Activity and Immunological Traits

Bacterial metabolites are crucial for the gut health of broilers through their impacts on the intestinal epithelium and immune system. Bacterial metabolites are produced in various parts of the digestive tract, with the crop, ileum and caeca being the primary locations. The production of microbial metabolites in broiler's gut may be affected by intrinsic factors such as age, breed, and sex, as well as extrinsic factors such as nutrition and environmental conditions. Probiotics and phytobiotics are added in broiler diets to improve broiler health and performance by regulating gut microbiota and immune response. However, these feed additives may independently or in association with host-related factors show different impacts on metabolite production and immune responses in the broilers gut (**Chapter 1**).

Chapter 2 provides a summary of the literature on the effects of host-related factors as well as probiotics and phytobiotics on microbial activity and immune response in different intestinal regions of broilers.

Chapters 3 and 4 examine the effects of age (day 7, 21, 35), breed (Ross308 and Cobb500), sex (male and female), as well as probiotics (*Bacillus* based probiotics) and phytobiotics (grape polyphenols) on bacterial metabolite concentration, cytokine expression, gut morphology, and goblet cell count in the ileum and caecum of broilers. The overall results showed that age had remarkable impacts on all the measurements in the ileum and caecum, while breed, sex, and dietary treatments show only slight impacts on these parameters.

In conclusion, the present thesis demonstrated that the alterations in bacterial activity and immunological trait in the gut of broilers are age- and region-specific. The co-maturation of gut microbiota and immune system results in modification to the quality and quantity of bacterial-derived products as well as immune response during broiler growth. These changes in the ileum and caecum differed, which could be influenced by their distinct gut functions. Biological influences related to breed and sex as well as the supplementation of *Bacillus* based probiotics and grape polyphenols had a limited impact on bacterial activity and immunological traits, regardless of intestinal region. Future research is required to investigate the changes in gut microbial activity during broiler growth and to comprehend the underlying mechanisms of interactions between bacterial metabolites and gut immune system, which will elucidate the

potential actions of these metabolites in influencing the immune system in the gut (**Chapter 5**).

7. Zusammenfassung

Zusammenfassung der PhD-Arbeit:

Phenotypisierung des Darms von Broilern: Einfluss von wirtsspezifischen Faktoren und Futtermittelzusatzstoffe auf die bakterielle Aktivität und immunologische Eigenschaften

Bakterielle Stoffwechselprodukte sind aufgrund ihrer Auswirkungen auf das Darmepithel und das Immunsystem entscheidend für die Darmgesundheit von Broilern. Bakterielle Stoffwechselprodukte werden in verschiedenen Teilen des Verdauungstrakts produziert, wobei Kropf, Ileum und Zäkum die wichtigsten Orte sind. Die Produktion von mikrobiellen Stoffwechselprodukten im Darm von Masthähnchen kann durch intrinsische Faktoren wie Alter, Rasse und Geschlecht sowie durch extrinsische Faktoren wie Ernährung und Umweltbedingungen beeinflusst werden. Probiotika und Phytobiotika werden dem Futter für Masthähnchen zugesetzt, um die Gesundheit und Leistung der Tiere zu verbessern, indem die Darmmikrobiota und die Immunantwort reguliert werden. Diese Futtermittelzusatzstoffe können jedoch unabhängig oder in Verbindung mit wirtsspezifischen Faktoren unterschiedliche Auswirkungen auf die Metabolitenproduktion und die Immunreaktionen im Darm der Masthähnchen haben (**Kapitel 1**).

Kapitel 2 enthält eine Zusammenfassung der Literatur über die Auswirkungen von wirtsspezifischen Faktoren sowie Probiotika und Phytobiotika auf die mikrobielle Aktivität und die Immunreaktion in verschiedenen Darmregionen von Masthähnchen. Die Ziele und Hypothesen der Studie werden in Kapitel 3 ausführlich erläutert.

In den **Kapiteln 3 und 4** werden die Auswirkungen von Alter (7., 21., 35. Tag), Rasse (Ross 308 und Cobb500), Geschlecht (männlich und weiblich) sowie Probiotika (Mischprodukt aus drei Stämmen) und Phytobiotika (Traubenpolyphenole) auf die Konzentration bakterieller Metaboliten, die Zytokinexpression, die Darmmorphologie und die Anzahl der Becherzellen im Ileum und Zäkum von Broilern untersucht. Die Gesamtergebnisse zeigten, dass das Alter die größte Auswirkung auf die Messungen sowohl im Ileum als auch im Zäkum hatte, während Rasse, Geschlecht und Nahrungsbehandlungen kaum Auswirkungen hatten.

Zusammenfassend lässt sich sagen, dass die vorliegende Arbeit gezeigt hat, dass die Veränderungen der bakteriellen Aktivität und der immunologischen Eigenschaften im Darm von Masthähnchen alters- und regionsspezifisch sind. Die Ko-Maturation von Darmmikrobiota und Immunsystem führt zu einer Veränderung der Qualität und Quantität der bakteriell erzeugten Produkte sowie der Immunantwort während des Wachstums von Masthähnchen. Diese Veränderungen im Ileum und Zäkum unterschieden sich, was durch ihre

unterschiedlichen Darmfunktionen beeinflusst werden könnte. Biologische Einflüsse im Zusammenhang mit Rasse und Geschlecht hatten unabhängig von der Darmregion einen begrenzten Einfluss auf die bakterielle Aktivität und die immunologischen Eigenschaften. Die Supplementierung von Probiotika auf Bacillus-Basis und Traubenpolyphenolen zeigte fast keine Auswirkungen auf die Messungen im Ileum und Zäkum. Zukünftige Forschungsarbeiten sind erforderlich, um die dynamischen Veränderungen der mikrobiellen Aktivität im Darm zu untersuchen und die zugrunde liegenden Mechanismen der Wechselwirkungen zwischen bakteriellen Metaboliten und dem Immunsystem des Darms bei Masthähnchen zu verstehen, um die potenziellen Auswirkungen dieser Metaboliten auf das Immunsystem im Darm aufzudecken (**Kapitel 5**).

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9. List of publications

9.1. Published and accepted papers (peer reviewed)

Duangnumsawang Y, Zentek J, Goodarzi Boroojeni F (**2021**). Development and Functional Properties of Intestinal Mucus Layer in Poultry. Front Immunol 12:1–18. https://doi.org/10.3389/fimmu.2021.745849

Duangnumsawang Y, Zentek J, Vahjen W, Tarradas J, Goodarzi Boroojeni F (**2022**). Alterations in bacterial metabolites, cytokines, and mucosal integrity in the caecum of broilers caused by feed additives and host-related factors. Front Physiol 13:1593. https://doi.org/10.3389/fphys.2022.935870

Duangnumsawang Y, Zentek J, Vahjen W, Tarradas J, Goodarzi Boroojeni F (**2023**). Impact of feed additives and host-related factors on bacterial metabolites, mucosal integrity and immune response in the ileum of broilers. Vet Res Commun. https://doi.org/10.1007/s11259-023-10135-9

9.2. Congress contributions

9.2.1. Oral presentations

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2021**). The effects of age, gender, breed and feed additives on ileal epithelium and gene expression of broilers. 75. digitale Tagung der Gesellschaft für Ernährungsphysiologie – 16.03.-18.03.2021. In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.), DLG-Verlag; 30, 101. ISBN: 978-3-7690-4114-9

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2021**). Impact of feed additives and host-related factors on bacterial metabolic activity, and immunological traits in the caecum of broilers. Tagung der DVG-Fachgruppe Tierernährung – 13.10 – 15.11.2022. Basel. Switzerland. In: Proceeding of the 68th Conference of German Small Animal Veterinary Association - Deutsche Veterinärmedizinische Gesellschaft e.V. (Hrsg.); 23-25. ISBN: 978-3-86345-633-7

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2022**). Impact of feed additives and host-related factors on bacterial

metabolic activity and immunological trait in the caecum of broilers. 76. digitale Tagung der Gesellschaft für Ernährungsphysiologie – 08.03.-10.03.2022. In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.), DLG-Verlag; 31, 147. ISBN: 978-3-7690-4115-6

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2022**). Impact of feed additives and host-related factors on bacterial metabolic activity, and immunological traits in the caecum of broilers. Kaesler Forum : antibiotic resistance : is livestock farming a health risk? - 30.03.-31.03.2022. Berlin. Germany

9.2.2. Poster presentations

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2021**). The effects of age, gender, breed and feed additives on ileal histomorphology and gene expression of broilers. Tagung der DVG-Fachgruppe Tierernährung – 18.11 – 20.11.2021. In: Proceeding of the 67th Conference of German Small Animal Veterinary Association (DVG); 61. ISBN: 978-3-86345-586-6

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2022**). Impact of feed additives and host-related factors on bacterial metabolic activity, and immunological traits in the caecum of broilers. 26th Congress of the European College of Veterinary and Comparative Nutrition (Hrsg.) – 09.09.- 11.09.2021. In: Congress Proceedings of the European Society of Veterinary and Comparative Nutrition; 148. ISBN: 978-3-033-09422-2

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2022**). Impact of feed additives and host-related factors on bacterial metabolic activity, and immunological traits in the caecum of broilers. 1st Applied HoloGenomics Conference – 13.10.-15.10.2022. Bilbao, Spain. In: Presentation abstracts for the 1st Applied HoloGenomics Conference – HoloFood Innovation Action (Hrsg.); 22

Duangnumsawang Y, Goodarzi Boroojeni F, Vahjen W, Tarradas J, Esteve E, Estibaritz A, Marcos S, Zentek J (**2023**). Influence of host-related factors and feed additives on spatiotemporal patterns of intestinal T cell distribution of broilers. 77. Tagung der Gesellschaft für Ernährungsphysiologie – 07.03.-09.03.2023. Göttingen. Germany. In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.), DLG-Verlag

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12. Conflict of Interest

The author declares no conflict of interest.

13. Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

Yada Duangnumsawang

Berlin, 27.09.2023