CHICKEN INTESTINAL MYCOBIOME:

BIOGEOGRAPHY, SUCCESSION, AND RESPONSE

TO IN-FEED ANTIBIOTICS

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Abstract:
In-feed antibiotics increase animal performance primarily through modulation of the intestinal microbiota. However, the exact mechanism of action is not understood. Furthermore, a majority of research on intestinal microbiota has focused solely on the intestinal bacterial population with very little being known about smaller populations such as fungi. Deep sequencing of the internal transcribed spacer 2 (ITS2) region of fungal rRNA genes was utilized in two studies to characterize the biogeography of the chicken intestinal fungal community, the mycobiome, along the gastrointestinal tract of broiler chickens on days 28 and 42 and its possible shift in response to bacitracin methylene disalicylate (BMD), a commonly used in-feed antibiotic. Intestinal luminal contents were also collected from the duodenum, jejunum, ileum, and cecum at seven different time points throughout an entire production cycle to determine the succession of the mycobiome. The phyla Ascomycota and Basidiomycota were found to be predominant regardless of age or GI location. Genera commonly associated with feed ingredients or soil such as *Microascus, Gibberella, Trichosporon,* and *Aspergillus* were the most abundant. However, their abundance varied greatly between studies indicating a strong environmental influence. A clear succession of the cecal mycobiome was observed, in which specific fungal consortia moved down the intestinal tract as birds aged indicating that this population is transient in nature. Dietary supplementation of BMD at a subtherapeutic level of 55 mg/kg resulted in a decrease in the cecal fungal diversity. To further elucidate effect of antibiotics on cecal bacterial populations, broiler chicks were supplemented with or without one of five commonly used antibiotics for 14 days followed by deep sequencing of the V3-V4 region of the bacterial 16S rRNA gene. Although each antibiotic modulated the gut microbiota differently, the closer the antibacterial spectrum, the more similarly the microbiota is regulated with treatments classified as ionophores having the greatest effect. Importantly, all antibiotics had a strong tendency to enrich butyrate- and lactic acid-producing bacteria, while reducing bile salt hydrolase-producing bacteria, suggesting in-feed antibiotics improve animal growth performance through enhanced metabolism and utilization of dietary carbohydrates and lipids and improved energy harvest.

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CHAPTER I

INTRODUCTION

Subtherapeutic antibiotics have been routinely included in livestock diets to improve animal performance. However, a rise in antibiotic resistance linked to livestock use has led to a ban on antimicrobial use for growth promotion and a change in consumer preference towards antibiotic-free animal products. This has, therefore, created a need to develop effective antibiotic alternatives to ensure animal health and growth performance.

The gastrointestinal (GI) tract is home to a unique ecosystem of microorganisms consisting of bacteria, fungi, viruses, archaea, and protozoa, and antibiotics are hypothesized to provide performance benefits through modulation of this ecosystem. These microbes play a vital role in host health and metabolism through their effects on feed digestion, nutrient absorption, vitamin synthesis, and immune system development. To date, the majority of research has focused solely on the bacterial population, referred to as the microbiota. Its composition, as well as spatial and temporal changes, have been elucidated in several species. A link between the intestinal microbiota and growth performance of livestock animals has been established with studies showing strong correlations between specific bacterial taxa and weight gain. Furthermore, studies have shown the ability of specific antibiotics to modulate the intestinal microbiota, but with varying results.

Far less is currently known about the remaining microbial populations within the GI tract. Fungi in particular are known to account for less than 0.01% of the human fecal microbiota of humans, but are thought to account for a large proportion of the microbial biomass due to their size. Traditional culture dependent studies have identified yeast in the intestinal tracts of humans and animals with most focusing on the role of specific fungi in disease. With the advent of next-generation sequencing technology, some have undertaken to characterize the fungal population, known as the mycobiome, in more detail. However, information is still scarce with the majority of it pertaining to humans.

In this dissertation, we summarized the latest progress on the intestinal mycobiome of humans and livestock animals (Chapter II). We characterized the biogeography of the chicken intestinal mycobiome on day 28 and its response to a common antibiotic, bacitracin methylene disalicylate (BMD), using Illumina sequencing of the internal transcribed spacer 2 (ITS2) region of fungal rRNA genes (Chapter III). We revealed an obvious biogeographic difference in the mycobiota composition along the GI tract, with the crop, ventriculus, and duodenum being more diverse than the jejunum, ileum, cecum, and colon. The intestinal mycobiome was dominated by Ascomycota and Basidiomycota, with three genera including *Microascus, Trichosporon,* and *Aspergillus* accounting for over 83% of the total fungal population in any given segment, except for the duodenum, which harbored the most diverse fungal community. Antibiotic supplementation at 55 mg/kg was shown to decrease cecal diversity on day 14.

A second study was further conducted to characterize the biogeography of the mycobiome at the market age, day 42 (Chapter IV). Additionally, luminal contents were collected from four GI locations (duodenum, jejunum, ileum, and cecum) at seven time points throughout an entire production cycle (days 3, 7, 14, 21, 27, 35, and 42) to evaluate the succession of the mycobiome. Diversity was once again shown to be highest in the upper GI tract. However, an age effect was observed as the jejunum displayed the greatest diversity on day 42. The genera *Gibberella* was the

most abundant in this study regardless of age or GI location, followed by *Aspergillus* and *Candida.* Evaluation of the fungal composition at the feature level revealed a clear passage of four fungal consortia down the GI tract as birds aged. In total, these results revealed the chicken intestinal mycobiome to be transient in nature and highly influenced by the environment.

A third study was conducted to better understand the effect of antibiotics on intestinal microbes (Chapter V). Broiler chicks were raised for 14 days and supplemented with or without one of five commonly used antibiotics including three classical antibiotics (bacitracin methylene disalicylate, tylosin, and virginiamycin) and two ionophores (monensin and salinomycin) that differ in antimicrobial spectrum and mechanisms. Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA gene revealed each treatment to modulate the gut microbiota differently with ionophores having the strongest effect. Importantly, all treatments had a strong tendency to enrich butyrate- and lactic acid-producing bacteria, while reducing bile salt hydrolase-producing bacteria, suggesting enhanced metabolism and utilization of dietary carbohydrates and lipids and improved energy harvest.

Collectively, we have comprehensively characterized the biogeography and succession of the chicken intestinal mycobiota throughout one entire production cycle, and its response to BMD. Furthermore, we have added to the knowledge regarding the effect of antibiotics on intestinal microbiota showing antibiotic-specific modulation of cecal bacteria. This data lays the foundation for future work investigating the potential of intestinal microbiota manipulation as a novel strategy to develop alternatives to antibiotics for livestock applications.

CHAPTER II

REVIEW OF LITERATURE

COMPOSITION AND MODULATION OF THE INTESTINAL MYCOBIOME OF HUMANS AND LIVESTOCK ANIMALS

ABSTRACT

The rise in antibiotic resistance in recent years has led to a ban on the use of medically important antibiotics in livestock diets. Additionally, consumer preference has shifted to a preference for antibiotic free products creating an urgent need for effective antibiotic alternatives. As subtherapeutic antibiotics are widely considered to improve animal performance through modulation of the intestinal microbiota, extensive work has been accomplished regarding characterization of the bacterial communities of humans and livestock and their response to antibiotic supplementation. However, very little is known regarding the smaller microbial populations including fungi, viruses, archaea, and protozoa. Fungi in particular are known to assist in the breakdown of dietary fiber and stimulate host immune function. This makes them an intriguing target for future research focused on antibiotic mechanism of action and the discovery of novel probiotics. However, overgrowth of several species is known to be pathogenic indicating a delicate balance within the intestinal ecosystem. In this review we summarized recent research regarding composition of the intestinal fungal population, known as the mycobiome, of humans, mice, and livestock animals as well its modulation by diet and antibiotic supplementation. While work in this area is increasing, very little has utilized advanced sequencing technology to comprehensively evaluate fungal communities. We believe that modulation of the intestinal mycobiota may be a novel alternative to antibiotics. However, additional work using deep sequencing technology is first needed to characterize the mycobiome in different species.

Keywords: mycobiome, mycobiota, fungal microbiome, antibiotic alternative, antimicrobial resistance

INTRODUCTION

Subtherapeutic antibiotics were first discovered to improve growth in livestock animals in the 1940's (1) and subsequently became common additives of livestock diets to improve production performance (2, 3). However, increased microbial resistance linked to antibiotic use in food animals has led to a ban on antimicrobial growth promoters and a change in consumer preference towards products from animals raised without antibiotics (2, 4, 5). This has, therefore, created a need to develop antibiotic alternatives to ensure animal health and growth performance.

Antibiotics are hypothesized to improve animal performance through modulation of the intestinal microbiota (3, 6-9), which is a unique ecosystem consisting primarily of bacteria with smaller populations of fungi, viruses archaea, and protozoa. The intestinal microbiota plays a vital role in host health and metabolism through its effects on feed digestion, nutrient absorption, vitamin synthesis, and immune system development (10-12). To date, research has focused almost exclusively on the bacterial portion of the intestinal microbiota with very little known regarding the smaller populations.

Fungi have been shown to account for less than 0.03% of the fecal microbiota of humans and are typically considered to be part of the "rare biosphere" (13). However, a typical fungal cell is >100 times larger than a bacterial cell and make up a large portion of the microbial biomass (14). Overgrowth of fungal species such as *Candida* and *Aspergillus* is known to cause disease in humans with recent work associating fungal dysbiosis with intestinal and extra-intestinal diseases (14-16). Nevertheless, yeasts species such as *Saccharomyces cerevisiae* have been shown to be beneficial and are commonly used as probiotics for human and livestock applications (17-19). A better understanding of the fungal community, known as the mycobiome, lays the groundwork for future studies investigating modulation of this community to improve animal growth and health.

The first step in understanding the mycobiome is characterization of its composition in different species. Here we provide a review of the current literature regarding the intestinal mycobiome of humans and livestock animals and its modulation by diet and antibiotic supplementation. While the phyla *Ascomycota* and *Basidiomycota* predominate, mycobiome composition varies dramatically between species with factors such as age and diet playing a significant role on composition in each specie.

FUNGI IN HUMANS AND MICE

The presence of fungi in the human intestinal tract was recorded as early as 1917 by H. W. Anderson (20). Culture studies have identified fungi in the stool of infants as early as 1 week old with *Candida* spp. being the primary member observed (21-23). More recent investigations using Illumina deep sequencing technology have expanded this knowledge by identifying *Candida* species such as *C. parapsilosis, C. tropicalis, C. orthopsilosis,* and *C. albicans,* as well as *Saccharomyces cerevisiae, Cladosporium velox, Cryptococcus pseudolongus, Debaryomyces hansenii,* and *Rhodotorula mucilagnosa* on the skin, oral cavity, and fecal samples of infants (24, 25). The infant mycobiome was observed to be relatively stable over the first month of life (25) while sampling from 10 days of age to 2 years old revealed an increase in alpha diversity with age (24). Several species were found to overlap between the maternal and infant mycobiomes with offspring mycobiomes beginning to converge toward maternal patterns at 1 and 2 years of age (24).

In adults, mycobiome composition is known to vary by sampling location. Illumina ITS1 sequencing of oral wash samples revealed the presence of 81 genera with *Candida, Aspergillus*, *Cladosporium, Aureobasidium, Penicillium, Schizophyllum, Rhodotorula,* and *Gibberella* being the most abundant (26-28). The stomach was originally thought to be incompatible with microbial growth due to the harsh environment. However, E. C. von Rosenvinge et al. (29) found between

19 and 81 genus-level fungal OTUs in the stomach of adults with *Candida* and *Phialemonium* being found in all samples. However, 77.5% of all sequences could not be classified indicating a need for better reference databases for this intestinal location (29).

An early culture-based study of the small intestine identified 26 and 20 species in the jejunum and ileum, respectively (30). Culture-independent studies of these sites are limited. However, a metatranscriptome analysis of two patients receiving an ileostomy revealed the presence of *Candida* and *Saccharomyces* (31) while 18S rDNA polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis of ileal effluent also identified *Coccidioides, Kluyveromyces, Fusarium, Ajellomyces, Aspergillus,* and *Cryptococcus* (32). The feces of humans harbors between 10^2 and 10^6 cfu/g of cultivable fungi (33). Culture-independent studies of the fecal mycobiome have revealed a high amount of inter- and intra-individual variation (13, 34-37). Nevertheless, the genera *Candida* and *Saccharomyces* were identified in every study with *Malassezia, Aspergillus, Mucor, Penicillium,* and *Debayomyces* also identified in multiple studies (13, 34-37). By following the fecal mycobiome of 147 volunteers across three timepoints, A. K. Nash et al. (34) identified three OTUs, namely *S. cereisiae, M. restricta,* and *C. albicans,* to be present at all sampling time points in 63.6 to 92.2% of volunteers. The authors speculated that they may represent a core mycobiome. However, additional work supporting the idea of a core mycobiome has not emerged.

As intestinal fungi are part of a larger ecosystem, a few studies have investigated the relationship between the relative abundance of bacteria and fungi in human feces. A. K. Nash et al. (34), found several positive and negative correlations with *Rikenellaceae* and *Botrytis* displaying the strongest positive correlation while the strongest negative correlation was between *Penicillium* and *Faecalibacterium.* Additional studies have revealed a positive correlation between *Aspergillus* and *Bifidobacterium* and a negative association between *Candida* and *Bacteroides* (35, 38).

In mice, fungal load was shown to increase from the ileum to distal colon (39-41). While several of the same genera were identified in both mice and humans, the murine mycobiome appeared surprisingly different from that of humans. Oligonucleotide fingerprinting of rRNA genes (OFRG) analysis of restricted flora and specific pathogen free mice revealed high abundance of *Monilinia, Acremonium, Alternaria, Fusarium,* and *Cryptococcus* (40). More recent studies using next-generation sequencing revealed the presence of approximately 50 genera with over 97% of sequences belonging to only 10 species (39). While I. D. Iliev et al. (39) identified *Candida, Saccharomyces,* and *Trichosporon* in fecal samples (39), additional studies revealed very low abundance of these genera with members of *Wickerhamomyces, Debaryomyces, Aspergillus,* and *Penicillium* being most dominant (41, 42).

The murine mycobiome was also observed to change drastically over time. S. Dollive et al. (42) revealed the fecal mycobiome to be highly diverse for the first eight days of the experiment with no genera being predominate. A bloom of *Whickerhamomyces anomalus* was observed on day 15 becoming the most abundant species until day 22 when fecal mycobiomes diverged by cage (42). Specifically, one cage returned to a heterogenous state while the other was dominanted by *Debaryomyces hansenii* on day 22 and *Eurotiales* spp. on day 75 (42).

FUNGI IN LIVESTOCK ANIMALS

Supplementation of livestock diets with yeast species has been shown to improve production performance (43-45). A few species have been found to be pathogenic with *Candida, Aspergillus,* and *Fusarium* being associated with abortions and disease in livestock (46-52). However, only a handful of studies provide information on mycobiome composition in healthy animals.

RUMINANTS

Anaerobic fungi were first detected in ruminant animals by C. G. Orpin (53) and considerable work has been accomplished since then to understand this community. Fungi have been detected

in the rumen of lambs as early as 8 days of age (54) but were not detected in dairy calves until after weaning (55). In both cases they were preceded by bacterial colonization (54, 55). Anaerobic fungi concentration is highest in the rumen and omasum with abundance decreasing in the small intestine before increasing again in the large intestine and feces (56). Studies using anaerobic fungi specific primers have found the genera *Neocallimastix, Orpinomyces, Piromyces,* and *Caecomyces* in the rumen of beef and dairy cattle (57-60). All four are part of the family *Neocallimastigaceae* which has been shown to strongly co-occur with *Bacteroidales, Prevotella, Clostridia,* and *Lachnospiraceae* in the rumen of dairy cattle (59). These fungi are efficient at degrading cellulose while displaying limited ability to degrade protein sources (61, 62). For this reason, anaerobic fungi are thought to be the first to attach to fibrous material and contribute more to the degradation of plant material than rumen bacteria (63, 64).

Sequencing of rumen content using universal fungal primers such as ITS or 18S has revealed the presence of aerobic fungi as well with approximately 200 fungal genera identified (65, 66). Anaerobic fungi were found to predominate the rumen of sheep, cattle, and deer from New Zealand (67), and were the only fungi identified in the feces and rumen of dairy cattle sampled from two weeks of age to the first lactation (55). The latter is contradictory to a study by A. N. Hristov et al. (65) who found *Neocallimastix* to account for less than 3.5% of rumen mycobiota in dairy cows. A more recent study found anaerobic *Neocallimastigaceae* along with *Orpinomyces* and *Piromyces* to be most abundant in Holstein cows fed a high fiber diet while an increase in aerobic fungi belonging to the phylum Ascomycota was observed following switch to high grain diet (66). The most abundant aerobic fungi observed in the rumen include *Eupenicillium, Pichia, Candida, Aspgergillus, Fusarium, Trichosporon,* and *Cladosporium* (65, 66).

POULTRY

While several studies have investigated the role of *Aspergillus* and *Fusarium* in poultry disease (49-52), few have sought to understand the commensal fungal population. In broilers, fungal burden has been found to range from 1.6×10^3 to 3.9×10^3 cfu/g digesta with load being lowest in the cecum followed by the proventriculus and jejunum (68). *Candida* has been most often identified by culture-dependent studies followed by *Saccharomyces, Geotrichum, Trichosporon,* and *Rhodotorula* (68-71). Contrary to these results, J. A. Byrd et al. (72) identified 88 fungal species from the ceca of broiler chickens with *Aspergillus* being the most frequently identified followed by *Penicillium, Verticillium,* and *Sporidiobolus*.

To date, only two studies have used culture-independent methods to investigate the poultry mycobiome. Using 454 Roche sequencing only two *Cladosporium* species were detected in the cecum of broilers fed unmedicated diet, although another 17 fungal species were detected in animals supplemented with essential oils (73). More recently, ITS2 deep-sequencing of the turkey ileum revealed *Sarocladium kiliense* to be most abundant followed by *Candida, Dothidea,* and *Cladosporium* species (74). While the same four genera were predominant over time, sequential sampling of birds from the same cage revealed an increase in ileal alpha diversity over time (74).

PIGS

Contrary to other species, pigs appear to harbor a distinct commensal mycobiome dominanted by *Kazachstania slooffiae* (75-81). While very little is known about this yeast, characterization of the cell contents revealed abundant concentrations of amino acids including aspartic acid, glutamic acid, alanine, valine, isoleucine, leucine, lysin, proline and tryptophan (82). Exogenous supplementation of *K. slooffiae* after weaning was associated with in an increase in short chain fatty acid production, primarily propionic and valeric acid, and increased bacterial diversity (78). Along with *K. slooffiae,* members of *Pichia, Saccharyomyces, Trichosporon,* and *Cryptococcus*

were identified by culture or PCR-DGGE (79-81), while *Hyphopichia* and *Wallemia* were identified as abundant using ITS2 deep sequencing (75).

Investigation of the fecal mycobiome as pigs age revealed a low fungal load and very little change in fungal composition from birth to weaning at 21 days of age (77). A dramatic shift was observed at weaning with diversity increasing and the predominate family *Cladosporiaceae* being replaced by *Saccharomycetaceae* (77). The community then remains stable through at least 35 days of age (76, 77). It is interesting to note that the dominant species *K. slooffiae* is not observed until after weaning (75, 77, 78). One study investigating biogeography of the piglet mycobiome on day 39 found fungal diversity to be highest in the stomach before decreasing in the small intestine and increasing again in the colon (77). While four genera were found to be abundant across all gastrointestinal locations, beta diversity analysis found stomach and feces samples to cluster independently of the small intestine segments (77).

Similar to humans and dairy cattle, members of the porcine intestinal mycobiome have been shown to correlate with bacterial members. Using PCR-DGGE, total fecal yeast counts were positively correlated with *Lactobacilli* and negatively correlated with *Enterobacteria* (79). More recently, ITS2 sequencing revealed *Kazachstanie* to be positively correlated with several genera including *Alloprevotella, Lactobacillus, Prevotella 9,* and *Subdoligranulum* (75). The genera *Aspergillus, Cladosporium, Hyphopichia,* and *Wallemia* were negatively correlated with multiple bacterial species with the most notable association being the negative correlation between *Aspergillus* and short chain fatty acid producing bacteria (75).

EFFECT OF DIET ON THE INTESTINAL MYCOBIOME

Diet is observed to be a major driver of intestinal mycobiome composition regardless of species. Characterization of the human mycobiome in association with the Human Microbiome Project did not reveal any significant associations between mycboiota composition and the metadata

collected (34). This led the authors to speculate that factors such as diet, environment, daily cycles, and host genetics probably play a larger role in influencing fungal composition (34). In agreement with this, fecal mycobiomes of individuals on a vegetarian diet were dominated by *Fusarium, Malassezia, Penicillium,* and *Aspergillus* species with only low levels of *Candida* observed (37). Furthermore, fecal pellets of mice fed a high fat diet had a high abundance of *C. albicans* while *S. cerevisiae* was predominant in mice fed a normal diet (83).

As mentioned previously, switching dairy cattle from a high fiber to a high grain diet was associated with increased fungal diversity (66). Specifically an increase in aerobic fungi belonging to the phyla Ascomycota was observed which was associated with a concomitant decrease in anaerobic fungi (66) and decrease in anaerobic fungal diversity and load (57, 58, 60, 84). This change in composition is most likely due to the introduction of new fungal species in high concentrate diets combined with a reduction in cellulose, a preferred substrate of anaerobic fungi.

In pigs, dietary modulation of the mycobiota is most strongly associated with weaning and the introduction of solid feed (77). However, supplementation of piglet diets with transgalacto-oligosaccharides post-weaning was able to increase fecal yeast burden as compared to piglets on a control diet or the control diet supplemented with fructos-oligosaccharides (85). Additionally, fungal burden increased in the digesta of growing pigs fed a fermented liquid diet versus a nonfermented liquid diet (86).

MODULATION OF INTESTINAL MYCOBIOTA BY ANTIBIOTICS

Sub-therapeutic antibiotic supplementation is known to improve performance of livestock animals with modulation of intestinal microbial communities being most often proposed as the mechanism of action (3, 6-9). The effect of antibiotics on the mycobiome, though, is still unclear. Evaluation of stomach fluid revealed a striking reduction in fungal richness in one patient taking

antibiotics (29). Conversely, diversity was shown to be higher in antibiotic induced remission of pouchitis and to decrease when patients were switched to a placebo or probiotic treatment (87). A similar increase in diversity was observed in. mice provided an antibiotic cocktail which further showed an ability of the mycobiota to return to normal following cessation of antibiotic treatment (42).

Only two studies have investigated the mycobiome of livestock animals in the presence of antibiotics. In cattle, supplementation of diets with either monensin or nisin reduced the total rumen fungal population *in vitro* (88). In turkeys, sub-therapeutic BMD supplementation had no effect on the ileal mycobiota (74). While it is unlikely that antibiotics attack intestinal fungi directly, their effects on the bacterial population could results in subsequent changes in the mycobiome. More work is needed in this area to fully elucidate the broader effects of antibiotics on the intestinal microbial populations as a whole.

CONCLUSION

While work has begun to be completed regarding the intestinal mycobiome, research on this population is currently scarce with large variations observed between studies. Traditional culture based studies often identified *Candida* as the most abundant species of humans and monogastric animals. However, application of next-generation sequencing technology has revealed mycobiota composition to be unique in each species. The observation that diet is a major driver of fungal composition, along with the lack of a “core mycobiome”, indicates that fungi may be transient in nature with very few capable of colonizing the intestinal tract. While some fungal species are currently used as probiotic supplements, it has yet to be determined if modulation of the community is a viable target for antibiotic alternatives. In total, more work is needed in this area to determine not only mycobiome composition but its role in health and disease.

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CHAPTER III

CHICKEN INTESTINAL MYCOBIOME: INITIAL CHARACTERIZATION AND ITS RESPONSE TO BACITRACIN METHYLENE DISALICYLATE

ABSTRACT

The gastrointestinal (GI) tract harbors a diverse population of microorganisms consisting of not only bacteria, but also fungi, viruses, archaea, and protozoa. While much work has been focused on the characterization of intestinal bacterial community, very little is known about the fungal community, or mycobiota, in different animal species and chickens in particular. Here we characterized the biogeography of the mycobiota along the GI tract of day-28 broiler chicks and further examined its possible shift in response to bacitracin methylene disalicylate (BMD), a commonly used in-feed antibiotic, through Illumina sequencing of the internal transcribed spacer 2 (ITS2) region of fungal rRNA genes. We revealed an obvious biogeographic difference in the mycobiota composition along the GI tract, with the crop, ventriculus, and duodenum being more diverse than the jejunum, ileum, cecum, and colon. The intestinal mycobiome was dominated by Ascomycota and Basidiomycota, with three genera including *Microascus, Trichosporon,* and *Aspergillus* accounting for over 83% of the total fungal population in any given segment, except for the duodenum, which harbored the most diverse fungal community. We also observed an obvious shift of the cecal mycobiota between day-14 and day-28 broilers. Dietary supplementation of BMD at a subtherapeutic level of 55 mg/kg resulted in a significant decrease in the cecal fungal diversity. Taken together, we provided a comprehensive biogeographic view and maturation of the chicken intestinal mycobiota and its influence by an in-feed antibiotic. A better understanding of intestinal mycobiota may lead to development of novel strategies to improve poultry health and productivity.

KEYWORDS: Mycobiome, chickens, bacitracin methylene disalicylate, microbiome

INTRODUCTION

The intestinal tract is home to a diverse microbial population consisting primarily of bacteria with smaller populations of fungi, archaea, protozoa, and viruses (1, 2). To date, extensive work has been performed to characterize the intestinal bacterial community. Dysbiosis of gut microbiota is known to be associated with a variety of intestinal and extra-intestinal diseases (1, 2). However, very little is known about non-bacterial populations of the intestinal tract. Recently, increased work has been conducted to understand the fungal community of the human intestinal microbiota, known as the mycobiota, and its role in health and disease (3, 4). While large individual variations have been observed, human oral and intestinal fungi are known to belong primarily to the phyla Ascomycota and Basidiomycota, with genera such as *Saccharomyces, Candida, Aspergillus, Cladosporium, Penicillium, Wallemia, Malassezia, Aureobasidium,* and *Epicoccum* being most often observed (3-5). Although fungal species comprise fewer than 1% of the microorganisms in the human intestinal tract, recent work has demonstrated their ability to modulate the innate and adaptive immune systems (6-8). Fungal dysbiosis has been linked to multiple diseases such as inflammatory bowel disease, allergic airway disease, atopic dermatitis, and alcoholic liver disease (3, 4, 9).

To date, most of the mycobiota research have focused on humans and mice. Little is known regarding the intestinal mycobiota of livestock animals and nonruminants in particular. Ruminant mycobiome research has primarily focused on anaerobic fungi belonging to the phylum Neocallimastigomycota (10-14). First discovered in the rumen of sheep (15), anaerobic fungi are thought to be the first to attach to fibrous material (16) and contribute more to the degradation of plan material than cellulolytic bacteria (17). In pigs, *Kazachstania sloofiae* is the most frequently detected species in weaned Landrace pigs in a culture-based study (18), whereas deep sequencing identified *Kazachstania telluris* as the dominant fungal species in a miniature breed of weaned pigs (19).

In limited poultry mycobiome studies, all but one were culture-based and most were restricted only to the cecum (20-25). *Candida* or *Aspergillus* was revealed to be the predominant in the chicken cecum in culture-dependent studies. Surprisingly, the study that employed a culture-independent pyrosequencing approach only detected two *Cladosporium* species in the cecum of broilers fed unmedicated diet, although another 17 fungal species were detected in animals supplemented with essential oils (20). Therefore, we sought to comprehensively characterize the biogeography of the intestinal mycobiome of broiler chickens using Illumina deep sequencing of the luminal contents throughout the GI tract. We further investigated the maturation and the effect of subtherapeutic and therapeutic supplementation of bacitracin methylene disalicylate (BMD), an in-feed antibiotic, on the cecal mycobiome composition.

MATERIALS AND METHODS

Animal trial

All animal trials were conducted in accordance with the Institutional Animal Care and Use Committee of Oklahoma State University under protocol number AG-173. Day-of-hatch male Cobb broiler chicks were obtained from Cobb-Ventress Hatchery (Siloam Springs, AR) and randomly assigned to one of three dietary treatments with 12 birds per cage and 6 cages per treatment. Birds were provided ad libitum access to feed and tap water for the entire duration of the trial. Dietary treatments included antibiotic-free standard corn-soybean starter mesh diet or the starter diet supplemented with BMD at subtherapeutic (55 mg/kg) or therapeutic (275 mg/kg) levels. Birds were raised on floor cages with fresh dry pine wood shavings under standard management in an environmentally controlled room with temperatures starting at 33^{o}C and decreasing 3^{o}C every 7 days. Lighting for this trial included the light to dark ratio of 23:1 from day 0 to 7, 16:8 from day 8 to 22, 17:7 on day 23, and 18:6 from day 24 to 28. To minimize cross-contamination, birds in different treatments were housed in separate rows with physical

barriers in between. On days 14 and 28, two birds per cage and 12 birds per treatment were euthanized via CO_2 asphyxiation, and the cecal contents were collected to evaluate the effect of BMD on the cecal mycobiome. Additionally, the luminal contents were collected from different segments of the GI tract (crop, ventriculus, mid-duodenum, mid-jejunum, mid-ileum, cecum, colon, and cloaca) on day 28 from 12 control birds for characterization of the chicken intestinal mycobiome. The samples were immediately frozen in liquid nitrogen and stored at -80ºC until further processing.

DNA isolation and sequencing

Microbial DNA was isolated from intestinal contents using the ZR Fecal 96-Well DNA Isolation Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. DNA quality and quantity were determined using Nanodrop ND-1000 and the absence of degradation was confirmed using agarose gel electrophoresis. High-quality DNA was shipped on dry ice to Novogene (Novogene, Beijing, CA) for next generation PE250 sequencing of the ITS2 gene using the ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) primers on an Illumina HiSeq platform. PCR amplification and library preparation were performed by Novogene using the NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs, Ipswich, MA).

Bioinformatic analysis and statistics

Illumina paired-end reads were analyzed using the Deblur program (52) in Qiime2 (ver 2019.7) (53). Deblur is capable of achieving single-nucleotide resolution based on error profiles within samples and produces denoised sequences, known as amplicon sequence variants (ASVs) or exact sequence variants (ESVs) which can be compared between studies. Fungal sequences were processed using the 'deblur denoise-other' option with positive alignment-based filtering against the UNITE reference database. Denoised sequences were classified into fungal features using the

WARCUP v2 fungal ITS database and the Ribosomal Database Projects (RDP) Bayesian Classifier (54, 55). A bootstrap confidence of 80% was used for taxonomic classification. Features with a classification of less than 80% were assigned the name of the last confidently assigned level followed by "_unidentified". Species classification of the top 20 OTUs was confirmed through BLASTN search of the nucleotide database of GenBank. Features appearing in less than 5% of samples were removed from downstream analysis. Data was normalized using cumulative sum scaling in the metagenomeSeq package of R (56). Analysis and visualization of the mycobiota composition was conducted in R version 3.5.1 (57). The α- and β-diversities were calculated with the phyloseq package version 1.24.2 (58). Plots were made using ggplot2 version 3.0.0 (59).

Normality of the data was determined using Shapiro Wilks test in R. Significant differences in the α-diversity and the relative abundance were determined using Kruskal-Wallis and multiple comparisons were performed using pairwise Wilcoxon Rank Sums test. The α-diversity was calculated using Shannon Index and Observed OTUs. Results were plotted using box and whisker plots, in which the middle line denotes the median value and the lower and upper hinges represent the first and third quartiles, respectively. Whiskers extend from the hinge to the highest or lowest value no farther than 1.5× the inter-quartile range. Points outside of this range are considered outliers. The β-diversity was calculated using the Bray-Curtis and Jaccard indices as dissimilarity measures of diversity and richness, respectively. Significant differences were determined using ANOSIM in mothur (60).

Accession number

The raw sequencing reads of this study have been deposited in NCBI under the accession number BioProject PRJNA512838.

RESULTS

Biogeography of the chicken intestinal mycobiota

Male Cobb broiler chicks were fed an antibiotic-free, non-medicated diet under standard management as recommended by Cobb-Vantress (Siloam Springs, AR). To characterize the biogeography of intestinal mycobiota, luminal contents were obtained from eight different segments of the GI tract (crop, ventriculus, duodenum, jejunum, ileum, cecum, colon, and cloaca) of 12 heathy chickens on day 28. Microbial DNA was isolated and sequenced on an Illumina HiSeq using the internal transcribed spacer 2 (ITS2) chemistry. Following quality control, 31,148,538 high-quality sequencing reads were obtained with an average of 249,188 sequences per sample. Sequences were denoised using deblur and rare reads were removed resulting in identification of 648 features at the single-nucleotide resolution.

The α-diversity of the intestinal mycobiota was determined using Shannon Index (Fig. 1A), Number of Observed Features (Fig. 1B), and Pielou's Evenness Index (Fig. 1C), respectively. All metrics revealed a significant difference in the α-diversity across the GI tract. Interestingly, the mycobiome in the crop, ventriculus, and duodenum displayed the highest levels of richness, evenness, and diversity. A sharp decrease in Shannon diversity was observed the jejunum and ileum, while the lowest α-diversity was observed in the cecum. The overall α-diversity was increased slightly in the colon and cloaca, both of which also display larger variations among individual animals. A similar trend was observed for richness with cecum and colon displaying the least number of features per sample. Evenness also followed a pattern similar to that of the Shannon index and richness measurements. However, jejunum and ileum were shown to have the least balanced communities followed by a slight increase in evenness in the cecum, colon, and cloaca.

To measure the difference in the mycobiota composition among the GI locations, β-diversity analysis was performed using the Bray-Curtis and Jaccard indices. ANOSIM analysis revealed a significant difference in the β-diversity for both metrics ($P < 0.001$) with R values of 0.418 and 0.461 for the Bray-Curtis and Jaccard indices, respectively. Visualization of the Bray-Curtis distance matrix using PCoA analysis revealed a tendency to form three clusters (Fig. 2A). The first was dominated by the crop, colon, and cloaca, while the second and third were comprised of ileum and cecum, respectively. Greater individual variations in the mycobiota composition were observed for the ventriculus, duodenum, and jejunum. Similar results were seen with the Jaccard index (Fig. 2B), with the exception of a tendency for the ventriculus samples to cluster with the ileum, while the jejunal samples clustered with the cecum. Pairwise comparisons revealed a significant difference in almost all comparisons regardless of index (Supplementary Table 1). However, it should be noted that R values for significant comparisons range from 0.088 to 0.874, indicating that some significant p-values do not reflect a true difference in composition.

To further evaluate the mycobiota composition of day-28 broiler chickens, the relative abundance was calculated at both the phylum and feature levels (Fig. 3). Ascomycota and Basidiomycota were two most dominant phyla throughout the entire GI tract, representing 89-99.9% of the total fungal population in any given segment (Fig. 3A). While Ascomycota accounted for 72.6-88.0% of the fungi, Basidiomycota amounted to 11.7-27.4% (Data not shown). Zygomycota and Glomeromycota were two minor phyla detected at an average of less than 0.1% in each GI location, and approximately 0.02% fungi failed to be classified. Collectively, the minor phyla and unidentified Fungi were denoted as “Others” (Fig. 3A).

Gut mycobiota appeared to be rather limited in diversity, with top 20 features accounting for 66.8-96.3% of total fungal population in any given intestinal segment (Supplementary Table 2). In fact, all of the top 20 features were represented by members of the *Microascus,*

Trichosporon, and *Aspergillus* genera. A BLAST search of the GenBank database further confirmed that eleven of the top 20 features showed a 97 – 99% identity to the *Microascus* member *Scopulariopsis brevicaulis*, while all four *Trichosporon* features showed a 97-100% identity to *T. asahii*. Due to the high resolution of the deblur algorithm, it is very likely that these features represent several strains of one species or several closely related species in the GI tract. Out of five *Aspergillus* species, F3, F10, F15, and F20 are 99-100% identical to several related species of *Aspergillus*, while F13 is 99-100% identical to *A. versicolor* (Supplementary Table 2). All of these top 20 features, showed a differential enrichment in different segments of the GI tract (Supplementary Table 2). Furthermore, many of the highly homologous features were enriched differently. For example, the relative abundance of *S. brevicaulis* F2, F5, and F8 varied greatly throughout the GI tract. While *S. brevicaulis* F12 and F17 were most abundantly colonized in the crop, colon and cloaca, *S. brevicaulis* F4 and F7 were found to consistently be absent from the ileum and cecum (Supplementary Table 2).

Maturation of the cecal mycobiome

Previous work in poultry has demonstrated a maturation of the cecal bacteriome as birds age (26-28). To evaluate developmental succession of the mycobiota, the cecal contents were collected from 12 healthy male Cobb broiler chickens at 14 and 28 days of age and analyzed for their mycobiota compositions. No significant difference was observed for either the Shannon Index (Fig. 4A), Observed Features (Fig. 4B), or Pielou's Evenness Index (Fig. 4C). A large inter-individual variation appeared to be present on day 14 versus day 28. This was especially true for sample richness. Additionally, β-diversity analysis revealed a significant separation between day 14 and day 28 for the Bray-Curtis (Fig. 5A) index, although ANOSIM analysis revealed the separation to be weak, as indicated by $R = 0.267$. Similar to α-diversity, the Jaccard (Fig. 5B) index found no difference in richness between day 14 and day 28.

Compositionally, the cecal mycobiome did appear to undergo a substantial change in composition. This was most clearly seen by a strong increase in Ascomycota, and subsequent decrease in Basidiomycota, abundance on day 28 relative to day 14 (Fig. 6A). Relative abundance of fungal features revealed two *Trichosporon asahii* features (F1 and F9) to dominate every sample on day 14 followed by members of *Scopulariopsis brevicaulis* (F8 and F11) and *Aspergillus sp.* (F3 and F15) (Fig. 6B). An increase in three *S. brevicaulis* features (F8, F11, and F21) was observed on day 21 along with an increase in the *Aspergillus* feature F3. Statistical analysis on the top 20 features using Kruskal-Wallis test did not reveal any significant differences at FDR ≤ 0.05 (Supplementary Table 3).

Effect of BMD on the cecal mycobiome

A number of studies have demonstrated the ability of antibiotics to modulate the chicken intestinal bacterial community (29-32). To determine the potential effect of antibiotics on intestinal mycobiome, day-of-hatch male Cobb chicks were fed unmedicated feed or feed supplemented with either a subtherapeutic (55 mg/kg) or therapeutic level (275 mg/kg) of BMD, a commonly used in-feed antibiotic, continuously for 14 days, followed by analysis of the cecal contents from 12 chickens in each of the three groups. Although we failed to observe any significance difference in growth performance of chickens (data not shown), the α-diversity analysis of the cecal mycobiome revealed a significant decrease in both the diversity and richness in response to BMD (Fig. 7). The Shannon index revealed a decrease in overall diversity in response to both doses of BMD. However, the shift was only found to be significant for the subtherapeutic group. Cecal richness appeared to dose dependently increase in response to BMD with the therapeutic group being significantly greater than both control and subtherapeutic BMD. A similar trend to the Shannon Index was observed for community evenness with no significant difference between groups (Fig. 7C). It should be noted that a high degree of variation in all indices was observed for both the control and therapeutic BMD groups. The β-diversity analysis

revealed a significant difference in the mycobiome composition as measured by both overall diversity ($P < 0.001$, R = 0.269) (Fig. 8A) and richness ($P = 0.001$, R = 0.448) (Fig. 8B). Both indices revealed two distinct clusters with both doses of BMD clustering largely separately from the controls. Pairwise comparisons revealed a significant difference between all three groups for the Jaccard index with the strongest difference observed between control and BMD55 (R = 0.570) (Supplementary Table 4). For the Bray-Curtis index, both doses of BMD were significantly different from control but not different from one another.

Investigation of the relative abundance of different fungal phyla in the chicken cecum revealed no significant difference ($FDR > 0.05$) in either the Basidiomycota or Ascomycota populations in response to BMD. Basidiomycota accounted for 69.1 – 81.5% of all fungi while Ascomycota relative abundance ranged from 18.5 – 30.8% (Data not shown). *Trichosporon, Aspergillus,* and *Microascus* were the dominant genera regardless of age (Fig. 9A). An increase in *Aspergillus* was observed in response to BMD treatment at the expense of *Microascus.* A number of features were shown to be significantly regulated by BMD with closely related features being regulated differently (Fig. 9B). For example, both doses of BMD had a tendency (FDR = 0.068) to increase the abundance of *T. asahii* F1 from approximately 27% in the control group to 75% and 72% in the subtherapeutic and therapeutic groups, respectively. However, *T. asahii* F9 was completely abolished in both BMD groups while another *T. asahii* feature (F34) remained largely unchanged (Supplementary table 5). Similar results were observed with *S. brevicaulis* features as F8, F11, and F21 disappeared in the presence of BMD while F2 and F5 increased in a dose-dependent manner (Supplementary table 5). Additional features significantly affected by BMD included enrichment of an unidentified *Aspergillus sp,* F3, *Aspergillus versicolor* F13, and *Debaryomyces hansenii* F424, as well as depletion of *Aspergillus sp* F15, *Stenocarpella maydis* F45, and *Debaryomyces hansenii* F43 (Supplementary table 5).

DISCUSSION

While extensive work has been performed in multiple animal species to characterize the intestinal bacterial population and its relationship with health and disease, very little is known about the intestinal mycobiota particularly in chickens. The fungal community is estimated to account for approximately 0.02% of the intestinal mucosa-associated microbiota and 0.03% of the fecal microbiota in humans, albeit with large variations among individuals (33). In humans and mice, members of Ascomycota and Basidiomycota predominate, with low abundance of Zygomycota and Chytridiomycota in the GI tract (3, 34). Culture-dependent studies have long identified members of the genus *Candida* to be prevalent in the human GI tract (3), while culture-independent techniques have typically revealed the presence of less than 100 fungal genera, with *Candida, Penicillium, Wallemia, Cladosporium*, *Cladosporium,* and *Saccharomyces* being the most prevalent in the stools of healthy individuals (3, 4). To date, at least 50 different fungal genera have been identified in mice, with *Candida, Saccharomyces*, and *Cladosporium* being the most common (4). Next generation sequencing of swine gut mycobiota has revealed the presence of three phyla (Ascomycota, Basidiomycota, and Zygomycota) and 67 genera (19). The phylum Ascomycota accounts for more than 97% of the sequence reads, while *Kazachstania*, a genus of Ascomycota, is represented by over 78% of the sequences (19).

Previous limited investigations into the poultry mycobiome have relied primarily on culture-based techniques. Using culture methods, most studies have reported fewer than 20 fungal species and *Candida* has been frequently described as the most abundant genus in the GI tract of chickens and turkeys, although dominant *Candida* species varies among different studies (21-24). Besides *Candida, Trichosporon, Geotrichum, Rhodotorula*, and *Saccharomyces* have been frequently isolated in chickens as well (21-23). However, one other culture-dependent study reported the identification of 88 fungal species from over 3,000 cecal samples of broiler and layer chickens, with *Aspergillus, Penicillium, Verticillium*, and *Sporidiobolus* being the top four genera

(25), whereas a culture-independent 454 pyrosequencing method only revealed two fungal species, *Cladosporium sp.* and *Cladosporium sphaerospermum*, in the cecum of broilers fed unmedicated diets (20), presumably due to a lack of the sequencing depth.

In this study, we identified 659 unique fungal features in the chicken GI tract. Consistent with previous studies in chickens and other animal species, our Illumina deep sequencing revealed a high abundance of Ascomycota throughout the GI tract of broiler chickens, followed by Basidiomycota in day-28 broiler chickens. Both phyla account for 90-99% of all fungal populations in any segment of the GI tract. Among all classifiable fungi, *Microascus, Trichosporon, Aspergillus,* and *Gibberella* are the top four most abundant genera (data not shown). Clearly, because of the depth of our sequencing approach, we have significantly expanded the repertoire of the fungal community in the GI tract of chickens in this study.

Using the deblur method of denoising in Qiime2, we were able to achieve single-nucleotide resolution in differentiating fungal features. Suprisingly, we identified *Microascus* as the most dominant genus on day-28 with 11 of the top 20 features in the chicken GI tract belonging to *S. brevicaulis.* This is in sharp contrast to previous studies. Furthermore, we did not observe any *Candida* species in our study while others found it to be a major genus. Such apparent discrepancies between our and several previously published studies are likely due to the dietary, environmental, and technical differences among different studies. Most poultry studies relied on culture-based methods, which are known to be biased towards fast-growing and non-fastidious species (3), which is not surprising why the *Candida* species are frequently observed. Additionally, it is well known that the microorganisms including fungi present in the diet and on the flooring and caging materials are also major contributors to the intestinal microbiota (35, 36). Furthermore, the age and genetic background of animals used in several studies could be different. Nevertheless, the remaining top features belong to the genera *Trichosporon* and *Aspergillus,* both of which have been reported in humans as well as in chickens (21-23).

It is noted that the top features in our study are most commonly associated with soil or cereal grains, so their presence in the GI tract of broiler chickens likely originated from feed ingredients or wood shavings used on the floor. For example, the most dominant *Microascus* member, *S. brevicaulis,* in our study is a saprophyte and commonly found in soil, plant litter, wood, dung, and animal remains (37). Other dominant *Trichosporon* and *Aspergillus* species are also ubiquitous in soil and commonly associated with decaying plants and food material (38-40), while *Gibberella* is most likely derived from the corn used in the feed as it often causes Gibberella ear rot, a fungal disease manifested by the red or pink color of the mold starting at the ear tip of corns (41).

Although they are likely to originate from the diet or the surrounding environment, most of these fungi appear to be able to survive in the GI tract, because their abundance remains relatively stable throughout the GI tract. In fact, *S. brevicaulis* has genetic potential to degrade a large variety of plant substrates (42), while *T. asahii* can assimilate a large variety of carbon and nitrogen sources (38), which may contribute to their ability to survive and thrive in the poultry GI tract. Whether these dominant fungal species play a beneficial role to the host and how they interact with the host remain unknown. Further work is warranted to better understand the role of these intestinal fungi in nutrient digestion, gut health, and disease resistance of poultry. It is worth noting that many of the *Microascus, Trichosporon,* and *Aspergillus* species are considered opportunistic pathogens particularly in immunocompromised humans (38, 39, 43). Therefore, these dominant intestinal fungi need to be monitored for possible contamination in poultry meat, litter, aerosol, and processing plant in order to ensure food safety and the safety of personnel.

Similar to the biography of animal intestinal bacteriome, different segments of the GI tract harbor rather a distinct fungal community in chickens. However, to our surprise, the upper GI tract (crop, ventriculus, and duodenum) hosts a more diverse mycobiota than the jejunum, ileum, and cecum. Specifically, the chicken duodenum exhibits the highest diversity, while the

cecum shows the least diversity in the fungal community. These observations are opposite to the intestinal bacteriome, which is the most abundant and diverse in the lower GI segments of chickens (44). However, our mycobiome results are consistent with a recent culture-based study showing that the fungal population is the lowest in the cecum of broiler chickens, relative to the jejunum and proventriculus, while layers have the highest fungal load in the cecum (21). In turkeys, 50% of all fungal strains were cultured from the crop, with 31% coming from the beak cavity and 19% from the cloaca (23). The biogeography of the intestinal mycobiota in humans remains elusive, but a direction comparison of fungal rRNA copy number among the ileum, cecum, and proximal and distal colon of mice showed a gradual increase in the fungal population along the GI tract (6). Apparently, additional larger-scale studies on the spatial distribution and absolute quantification of the fungal community in the GI tract of different animal species are warranted in order to derive a more definitive conclusion, given large variations in the mycobiota among individuals (45) and the paramount role of the diet and environment in shaping the intestinal microbial community (36).

Intestinal bacterial community typically undergoes age-dependent succession and becomes more diversified and stabilized as animals mature (13, 26, 27). While a difference in bacterial composition was observed between day 14 and day 28 as measured by the Bray-Curtis index, no significant difference was seen in richnes or cecal alpha diversity. This is most likely due to the large variation observed on day 14. A numerical difference in fungal composition was observed as birds aged. In particular, day 28 was marked by an increase in Ascomycota, driven by *S. brevicaulis* features belonging to the *Microascus* genus, and a concomitant decrease in Basidiomycota, primarily *T. asahii* features. However, statistical analysis did not find any of these changes to be significant. Human fecal mycobiota compositions are obviously different among healthy individuals of different age groups (46). Specifically, an inverse relationship between the intestinal fungal α-diversity and age was observed in an earlier human study showing

that infants and children harbor more fungal species than adults (46). This is consistent with the numerical, though not statistically significant, decrease in the number of features observed on day 28 versus day 14. Obviously, sampling beyond 28 days of age is needed to determine whether the chicken intestinal mycobiota could eventually reach the stability and "maturation" stage. Additionally, absolute quantification of the fungi throughout the GI tract of chickens of different ages is also warranted to assess temporal changes of the overall fungal abundance.

Antibiotics are known to significantly alter gut microbiota of chickens (29-32) and other animal species including humans (47); however, its impact on the fungal community has not been extensively explored. BMD is a common in-feed antibiotic and a derivative of bacitracin that is mainly active against Gram-positive bacteria by interfering with the synthesis of peptidoglycan, a major bacterial cell wall component (48). In this study, subtherapeutic inclusion of BMD causes a significant decrease in the α-diversity of the chicken cecal mycobiota but has no effect on cecal richness, whereas a therapeutic dose of BMD surprisingly has no obvious impact on overall diversity but causes a significant increase in cecal richness. Additionally, both BMD doses induce an obvious shift in the cecal fungal community composition. An increase in the fecal fungal α-diversity was observed in both humans and mice in response to antibiotic treatment (49, 50), although a decrease in fungal richness was seen in a human patient treated prophylactically with antibiotics (51). Additional studies on the temporal and spatial impact of antibiotics on the absolute and relative abundance of the intestinal mycobiota are warranted.

In summary, we comprehensively studied the biogeography of the chicken intestinal mycobiota by deep sequencing and revealed by surprise that the upper GI tract harbors a more diverse fungal community than the lower GI tract. Furthermore, the chicken gut mycobiota is dominated only by a few major fungal species, showing a much reduced complexity and greater individual variations than the bacteriome. Similar to the gut bacteriome, the mycobiome undergoes a compositional shift as chickens age. Additionally, in-feed antibiotics are capable of

altering the fungal community structure, although it is currently unknown whether it is a direct or indirect effect. A better understanding of the intestinal mycobiome provides a foundation for future work into the relationship between the mycobiome and gut health and physiology in poultry, making it possible to modulate gut mycobiota to improve animal performance and prevent disease.

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Table 3.S1. Pairwise ANOSIM of Biogeography Beta Diversity Indices

	Crop	Ventriculus	Duodenum	Jejunum	Ileum	Cecum	Colon	Cloaca
Crop		*0.005*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*0.001*	*0.004*
		(0.280)	*(0.414)*	*(0.545)*	*(0.874)*	*(0.693)*	*(0.423)*	*(0.381)*
Ventriculus	0.002		*0.020*	*0.007*	*<0.001*	*< 0.001*	*< 0.001*	*0.001*
	(0.307)		*(0.209)*	*(0.339)*	*(0.295)*	*(0.561)*	*(0.519)*	*(0.345)*
Duodenum	< 0.001	0.049		*0.048*	*< 0.001*	*0.002*	*< 0.001*	*0.005*
	(0.388)	(0.104)		*(0.126)*	*(0.588)*	*(0.306)*	*(0.446)*	*(0.273)*
Jejunum	< 0.001	0.001	0.186		*0.001*	*0.045*	*< 0.001*	*< 0.001*
	(0.524)	(0.277)	(0.034)		*(0.492)*	*(0.088)*	*(0.551)*	*(0.483)*
Ileum	< 0.001	0.001	< 0.001	< 0.001		*< 0.001*	*< 0.001*	*< 0.001*
	(0.874)	(0.341)	(0.374)	(0.547)		*(0.823)*	*(0.807)*	*(0.738)*
Cecum	< 0.001	< 0.001	0.016	0.188	< 0.001		*< 0.001*	*< 0.001*
	(0.693)	(0.552)	(0.175)	(0.031)	(0.760)		*(0.639)*	*(0.577)*
Colon	0.002	< 0.001	< 0.001	< 0.001	0.001	< 0.001		*0.399*
	(0.348)	(0.467)	(0.432)	(0.731)	(0.508)	(0.600)		*(0.005)*
Cloaca	0.010	0.007	0.005	< 0.001	< 0.001	< 0.001	0.426	
	(0.355)	(0.262)	(0.249)	(0.450)	(0.523)	(0.597)	(0.006)	

Note: P values for the Jaccard index are italicized. R values for each comparison are shown in parenthesis.

Table 3.S2. Biogeography of the mycobiota in the gastrointestinal tract of day-28 chickens

Feature	Crop	Ventriculus	Duodenum	Jejunum	Ileum	Cecum	Colon	Cloaca	*P*-value	FDR
***S. brevicaulis*_F2**	0.00^{a}	18.10^{b}	7.20^{bd}	6.22^{ad}	38.95^{c}	0.76^{ad}	4.51^{ad}	11.06^{ab}	<0.001	**<0.001**
***S. brevicaulis*_F8**	0.00^{a}	0.00^{a}	11.54^{bc}	28.35^{cd}	0.00^{a}	36.55^{c}	4.09^{ab}	0.00^{a}	<0.001	**<0.001**
***T. asahii*_F1**	0.00^{a}	6.62^{b}	10.48^{bc}	8.29^{ab}	14.49^{c}	9.35^{ab}	0.63^{a}	8.13^{ab}	<0.001	**<0.001**
***S. brevicaulis*_F12**	8.96^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	26.48^{b}	22.29^{b}	<0.001	**<0.001**
***S. brevicaulis*_F4**	20.62^{a}	13.76^{ab}	2.73^{bc}	3.22^{c}	0.00^{c}	0.00^{c}	7.48^{bc}	1.64^{bc}	<0.001	**<0.001**
***Aspergillus sp*_F3**	0.00^{a}	13.19^{bc}	5.66^{bd}	4.70^{abd}	17.53^{c}	5.22^{ab}	0.16^{ad}	2.83^{ab}	<0.001	**<0.001**
***S. brevicaulis*_F5**	0.00^{a}	14.85^{bd}	5.07^{cd}	2.82^{ac}	16.74^{b}	0.43^{ac}	2.35^{ac}	5.00^{acd}	<0.001	**<0.001**
***S. brevicaulis*_F11**	0.00^{c}	0.00^{c}	8.64^{ab}	15.64^{a}	0.00^{c}	18.92^{a}	1.41^{bc}	0.00^{c}	<0.001	**<0.001**
***T. asahii*_F6**	19.23^{a}	5.02^{b}	2.42^{bc}	0.75^{bc}	0.00^{c}	0.00^{c}	2.86^{bc}	4.31^{bc}	<0.001	**<0.001**
***S. brevicaulis*_F7**	12.91^{a}	11.05^{ab}	1.91^{bc}	1.79^{bc}	0.00^{c}	0.00^{c}	2.62^{bc}	0.72^{bc}	<0.001	**<0.001**
***T. asahii*_F9**	0.00^{a}	0.00^{a}	6.13^{bc}	3.43^{b}	0.00^{a}	17.87^{d}	1.08^{ac}	0.00^{a}	<0.001	**<0.001**
***T. asahii*_F16**	1.61^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	13.49^{b}	11.47^{b}	<0.001	**<0.001**
***S. brevicaulis*_F17**	6.38^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	10.91^{ab}	9.13^{ab}	<0.001	**<0.001**
***A. sp*_F10**	14.19^{a}	1.96^{b}	0.17^{bc}	5.32^{bc}	0.00^{c}	0.00^{c}	0.93^{bc}	1.64^{bc}	<0.001	**<0.001**
***A. sp*_F20**	0.72^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	9.88^{b}	8.13^{b}	<0.001	**<0.001**
***A. sp*_F15**	0.00^{a}	0.00^{a}	1.90^{bd}	10.09^{c}	0.00^{a}	2.42^{bc}	1.00^{ad}	0.00^{a}	<0.001	**<0.001**
***S. brevicaulis*_F14**	0.00^{a}	2.12^{b}	1.23^{bc}	0.54^{ac}	4.36^{d}	0.07^{ac}	0.30^{ac}	1.41^{ab}	<0.001	**<0.001**
***S. brevicaulis*_F21**	0.00^{a}	0.00^{a}	1.53^{bd}	3.40^{bc}	0.00^{a}	4.47^{c}	0.25^{ad}	0.00^{a}	<0.001	**<0.001**
***A. versicolor*_F13**	0.00^{a}	1.70^{b}	0.17^{a}	0.89^{ab}	4.20^{c}	0.34^{a}	0.29^{a}	1.24^{ab}	<0.001	**<0.001**
***S. brevicaulis*_F29**	0.85^{ac}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	2.98^{b}	2.65^{bc}	<0.001	**<0.001**

Note: Mean relative abundance (%) of the phylum, top 20 fungal features are shown, with 12 samples per intestinal segment. Statistical significance was determined using non-parametric Kruskal Wallis test and p values were corrected for multiple comparisons using the Benjamini-Hochberg correction. For columns with an FDR $\leq$ 0.05, means were separated using pairwise Wilcoxon Rank Sums test. Columns not sharing a common superscript are considered significantly different ($P < 0.05$). It is noted that, among the top 20 features, only *Trichosporon* belongs to the phylum Basidiomycota, while all the remaining fungi belong to Ascomycota.

Table 3.S3. Maturation of the chicken cecal mycobiome

	Day 14	Day 28	*P*-Value	FDR
***T. asahii*_F9**	41.55	17.87	0.172	0.448
***S. brevicaulis*_F8**	6.98	36.55	0.003	0.261
***T. asahii*_F1**	26.95	9.35	0.232	0.452
***S. brevicaulis*_F11**	4.19	18.92	0.003	0.261
***Aspergillus sp.*_F3**	3.21	5.22	0.473	0.598
***Aspergillus sp.*_F15**	4.27	2.42	0.619	0.721
***S. brevicaulis*_F21**	0.60	4.47	0.003	0.261
***Stenocarpella maydis*_F19**	3.10	0.07	0.176	0.448
***S. maydis*_F45**	1.78	0.18	0.291	0.452
***A. versicolor*_F33**	0.45	1.11	0.015	0.448
***D. hansenii*_F43**	1.00	0.51	0.901	0.937
***S. brevicaulis*_F2**	0.29	0.76	0.381	0.514
***Cephaliophora sp.* _F65**	0.54	0.34	0.238	0.452
***A. versicolor*_F13**	0.29	0.34	0.339	0.461
***S. brevicaulis*_F5**	0.15	0.43	0.473	0.598
***Aspergillus sp.* _F93**	0.36	0.03	0.118	0.448
***Gibberella sp.* _F44**	0.37	0.02	0.265	0.452
***Aspergillus sp.* _F27**	0.31	0.00	0.059	0.448
***Gibberella sp.* _F140**	0.29	0.02	0.321	0.461
***T. asahii* _F34**	0.22	0.06	0.202	0.449

Note: Mean relative abundance (%) of the phylum, top 20 fungal features are shown, with 12 samples per intestinal segment. Statistical significance was determined using non-parametric Kruskal Wallis test and p values were corrected for multiple comparisons using the Benjamini-Hochberg correction. For columns with an FDR $\leq$ 0.05, means were separated using pairwise Wilcoxon Rank Sums test. Columns not sharing a common superscript are considered significantly different ($P < 0.05$).

Table 3.S4. Pairwise ANOSIM of Beta Diversity Indices

	Control	BMD55	BMD275
Control		*< 0.001 (0.570)*	*< 0.001 (0.392)*
BMD55	< 0.001 (0.365)		*< 0.001 (0.369)*
BMD275	0.001 (0.386)	0.058 (0.088)	

Note: P values for the Jaccard index are italicized. R values are shown in parenthesis.

Table 3.S5. Effect of BMD on the chicken cecal mycobiome composition

Features	Control	BMD55	BMD275	*P*-Value	FDR
***T. asahii*_F1**	26.95	75.49	72.33	0.021	0.068
***T. asahii*_F9**	41.55[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***Aspergillus sp.*_F3**	3.21[a]	10.04[b]	14.73[b]	0.004	**0.034**
***S. brevicaulis*_F8**	6.98[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***Stenocarpella maydis*_F19**	3.10	1.19	1.60	0.206	0.299
***T. asahii*_F6**	0.00	5.50	0.00	0.384	0.408
***Aspergillus sp.*_F15**	4.27[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***S. brevicaulis*_F11**	4.19[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***S. brevicaulis*_F2**	0.29[a]	0.94[b]	2.50[c]	<0.001	**0.003**
***S. brevicaulis*_F5**	0.15[a]	0.56[b]	1.82[c]	<0.001	**0.002**
***A. versicolor*_F13**	0.29[a]	0.40[a]	1.83[b]	0.001	**0.016**
***D. hansenii*_F24**	0.22[a]	0.90[b]	1.07[b]	0.013	**0.049**
***Stenocarpella maydis*_F45**	1.78[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***D. hansenii*_F43**	1.00[a]	0.00[b]	0.00[b]	<0.001	**0.002**
***T. asahii* _F34**	0.22	0.38	0.38	0.529	0.550
***Aspergillus sp.*_F10**	0.00	0.92	0.00	0.384	0.408
***Aspergillus sp.*_F27**	0.31	0.10	0.37	0.405	0.427
***D. hansenii*_F28**	0.00	0.66	0.00	0.384	0.408
***Gibberella sp.* _F44**	0.37	0.12	0.15	0.038	0.096
***S. brevicaulis*_F21**	0.60[a]	0.00[b]	0.00[b]	<0.001	**0.002**

Note: Mean relative abundance (%) of the phylum, top 20 fungal features are shown, with 12 samples per intestinal segment. Statistical significance was determined using non-parametric Kruskal Wallis test and p values were corrected for multiple comparisons using the Benjamini-Hochberg correction. For columns with an FDR $\leq$ 0.05, means were separated using pairwise Wilcoxon Rank Sums test. Columns not sharing a common superscript are considered significantly different ($P < 0.05$). It is noted that, among the top 20 features, only *Trichosporon asahii* belongs to the phylum Basidiomycota, while all the remaining fungi belong to Ascomycota.

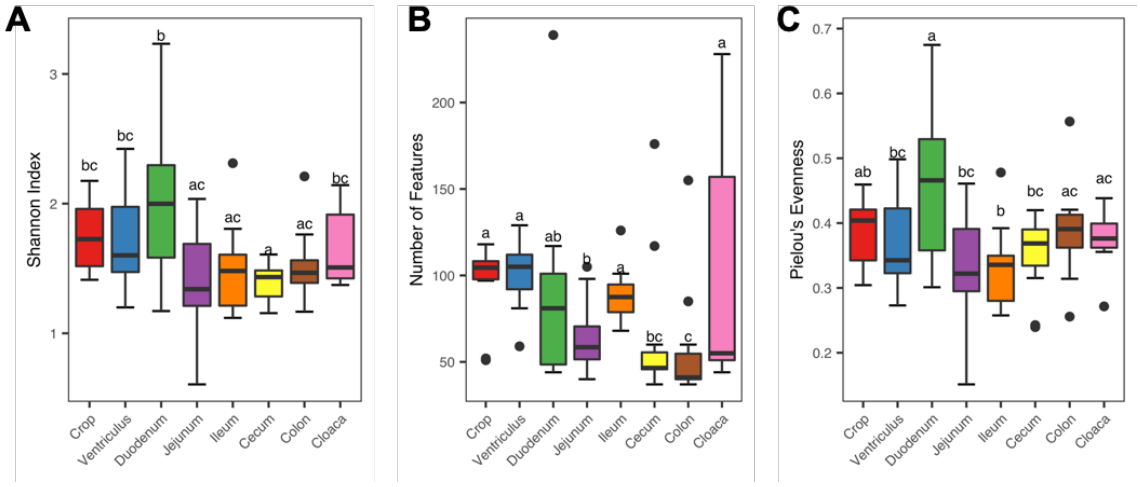

Fig. 3.1. Variation in the α-diversity of the mycobiome along the gastrointestinal tract of day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet for 28 days before the luminal content samples were collected from each gastrointestinal segment. The diversity and richness were calculated from 12 samples of each segment and indicated by the Shannon Index (A), Observed OTUs (B), and Pielou's Evenness (C) respectively. Kruskal Wallis was performed, and the means were separated with pairwise Wilcoxon Rank Sums with the segments not sharing a common superscript considered significantly different ($P < 0.05$).

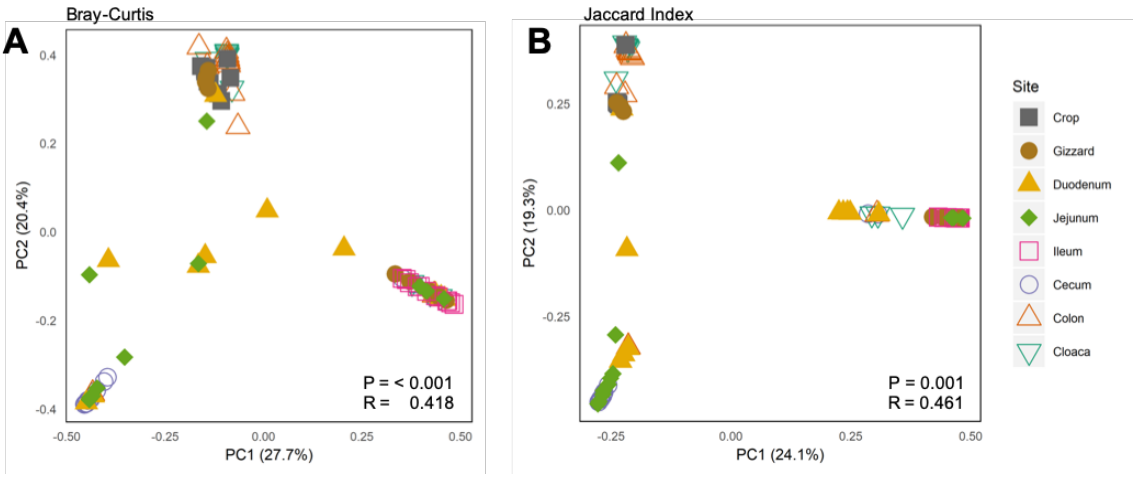

Fig. 3.2. Principal coordinate analysis (PCoA) of the mycobiome along the gastrointestinal tract of day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet for 28 days before the luminal content samples were collected from each gastrointestinal segment. Each data point represents an individual sample. PCoA was calculated from 12 samples of each segment using Bray-Curtis (A) and the Jaccard indicews (B), respectively. Statistical significance was determined using ANOSIM and indicated in each plot.

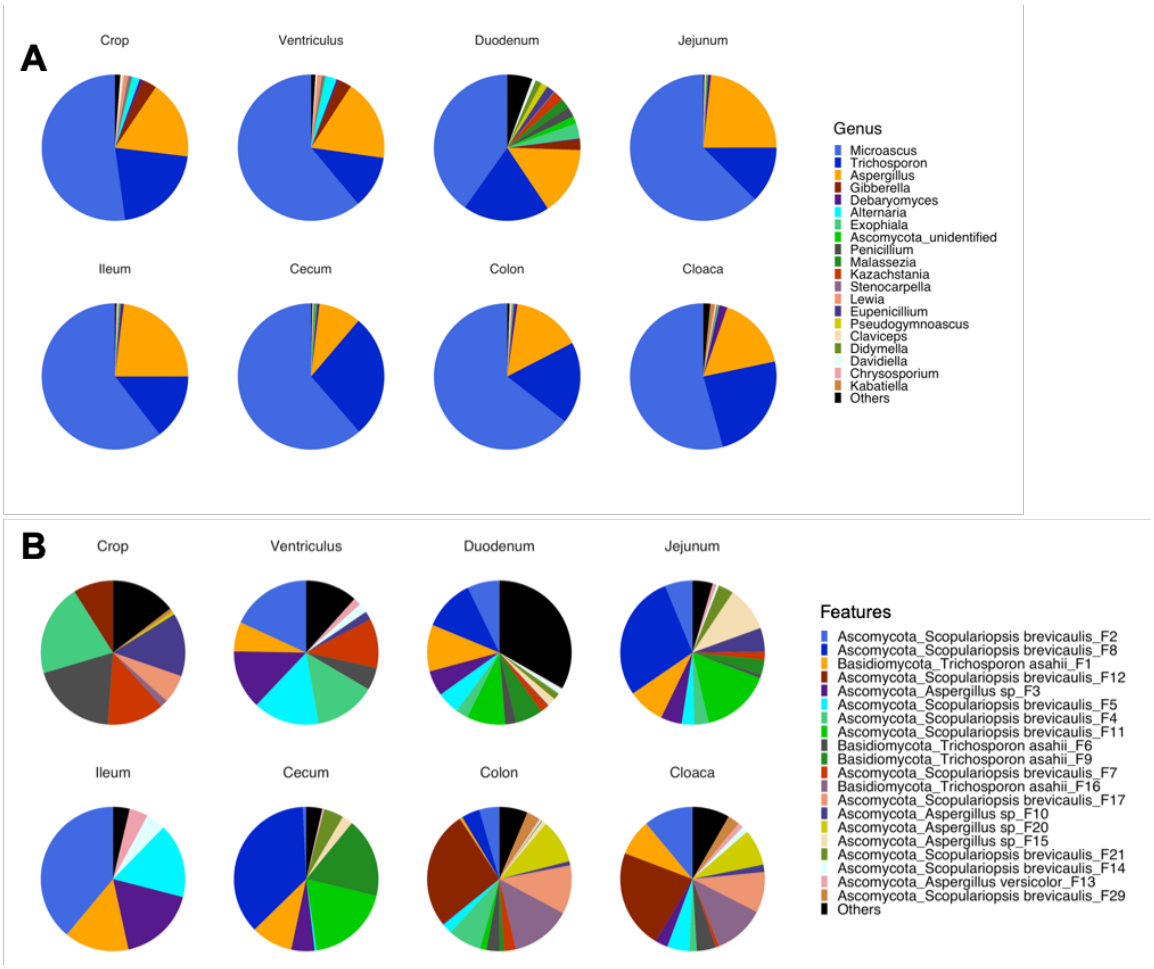

Fig. 3.3. The mycobiota composition in different segments of the gastrointestinal tract of day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet for 28 days before the luminal content samples were collected from each gastrointestinal segment. The relative abundance of OTUs was used to determine the fungal composition at the genus (A) and feature (B) levels with 12 samples of each segment. Only the top 20 genera and features are shown, with unidentified and lowly abundant fungi being collectively denoted as “Others”.

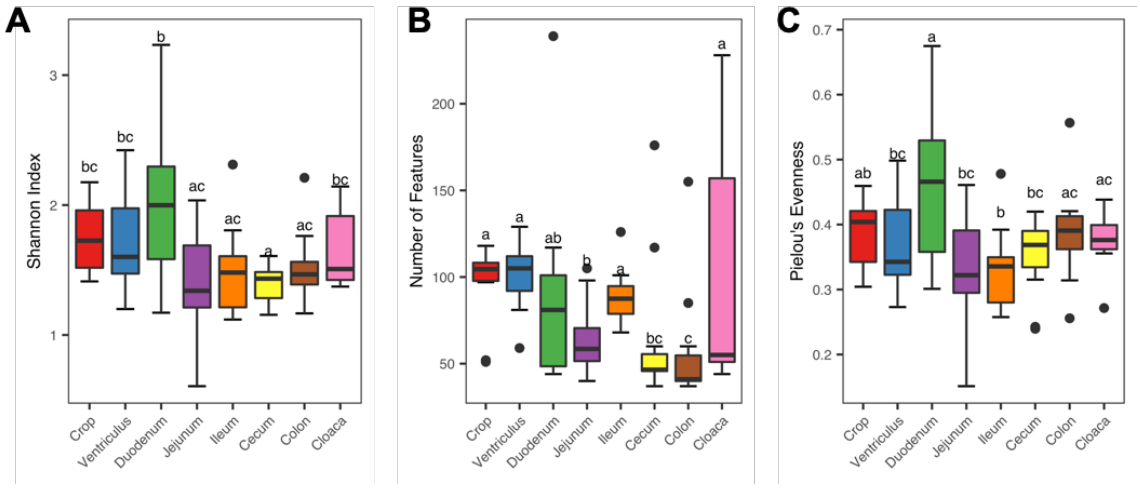

Fig. 3.4. Changes in the α-diversity of the cecal mycobiome between day-14 and day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet before the cecal content samples were collected on days 14 and 28. The diversity and richness were calculated from 12 samples on each sampling day and indicated by the Shannon Index (A), Observed OTUs (B), and Pielou's Evenness (C), respectively. *$P < 0.05$ and *** $P < 0.001$ as determined by Wilcoxon Rank Sums.

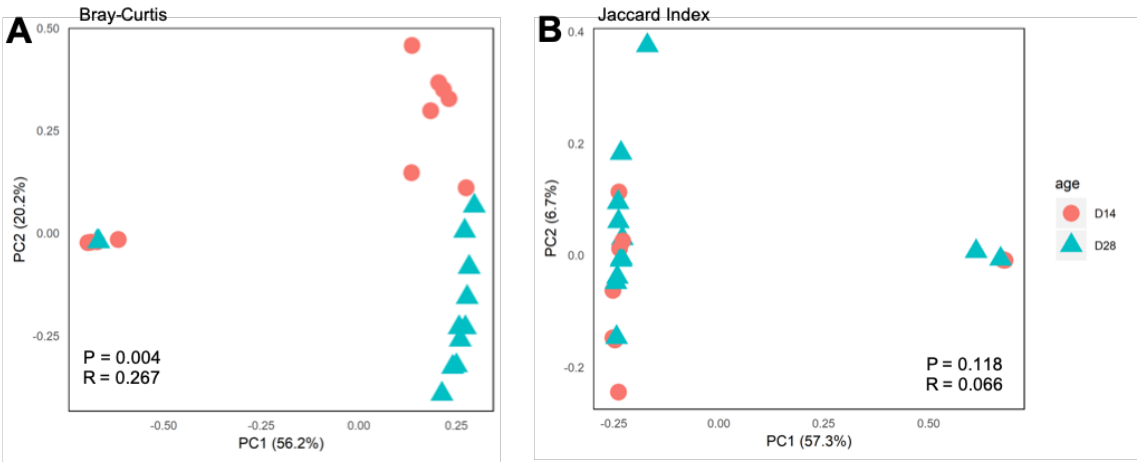

Fig. 3.5. Principal coordinate analysis (PCoA) of the cecal mycobiome in day-14 and day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet before the cecal content samples were collected on days 14 and 28. Each data point represents an individual sample. PCoA was calculated from 12 samples on each sampling day using Bray-Curtis (A) and the Jaccard indices (B), respectively. Statistical significance was determined using ANOSIM and indicated in each plot.

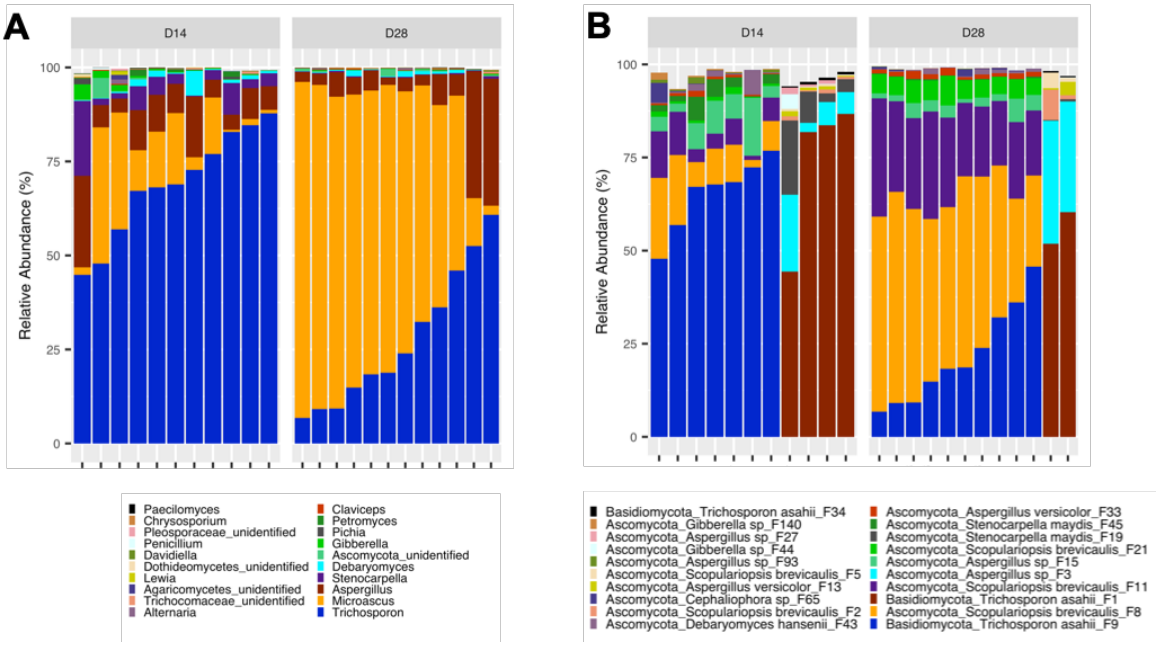

Fig. 3.6. The mycobiota composition in the cecum of day-14 and day-28 chickens. Newly hatched chicks were fed an antibiotic-free diet before the cecal content samples were collected on days 14 and 28. The relative abundance of OTUs was used to determine the fungal composition at the genus (A) and feature (B) levels with 12 samples on each sampling day. Only the top 20 genera and features are shown, with unidentified fungi and low abundance taxa being collectively denoted as 'Others'.

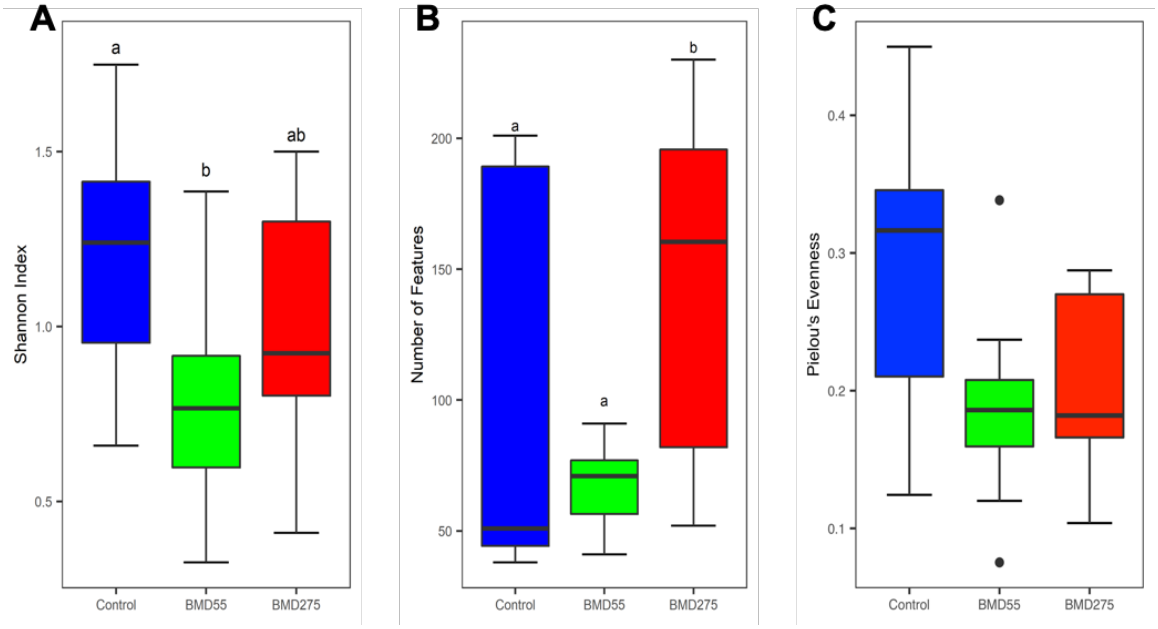

Fig. 3.7. Effect of BMD on the α-diversity of the chicken cecal mycobiota. The cecal content samples were collected 14 days after day-of-hatch chickens were fed an antibiotic-free diet or the diet supplemented with 55 or 275 mg/kg of BMD. The diversity and richness were calculated from 12 samples in each treatment and indicated by the Shannon Index (A), Observed OTUs (B), and Pielou's Evenness (C), respectively. Differences were determined using Kruskal Wallis and means were separated using Wilcoxon Rank Sums. Treatments not sharing a common superscript are considered significantly different ($P < 0.05$).

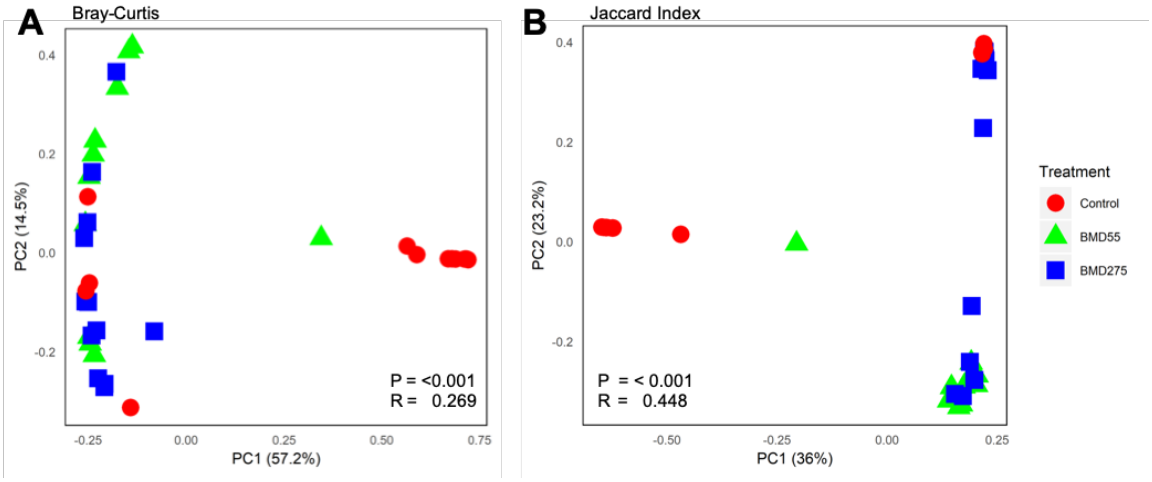

Fig. 3.8. Effect of BMD on the β-diversity of the chicken cecal mycobiota. The cecal content samples were collected 14 days after day-of-hatch chickens were fed an antibiotic-free diet or the diet supplemented with 55 or 275 mg/kg of BMD. Principal coordinate analysis (PCoA) was performed with 12 samples in each treatment using Bray-Curtis (A) and the Jaccard indices (B), respectively. Each data point represents an individual sample. Statistical significance was determined using ANOSIM and indicated in each plot.

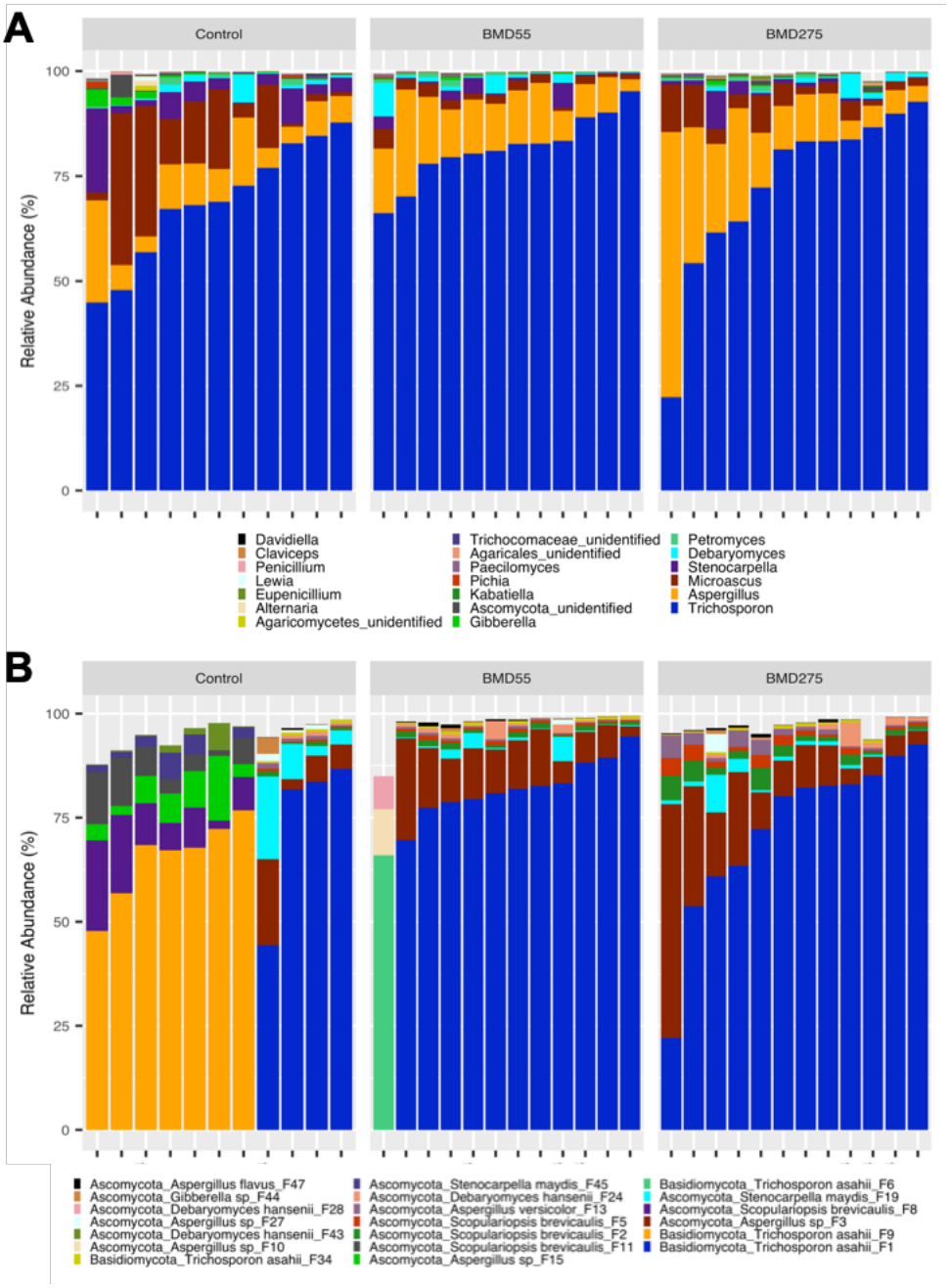

Fig. 3.9. Effect of BMD on the chicken cecal mycobiota composition. Cecal content samples were collected 14 days after day-of-hatch chickens were fed an antibiotic-free diet or the diet supplemented with 55 or 275 mg/kg of BMD. The relative abundance of each OTU was calculated and combined at the genus level (A) or feature level (B) for 12 individual samples in each treatment.

CHAPTER IV

SPATIOTEMPORAL DEVELOPMENT OF THE CHICKEN INTESTINAL MYCOBIOME

ABSTRACT

Extensive work has been accomplished in recent years to characterize the intestinal bacterial community and its association with health and disease. However, very little has been done to describe the smaller intestinal microbial communities such as fungi, viruses, archaea, and protozoa. Here we comprehensively characterized the biogeography of the intestinal mycobiota of day-42 broiler chickens and its succession over the entire production cycle. Combining Illumina sequencing of the internal transcribed spacer 2 (ITS2) region of fungal rRNA genes with absolute quantification of bacterial and fungal populations, we revealed significant spatial and temporal differences in the chicken intestinal mycobiota. The intestinal mycobiome was dominated by Ascomycota and Basidiomycota, representing over 98% of the data. Three genera commonly associated with corn and soil, *Gibberella* (30 – 87%)*, Aspergillus* (0.5 – 16.6%)*,* and *Candida* (0.8 – 14.2%), were found to be most abundant. Feature analysis revealed an obvious age-dependent transition of four distinct fungal consortia along the GI tract. In total, these results reveal a highly transient fungal population shaped primarily by the environment.

KEYWORDS: Mycobiome, chicken, microbiome, fungi

INTRODUCTION

The intestinal bacterial community is known to be associated with multiple intestinal and extra-intestinal diseases (1-6). However, less studies have been performed to investigate smaller microbial communities within the gastrointestinal (GI) tract, which include fungi, viruses, archaea, and protozoa. Recently, studies have been undertaken to understand the fungal portion of the human intestinal microbiota and its role in health and disease (7, 8).

In humans, the fungal community, known as mycobiota, is estimated to account for approximately 0.02% of the intestinal mucosa-associated microbiota and 0.03% of the fecal microbiota (9). Investigations into the human oral and intestinal fungal residents have revealed the phyla Ascomycota and Basidiomycota to be dominant, and *Saccharomyces, Candida, Aspergillus, Cladosporium, Penicillium, Wallemia, Malassezia, Aureobasidium,* and *Epicoccum* are among the most common genera that have been identified (7, 8, 10). Fungal dysbiosis has been linked to multiple diseases such as inflammatory bowel disease, allergic airway disease, atopic dermatitis, and alcoholic liver disease (7, 8, 11). Although Ascomycota and Basidiomycota have also been identified as the major phyla in mice, *Candida, Saccharomyces, Trichosporon, Aspergillus, Penicillium, Wickerhamomyces, Cladosporium,* and *Fusarium* are among the top genera identified (12-15).

In livestock animals, the mycobiome research has primarily focused on ruminant animals with special attention given to anaerobic fungi in the phylum Neocallimastigomycota (16-20). First discovered in the rumen of sheep (21), anaerobic fungi are thought to be the first to attach to fibrous material (22) and contribute more to the degradation of plan material than cellulolytic bacteria (23). A culture-based study with weaned Landrace pigs revealed *Kazachstania sloofiae* to be the most frequently detected species (24), whereas deep sequencing identified *K. telluris* as the dominant fungal species in a miniature breed of weaned pigs (25).

In limited poultry mycobiome studies, all but one were culture-based and most were restricted only to the cecum (26-31). *Candida* or *Aspergillus* was revealed to be the predominant in the chicken cecum in culture-dependent studies. Surprisingly, the study that employed a culture-independent pyrosequencing approach only detected two *Cladosporium* species in the cecum of broilers fed unmedicated diet, although another 17 fungal species were detected in animals supplemented with essential oils (26). Previous work in our lab used next generation Illumina sequencing to investigate the biogeography of the chicken intestinal mycobiome on day 28. We revealed *Microascus, Trichosporon, Aspergillus,* and *Gibberella* to be the top four genera and revealed an obvious development-associated shift in the cecal mycobiome composition (unpublished). However, only two time points (day 14 and day 28) were included in the study making it difficult to fully determine any successional changes in mycobiome composition. Also, because of no obvious maturation pattern up to day 28, there is a need to expand the study to include the broiler chickens up to the market age (day 42-49). Therefore, we sought to expand our previous study by using Illumina deep sequencing of the luminal contents from four GI locations (duodenum, jejunum, ileum, and cecum) from day 3-42 to characterize the succession of the chicken mycobiome throughout the entire production cycle. Furthermore, we also sequenced the luminal contents throughout the GI tract of day-42 broilers to determine the biogeography of the chicken mycobiome at market age.

MATERIALS AND METHODS

Animal Trial

An animal trial was conducted in accordance with the Institutional Animal Care and Use Committee of Oklahoma State University under protocol number AG-173. Day-of-hatch male Cobb broiler chicks were obtained from Cobb-Vantress (Siloam Springs, AR) and randomly assigned to 12 cages with 10 birds per cage and *ad libitum* access to feed and tap water for the entire duration of the trial. Birds were raised on floor cages with fresh dry pine wood shavings in

an environmentally controlled room with temperatures starting at 33°C and decreasing 3°C every 7 days. Lighting included the light to dark ratio of 23:1 from day 0 to 7 and 16:8 from day 8 to 42. Non-medicated starter, grower, and finisher diets were formulated to meet or exceed NRC recommendation with no in-feed antibiotics or coccidiostat. Diet was switched from starter to grower and then to finisher on days 14 and 27, respectively. On days 3, 7, 14, 21, 27, 35, and 42, one bird per cage was euthanized via CO_2 asphyxiation and luminal contents were aseptically collected from the mid-duodenum, mid-jejunum, mid-ileum, and cecum. Sample collection on days 14 and 27 was performed prior to feed transitions. Additionally, luminal contents were collected on day 42 from the crop, ventriculus, and colon of 12 broilers with one per cage for characterization of spatial differences in the chicken intestinal mycobiome.

DNA isolation and sequencing

Microbial DNA was isolated from intestinal contents using ZR *Quick*-DNA Fecal/Soil Microbe Microprep or Miniprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. DNA quality and quantity were determined using Nanodrop ND-1000 and the absence of degradation was confirmed using agarose gel electrophoresis. High-quality DNA was subjected to PE250 sequencing of the ITS2 region using ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) primers on an Illumina HiSeq platform by Novogene (Novogene, Beijing, CA). PCR amplification and library preparation were performed using the NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs, Ipswich, MA).

Absolute quantification of bacterial and fungal abundances

Bacterial and fungal abundances in the GI tract were quantified by qPCR using the Femto Bacterial and Fungal DNA Quantification kits (Zymo Research), respectively.

Bioinformatic analysis and statistics

Raw fungal sequences were imported into QIIME 2 and processed using the Deblur program (32, 33). The 'deblur denoise-other' option was utilized, which included truncating sequences to 340 nucleotides and positive-alignment based filtering against the UNITE reference database (34). Deblur uses error profiles within samples to achieve single-nucleotide resolution. Denoised sequences are often referred to as features, amplicon sequence variants (ASVs), or exact sequence variants (ESVs). Features appearing in less than 5% of samples were removed from downstream analysis. Data was normalized using cumulative sum scaling in the metagenomeSeq package of R (35). The remaining features were classified using the WARCUP v2 fungal ITS database and the Ribosomal Database Projects (RDP) Bayesian Classifier (36, 37). Bootstrap confidence was set to 80% for every taxonomic level, and features with a classification of less than 80% were assigned the name of the last confidently assigned level followed by "_unidentified". Species classification of the top 20 OTUs was confirmed through BLASTN search of the nucleotide database of GenBank. Analysis and visualization of the mycobiota composition was conducted in R version 3.5.1 (38).

The α-diversity was calculated using Shannon index, Observed Features (Observed OTUs), and Pielou's Evenness index in the phyloseq package version 1.24.2 (39). Plots were made using ggplot2 version 3.0.0 (40). Results were plotted using box and whisker plots, in which the middle line denotes the median value and the lower and upper hinges represent the first and third quartiles, respectively. Whiskers extend from the hinge to the highest or lowest value no farther than 1.5× the inter-quartile range. Points outside of this range are considered outliers. The β-diversity was calculated using the Bray-Curtis and Jaccard indices in the phyloseq package of R. Dissimilarities were plotted as PCoA plots using phyloseq and ggplot2 in R.

Prior to statistical analysis, normality of the data was determined using the Shapiro-Wilks test in R. Significant differences in α-diversity and relative abundance were determined using

Kruskal-Wallis and multiple comparisons were performed using pairwise Wilcoxon rank-sum test. Significant differences in β-diversity were determined using ANOSIM in mothur (41).

RESULTS

Biogeography of the chicken intestinal mycobiome

To study the biogeography of the intestinal mycobiome in market-age broilers, intestinal contents were collected from seven different GI locations (crop, ventriculus, duodenum, jejunum, ileum, cecum, and colon) on day 42. Microbial DNA was isolated and sequenced on an Illumina HiSeq using the internal transcribed spacer 2 (ITS2) chemistry. A total of 26,364,044 quality-filtered sequences, with an average of 62,622 sequences per sample, were obtained and submitted to the deblur program. Following denoising and removal of rare features (present in less than 5% of samples), a total of 468 features were identified and included in downstream analysis.

Overall α-diversity was determined using the Shannon index (Fig. 1A), while richness and evenness were calculated using Observed Features (Fig. 1B) and Pielou's Evenness (Fig. 1C), respectively. Shannon index revealed diversity to be lowest in the crop and increase in the ventriculus before reaching a maximum in the duodenum and jejunum. A decrease was observed in the lower GI tract with the cecum and colon returning to the levels similar to crop. This trend remained similar with richness (Fig 1.B) and evenness measurements (Fig 1.C) as well. Differences in fungal composition along the GI tract were measured with β-diversity analysis using the Bray-Curtis (Fig. 2A) and Jaccard (Fig. 2B) indices. Statistical analysis using ANOSIM revealed a significant difference in composition for both metrics ($P = 0.001$), with R values of 0.745 and 0.685 for the Bray-Curtis and Jaccard indices, respectively. Both measurements indicated a separation of samples into three distinct clusters. The first was comprised entirely of the crop, ileum, colon samples, while the second consisted of the ventriculus, duodenum, and jejunum samples. In both metrics, the cecum was shown to cluster separately from all other GI

locations with the exception of one ventriculus sample located within the cluster. The presence of three clusters was further confirmed by pairwise comparisons using ANOSIM (Supplementary table 1). Large R values (> 0.800) were consistently seen when comparing GI locations in different clusters, regardless of the measurement used. For the Jaccard index, comparison of samples within the same cluster revealed moderate R values (0.334 – 0.765) indicating some difference in richness between GI locations. However, overall diversity between GI locations within the same cluster showed greater similarity with Bray-Curtis R values ranging from 0.009 to 0.304.

Fungal composition revealed Ascomycota to be the most dominant phylum accounting for 97 to 99% of sequences in each GI location (Data not shown). A total of 65 genera were identified in the entire dataset with 56 of those present in the GI tract at 42 days of age. The most abundant genus at this time point was *Gibberella* accounting for 49.06% to 86.7% of all sequences, followed by *Aspergillus* (1.8% – 16.6%), and *Candida* (1.8 – 7.7%). Top 20 genera captured greater than 98% of the sequences in every intestinal location (Fig. 3A). While relative abundance varied throughout the intestinal tract, a specific bloom of *Kabatiella, Davidiella,* and *Alternaria* was observed in the duodenum and jejunum, which is in accordance with the increased α-diversity in those locations.

Investigation into fungal features revealed a significant difference in fungal taxa dominating each intestinal location (Fig 3. B). The top 20 fungal features accounted for more than 75% of the sequences in each GI location except for the duodenum, where only 57% was captured. Consistent with β-diversity analysis, the most dominant features were shown to be similar between the crop, ileum, and cecum as well as between the ventriculus, duodenum, and jejunum. An unidentified *Gibberella* feature (F4) was dominant in the crop, ileum, and colon followed by *Candida albicans* F6 and *Aspergillus flavus* F8. Lower abundances of *Trichosporon asahii* F15 and *Aspergillus amstelodami* F20 were also present in these segments. A noticeable

shift was observed in the upper GI tract with the ventriculus, duodenum, and jejunum samples all dominated by one of two *Fusarium pseudonygamai* features (F1 and F2). However, similar to other GI locations, less abundant features were identified as *C. albicans* F7*, T. asahii* F17*,* and *A. flavus* F12, respectively. A difference in relative abundance of different features of the same species at different intestinal sites may indicate the presence of multiple sub-species within the GI tract.

The day-42 cecal mycobiome clustered separately from all other sample types in β-diversity analyses. Consistently, they were shown to primarily consist of a unique *Gibberella* feature (F3) along with *C. albicans* (F5), *A. flavus* (F14), *A. amstelodami* (F16), and *T. asahii* (F21) (Fig. 3B). While these are all closely related to the *Gibberella* features observed in the crop, ileum, and colon, the difference in subtype residing in the cecum may be an indication of the fermentation process or transit time within the cecum. Statistical analysis with top 20 features revealed a significant difference among the GI location in all cases (Supplementary table 2), consistent with the fact that most features are only present in specific GI locations.

Developmental changes of the chicken intestinal mycobiome

To evaluate the development of the intestinal mycobiome throughout the growth cycle of broiler chickens, luminal contents were obtained from the duodenum, jejunum, ileum, and cecum on days 3, 7, 14, 21, 27, 35, and 42. Measurement of α-diversity using the Shannon index revealed a significant change in overall diversity in the jejunum, ileum, and cecum as birds aged (Fig. 4A). In the ileum and cecum, diversity appeared to be relatively consistent on days 3, 7, and 14 before increasing significantly on day 21. A steady decrease was then observed from day 27 to 42 (Fig. 4A). This pattern was less clear in the jejunum, however, a sharp increase in diversity was noted on day 27 followed by a decrease on day 35. Diversity in the jejunum recovered slightly on day 42. No significant difference in the Shannon index was observed for the duodenum.

In the duodenum, richness of the fungal community, measured by Observed Features (Fig. 4B), remained relatively low from day 3 to day 21 before increasing significantly on days 27 to 42. However, Pielou's Evenness Index revealed a strong drop in evenness beginning on day 27 and becoming even more pronounced on days 35 and 42 (Fig. 4C). In the jejunum, richness experienced a more gradual increase as birds aged (Fig. 4B), while evenness remained stable except for a significant increase on day 27 (Fig. 4C). While richness increased in the duodenum and jejunum with age, the number of observed features in the ileum decreased significantly from day 3 to day 7 and remained stable for the rest of the trial. Additionally, cecal richness varied only slightly throughout the entire growth cycle (Fig. 4B). Pielou's Evenness index more closely resembled that of the Shannon index for both the ileum and cecum, indicating that diversity in these samples is driven by changes in species evenness rather than richness (Fig. 4C).

The β-diversity analysis revealed significant changes in fungal composition as birds aged. However, age dependent changes varied by GI location. In the duodenum, samples formed three primary clusters, in which the first was comprised solely of day 3 samples and the second of days 7 and 14 as shown by Bray-Curtis analysis (Fig. 5A). The remaining samples were contained within the third cluster with diversity changing along a gradient from day 27 to 42. Ileum contents clustered similar to that of the duodenum with exception that days 21 to 42 were split into 2 distinct clusters containing days 21 and 27, and days 35 and 42, respectively (Fig. 5C). Jejunum samples clustered into four distinct groups with the first being comprised of day 3 and day 7, the second primarily of day 14, the third of day 27, and the fourth of samples from day 42 (Fig. 5B). Samples from day 21 were dispersed between the day 14 and day 27 clusters while day 35 was split between the day 21 and day 42 clusters. For the cecum, day 3 and 7, day 21, and days 35 and 42 formed three distinct clusters while day 14 and day 27 samples were dispersed between groups (Fig. 5D). Similar results were observed for the Jaccard index for every location (Supplementary figure 1). Pairwise ANOSIM further confirmed the presence of clusters at each

site with samples within the same cluster either not being statistically different from one another ($P > 0.05$) or having low R values indicating large overlap ($R < 0.250$).

Ascomycota was the most dominant phyla in every segment regardless of age. However, a slight bloom of Basidiomycota was observed on days 14 to 27 (data not shown). At the genus level, *Gibberella* remained the most abundant, followed by *Aspergillus* and *Candida*, although relative abundance of each varied widely between locations and across ages (Fig. 6). Additionally, members of *Trichosporon* were shown to be moderately abundant in each GI segment with relative abundance ranging from 0.03 to 17.3% (data not shown). Investigation of bacterial features revealed obvious changes in fungal composition in each GI location as birds aged (Fig. 7). Four fungal features were predominant throughout the intestinal tract including two unidentified *Gibberella* (F3 and F4) and two *F. pseudonygamai* variants (F1 and F2). It is interesting to note that none of the four major features were observed to coexist in the same sample despite their similarity in sequence structure. This indicates that they are more likely to be different strains of the same species rather than the result of sequencing error. Consistently, less abundant features classified to the same species were absent.

Transition and co-inhabitance of the chicken intestinal fungal consortia

Compositional data also revealed a strong tendency for certain fungal features to co-inhabit in the GI tract. For example, *C. albicans* F7 was only found in conjunction with *F. pseudonygamai* F1, while *C. albicans* F9, F5, and F6 were solely associated with *F. pseudonygamai* F2, *Gibberella sp.* F3, and *Gibberella sp.* F4, respectively (Fig. 7). Statistical analysis of the top 20 genera in each GI location revealed a significant difference in almost every instance (Supplementary Table 3). Similar to the biogeography data above, this is primarily driven by the presence of specific features at only a few time points in each GI location.

The appearance order and timing of the intestinal mycobiome is especially noteworthy and was summarized in Fig. 8. No features were shown to persist in a single GI segment up to day 42, indicating a lack of a "core" mycobiome. Instead, the mycobiota is observed to be transient in nature with specific compositions moving down the GI tract in an orderly manner. *Gibberella* F3 was highly abundant in the duodenum on day 3, accounting for 46.4% of the population, but was not observed in this segment on any other sampling day. Rather, F3 began to appear in the jejunum on day 7 and became predominant on days 14 and 21. It then appeared in the ileum on days 21 and 27 before moving to the cecum on days 27 through 42. The same pattern of transition was observed for *Gibberella* F4 and *F. pseudonygamai* F2 (Fig. 8). Interestingly, *F. pseudonygamai* F1 was abundant in the cecum on days 3, 7, and 14, but did not appear in the duodenum on day 21. It then began to follow the pattern of succession seen above, moving to the jejunum on days 35 and 42. This is most likely an indication of a recycling of the microorganisms in the litter. As certain fungi are excreted into the environment, birds are reinoculated through ingestion of them from bedding materials, feed, and/or the cage.

DISCUSSION

Extensive work has been undertaken in the past ten years to characterize the intestinal microbiome of humans, mice, and livestock animals, as well as its relationship with health and disease (5, 6). However, very little is known about the intestinal mycobiota particularly in chickens. Fewer than 20 fungal species are typically reported using culture-based methods and *Candida* has been frequently described as the most abundant genus in the GI tract of chickens and turkeys, although dominant *Candida* species varies among different studies (27-30). Besides *Candida, Trichosporon, Geotrichum, Rhodotorula*, and *Saccharomyces* have been isolated in chickens as well (27-29). However, one other culture-dependent study reported the identification of 88 fungal species from over 3,000 cecal samples of broiler and layer chickens, with *Aspergillus, Penicillium, Verticillium*, and *Sporidiobolus* being the top four genera (31), whereas

a culture-independent 454 pyrosequencing method only reported two fungal species, *Cladosporium sp.* and *Cladosporium sphaerospermum*, in the cecum of broilers fed unmedicated diets (26), presumably due to a lack of the sequencing depth.

A preliminary investigation into the chicken mycobiome by us used Illumina HiSeq deep sequencing of the ITS2 region revealed the presence of 659 unique fungal features with *Scopulariopsis brevicaulis, Trichosporon asahii,* and *Aspergillus* being predominant. In this study, we sought to follow up on our previous work by characterizing the biogeography of the chicken mycobiome on day 42, while comprehensively investigating successional changes in the intestinal mycobiome throughout the entire production cycle. Consistent with previous studies, we revealed Ascomycota and Basidiomycota to be abundant throughout the GI tract regardless of age or location. *Gibberella* was observed to be the most abundant genus followed by *Candida* and *Aspergillus.* Both *Candida* and *Aspergillus* are known to be abundant in humans, mice, and chickens (7, 15, 27, 29, 31, 42), while *Gibberella* has been identified as part of the oral mycobiome in humans (43). Previously, although we identified *Gibberella* and *Aspergillus* to be among the most abundant genera, *Microascus* was the predominant member in broiler chickens on day 28, accounting for 40 – 62% of all sequences. While *Microascus* was identified in this study, its relative abundance was much lower, ranging from less than 0.01% to 4.4% (data not shown).

While specific features varied throughout the intestinal tract, overall composition remained relatively stable. One of four *Gibberella* features was found to be most abundant regardless of bird age or GI location. Two were unable to be classified beyond the genus level (F4 and F3), while two were identified as *Fusarium pseudonygamai* (F1 and F2). Along with each major feature, there was a specific set of minor species identified as *C. albicans, A. flavus, A. amstelodami, S. fibuligera,* and *Trichosporon*. Interestingly, the same set of specific features were found to co-exist in all cases forming four distinct mycobiome compositions. When four different

GI locations were sampled over the production cycle of a broiler, the intestinal mycobiome was found to be highly transient descending down the GI tract as birds age.

While *Candida* is a known resident of the human mycobiome (7), the genera *Gibberella, Aspergillus,* and *Trichosporon* are commonly found in the soil or in association with plants and food material (44-47). Therefore, it is most likely that these features originate directly from the environment. This hypothesis is further strengthened by the observation that the fungal composition present in the lower GI tract (ileum and cecum) on days 3 and 7 reappeared in the duodenum on day 21. It is very likely that fungi present in the lower GI tract early in life were excreted into the litter and then re-introduced to the upper GI tract following their uptake from the environment. However, further study is needed to confirm the source of the intestinal mycobiota at hatch.

In our previous study, fungal composition was shown to vary significantly along the GI tract with the upper GI tract (crop, ventriculus, and duodenum) hosting a more diverse mycobiota than the jejunum, ileum, and cecum. Similar results were seen with this study; however, an age dependent process was present in the form of decreased crop diversity and increased jejunum diversity on day 42 versus day 28. Moreover, a significant difference in both α- and β-diversity was observed in the duodenum, jejunum, ileum, and cecum over time, although the particulars of that change were site-specific. Our initial work also indicated an obvious switch in cecal composition between days 14 and 28, as noted by an increase *S. brevicaulis* features at the expense of *T. asahii,* which was speculated to indicate a maturation of the intestinal mycobiome. However, more thorough investigation into the dynamics of the mycobiome in this study has revealed that the switch was most likely an indication of the successional changes in cecal mycobiome composition as birds age. In humans, fecal mycobiota compositions have also been reported to be obviously different among healthy individuals of different age groups (48).

Specifically, an inverse relationship between the intestinal fungal α-diversity and age was observed, with infants and children harboring more fungal species than adults (48).

In summary, we comprehensively studied the succession of the intestinal mycobiota throughout the production cycle of broiler chickens including the biogeography of the chicken intestinal mycobiome on day 42. Similar to previous studies, α-diversity was shown to be higher in the upper GI tract than the lower GI tract, while β-diversities varied significantly by age. Consistent with previous studies, the gut mycobiota was dominated by only a few fungal features. However, deep sequencing of samples from four adjacent GI locations over the entire production cycle revealed successional changes in fungal composition indicating the chicken gut mycobiome to be highly transient.

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Table 4.S1. Pairwise ANOSIM of Biogeography Beta Diversity Indices

	Crop	Ventriculus	Duodenum	Jejunum	Ileum	Cecum	Colon
Crop		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*
		(0.918)	*(0.802)*	*(1.000)*	*(0.765)*	*(1.000)*	*(0.334)*
Ventriculus	< 0.001		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*
	(0.918)		*(0.399)*	*(0.605)*	*(0.926)*	*(0.859)*	*(0.918)*
Duodenum	< 0.001	0.020		*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*
	(0.802)	(0.157)		*(0.668)*	*(0.824)*	*(0.802)*	*(0.802)*
Jejunum	< 0.001	0.007	0.003		*< 0.001*	*< 0.001*	*< 0.001*
	(1.000)	(0.178)	(0.277)		*(1.00)*	*(1.000)*	*(1.000)*
Ileum	0.001	< 0.001	< 0.001	< 0.001		*< 0.001*	*< 0.001*
	(0.304)	(0.926)	(0.824)	(1.000)		*(1.000)*	*(0.525)*
Cecum	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		*< 0.001*
	(1.000)	(0.784)	(0.802)	(1.000)	(1.000)		*(1.000)*
Colon	0.019	< 0.001	< 0.001	< 0.001	0.458	< 0.001	
	(0.181)	(0.918)	(0.802)	(1.000)	(0.009)	(1.000)	

Note: P values for the Jaccard index are italicized. R values for each comparison are shown in parenthesis.

Table 4.S2. Biogeography of the chicken mycobiome at market age

Feature	Crop	Vetriculus	Duodenum	Jejunum	Ileum	Cecum	Colon	P Value	FDR
F. *pseudonygamai*_F1	0.00^{a}	71.24^{b}	38.33^{c}	67.21^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
Gibberella sp. F4	84.89^{a}	0.00^{c}	0.00^{c}	0.00^{c}	75.43^{b}	0.00^{c}	81.37^{ab}	<0.001	**<0.001**
*Gibberella sp.*_F3	0.00^{a}	4.42^{a}	0.00^{a}	0.00^{a}	0.00^{a}	85.82^{b}	0.00^{a}	<0.001	**<0.001**
F. *pseudonygamai*_F2	0.00^{ab}	0.00^{ab}	14.12^{a}	0.00^{b}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.001	**0.001**
*C. albicans*_F5	0.00^{a}	0.01^{a}	0.00^{a}	0.00^{a}	0.00^{a}	5.03^{b}	0.00^{a}	<0.001	**<0.001**
*C. albicans*_F6	5.80^{a}	0.00^{c}	0.00^{c}	0.00^{c}	6.35^{b}	0.00^{c}	6.66^{b}	<0.001	**<0.001**
*C. albicans*_F7	0.00^{a}	4.21^{b}	1.27^{b}	1.76^{b}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
*A. flavus*_F8	1.51^{a}	0.00^{c}	0.00^{c}	0.00^{c}	7.85^{b}	0.00^{c}	4.25^{b}	<0.001	**<0.001**
*C. albicans*_F9	0.00^{ab}	0.00^{ab}	0.83^{a}	0.00^{b}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.001	**0.001**
*Trichosporon sp.*_F15	0.07^{ac}	0.00^{b}	0.00^{b}	0.00^{b}	0.41^{c}	0.00^{b}	0.03^{a}	<0.001	**<0.001**
*Trichosporon sp.*_F17	0.00^{a}	0.15^{b}	0.72^{bd}	0.02^{c}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
*S. fibuligera*_F28	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.15^{b}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
*A. flavus*_F13	0.00^{ab}	0.00^{ab}	0.76^{a}	0.00^{b}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.001	**0.001**
*A. flavus*_F12	0.00^{a}	2.14^{b}	1.24^{b}	4.51^{c}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
*A. amstelodami*_F16	0.00^{a}	0.08^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.60^{b}	0.00^{a}	<0.001	**<0.001**
*S. fibuligera*_F32	0.00^{a}	0.00^{a}	0.07^{b}	1.42^{c}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	**<0.001**
*A. flavus*_F14	0.00^{a}	0.23^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.87^{b}	0.00^{a}	<0.001	**<0.001**
*Hypocreales sp.*_F34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.201	0.201
*A. amstelodami*_F20	0.51^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.71^{b}	0.00^{c}	0.62^{ab}	<0.001	**<0.001**
Trichosporon sp _F21	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.23^{b}	0.00^{a}	<0.001	**<0.001**

Note: Mean relative abundance (%) of the phylum, top 20 fungal features are shown, with 10 to 12 samples per intestinal segment. Statistical significance was determined using non-parametric Kruskal Wallis test and p values were corrected for multiple comparisons using the Benjamini-Hochberg correction. For columns with an FDR $\leq$ 0.05, means were separated using pairwise Wilcoxon Rank Sums test. Columns not sharing a common superscript are considered significantly different ($P < 0.05$). Note: Among the top 20 features, only three unclassified *Trichosporon* species belonged to the phylum Basidiomycota. The rest belonged to Ascomycota.

Table 4.S3. Pairwise ANOSIM of Beta Diversity Indices by Age and Location

	Bray-Curtis R value	Bray-Curtis *P* value	Jaccard R value	Jaccard *P* value
Duodenum				
D14-D21	1.000	<0.001	1.000	<0.001
D14-D27	1.000	<0.001	1.000	<0.001
D14-D3	0.431	0.001	0.369	0.006
D14-D35	1.000	<0.001	1.000	<0.001
D14-D42	0.750	<0.001	0.750	<0.001
D14-D7	0.064	0.147	0.137	0.031
D21-D27	0.221	0.007	0.874	<0.001
D21-D3	0.767	<0.001	0.767	<0.001
D21-D35	0.307	<0.001	0.947	<0.001
D21-D42	0.269	0.002	0.591	<0.001
D21-D7	1.000	<0.001	1.000	<0.001
D27-D3	0.741	<0.001	0.741	<0.001
D27-D35	0.111	0.036	0.071	0.101
D27-D42	0.106	0.057	0.163	0.009
D27-D7	1.000	<0.001	1.000	<0.001
D3-D35	0.767	<0.001	0.767	<0.001
D3-D42	0.519	<0.001	0.519	<0.001
D3-D7	0.575	<0.001	0.438	0.002
D35-D42	0.083	0.078	0.012	0.315
D35-D7	1.000	<0.001	1.000	<0.001
D42-D7	0.824	<0.001	0.824	<0.001
Jejunum				
D14-D21	0.109	0.054	0.306	<0.001
D14-D27	1.000	<0.001	1.000	<0.001
D14-D3	1.000	<0.001	1.000	<0.001
D14-D35	0.795	<0.001	0.795	<0.001
D14-D42	1.000	<0.001	1.000	<0.001
D14-D7	0.387	<0.001	0.417	0.001
D21-D27	0.464	<0.001	0.314	0.002
D21-D3	0.758	<0.001	0.758	<0.001
D21-D35	0.275	0.002	0.260	0.003
D21-D42	0.758	<0.001	0.758	<0.001
D21-D7	0.306	0.002	0.313	<0.001
D27-D3	1.000	<0.001	1.000	<0.001
D27-D35	0.252	0.001	0.140	0.008
D27-D42	1.000	<0.001	1.000	<0.001
D27-D7	0.736	<0.001	0.736	<0.001
D3-D35	0.795	<0.001	0.795	<0.001
D3-D42	1.000	<0.001	1.000	<0.001
D3-D7	0.069	0.102	0.107	0.034
D35-D42	0.571	<0.001	0.720	<0.001

D35-D7	0.553	<0.001	0.553	<0.001
D42-D7	0.758	<0.001	0.758	<0.001
Ileum				
D14-D21	1.000	<0.001	1.000	<0.001
D14-D27	1.000	<0.001	1,000	<0.001
D14-D3	1.000	<0.001	1.000	<0.001
D14-D35	1.000	<0.001	1.000	<0.001
D14-D42	1.000	<0.001	1.000	<0.001
D14-D7	0.024	0.666	0.085	0.054
D21-D27	0.057	0.142	0.131	0.003
D21-D3	1.000	<0.001	1.000	<0.001
D21-D35	1.000	<0.001	1.000	<0.001
D21-D42	1.000	<0.001	1.000	<0.001
D21-D7	1.000	<0.001	1.000	<0.001
D27-D3	1.000	<0.001	1.000	<0.001
D27-D35	1.000	<0.001	1.000	<0.001
D27-D42	1.000	<0.001	1.000	<0.001
D27-D7	1.000	<0.001	1.000	<0.001
D3-D35	1.000	<0.001	1.000	<0.001
D3-D42	1.000	<0.001	1.000	<0.001
D3-D7	1.000	<0.001	1.000	<0.001
D35-D42	0.013	0.546	0.069	0.92
D35-D7	1.000	<0.001	1.000	<0.001
D42-D7	1.000	<0.001	1.000	<0.001
Cecum				
D14-D21	0.074	0.112	0.077	0.090
D14-D27	0.082	0.096	0.077	0.075
D14-D3	0.315	0.001	0.382	0.001
D14-D35	0.685	<0.001	0.685	<0.001
D14-D42	0.711	<0.001	0.711	<0.001
D14-D7	0.321	0.002	0.400	0.001
D21-D27	0.022	0.291	0.122	0.020
D21-D3	1.000	<0.001	1.000	<0.001
D21-D35	1.000	<0.001	1.000	<0.001
D21-D42	1.000	<0.001	1.000	<0.001
D21-D7	1.000	<0.001	1.000	<0.001
D27-D3	0.812	<0.001	0.811	<0.001
D27-D35	0.465	0.001	0.582	<0.001
D27-D42	0.475	<0.001	0.551	<0.001
D27-D7	0.790	<0.001	0.790	<0.001
D3-D35	1.000	<0.001	1.000	<0.001
D3-D42	1.000	<0.001	1.000	<0.001
D3-D7	0.021	0.575	0.047	0.104
D35-D42	0.102	0.059	0.110	0.039
D35-D7	1.000	<0.001	1.000	<0.001

Table 4.S4. Temporal shifts in fungal feature composition

	D3	D7	D14	D21	D27	D35	D42	P Value	FDR
Duodenum									
*F. pseudonygamai*_F1	0.00^{a}	0.00^{a}	0.00^{b}	26.81^{c}	38.10^{cd}	56.04^{d}	38.33^{cd}	< 0.001	**< 0.001**
*Gibberella sp.*_F3	46.44^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*F. pseudonygamai*_F2	0.00^{ab}	0.00^{a}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	14.12^{b}	0.002	**0.002**
*Gibberella sp.*_F4	9.83^{a}	46.87^{b}	35.72^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F5	0.34^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*C. albicans*_F6	0.42^{ac}	1.86^{b}	4.51^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F7	0.00^{a}	0.00^{a}	0.00^{a}	13.21^{b}	8.07^{b}	13.63^{b}	1.27^{c}	< 0.001	**< 0.001**
*A. flavus*_F8	0.19^{a}	3.65^{b}	1.91^{c}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F9	0.00^{ab}	0.00^{a}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.83^{b}	0.002	**0.002**
Trichosporon sp _F15	0.39^{a}	2.20^{b}	10.10^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
Trichosporon sp _F17	0.00^{a}	0.00^{a}	0.00^{a}	11.43^{bc}	12.39^{c}	0.58^{b}	0.72^{b}	< 0.001	**< 0.001**
*S. fibuligera*_F28	3.24^{a}	10.18^{b}	9.55^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*A. flavus*_F13	0.00^{ab}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.76^{a}	0.002	**0.002**
*A. amstelodami*_F16	0.20^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*S. fibuligera*_F32	0.00^{a}	0.00^{a}	0.00^{a}	16.78^{b}	2.63^{c}	0.01^{c}	0.07^{c}	< 0.001	**< 0.001**
*A. flavus*_F12	0.00^{a}	0.00^{a}	0.00^{a}	0.65^{b}	1.29^{bc}	3.20^{c}	1.24^{bc}	< 0.001	**< 0.001**
Hypocreales sp. _F34	1.83^{a}	9.45^{a}	8.26^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*A. flavus*_F14	0.55^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
Trichosporon sp _F21	0.19^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*A. amstelodami*_F20	0.18^{a}	5.78^{a}	2.15^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
Jejunum									
*F. pseudonygamai*_F1	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	18.91^{a}	67.21^{b}	< 0.001	**< 0.001**
Gibberella sp. _F3	0.00^{a}	26.17^{b}	69.12^{b}	45.93^{b}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*F. pseudonygamai*_F2	73.61^{a}	53.53^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
Gibberella sp. _F4	0.00^{a}	0.00^{a}	0.00^{a}	23.51^{b}	55.10^{bc}	55.58^{c}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F5	0.00^{a}	2.00^{b}	9.89^{c}	9.25^{c}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F6	0.00^{a}	0.00^{a}	0.00^{a}	4.63^{b}	11.87^{c}	8.55^{bc}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F7	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	2.83^{a}	1.76^{b}	< 0.001	**< 0.001**
*A. flavus*_F8	0.00^{a}	0.00^{a}	0.00^{a}	0.49^{b}	2.95^{c}	3.33^{c}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F9	1.32^{a}	0.04^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	< 0.001	**< 0.001**
Trichosporon sp _F15	0.00^{a}	0.00^{a}	0.00^{a}	0.70^{ac}	9.29^{b}	0.85^{bc}	0.00^{a}	< 0.001	**< 0.001**
Trichosporon sp _F17	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.02^{b}	< 0.001	**< 0.001**
*S. fibuligera*_F28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*A. flavus*_F13	4.65^{a}	3.59^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*A. amstelodami*_F16	0.00^{a}	0.36^{b}	2.39^{c}	2.70^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*S. fibuligera*_F32	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.42^{b}	< 0.001	**< 0.001**
*A. flavus*_F12	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.13^{a}	4.51^{b}	< 0.001	**< 0.001**
Hypocreales sp. _F34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.433	0.433
*A. flavus*_F14	0.00^{a}	1.64^{b}	3.63^{c}	0.92^{b}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
Trichosporon sp _F21	0.00^{a}	0.14^{b}	2.22^{c}	2.48^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*A. amstelodami*_F20	0.00^{a}	0.00^{a}	0.00^{a}	0.75^{b}	3.53^{c}	0.55^{b}	0.00^{a}	< 0.001	**< 0.001**
Ileum									
*F. pseudonygamai*_F1	78.91^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*Gibberella sp*_F3	0.00^{a}	0.00^{a}	0.00^{a}	56.25^{b}	66.75^{b}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*F. pseudonygamai*_F2	0.00^{a}	75.39^{b}	77.27^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
Gibberella sp. _F4	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	67.75^{b}	75.43^{b}	< 0.001	**< 0.001**
*C. albicans*_F5	0.00^{a}	0.00^{a}	0.00^{a}	17.12^{b}	11.45^{b}	0.00^{a}	0.00^{a}	< 0.001	**< 0.001**
*C. albicans*_F6	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	10.90^{b}	6.35^{b}	< 0.001	**< 0.001**
*C. albicans*_F7	1.09^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.001	**< 0.001**
*A. flavus*_F8	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	8.44^{b}	7.85^{b}	< 0.001	**< 0.001**

*C. albicans*_F9	0.00[a]	1.19[b]	4.65[b]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
Trichosporon sp _F15	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.24[b]	0.41[b]	< 0.001	**< 0.001**
Trichosporon sp _F17	0.11[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	< 0.001	**< 0.001**
*S. fibuligera*_F28	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.03[b]	0.15[c]	< 0.001	**< 0.001**
*A. flavus*_F13	0.00[a]	4.03[b]	5.37[b]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
*A. amstelodami*_F16	0.00[a]	0.00[a]	0.00[a]	9.38[b]	2.24[c]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
*S. fibuligera*_F32	0.08[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	< 0.001	**< 0.001**
*A. flavus*_F12	6.03[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	< 0.001	**< 0.001**
Hypocreales sp. _F34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.423	0.423
*A. flavus*_F14	0.00[a]	0.00[a]	0.00[a]	3.53[b]	5.47[b]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
Trichosporon sp _F21	0.00[a]	0.00[a]	0.00[a]	3.53[b]	4.47[b]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
*A. amstelodami*_F20	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]	1.49[b]	0.71[c]	< 0.001	**< 0.001**
Cecum									
*F. pseudonygamai*_F1	91.62[a]	90.65[a]	35.59[b]	0.00[c]	0.00[c]	0.00[c]	0.00[c]	< 0.001	**< 0.001**
Gibberella sp. _F3	0.00[a]	0.00[a]	0.00[a]	0.00[a]	24.87[a]	85.00[b]	85.82[b]	< 0.001	**< 0.001**
*F. pseudonygamai*_F2	0.00[a]	0.00[a]	45.40[b]	66.83[b]	55.21[b]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
Gibberella sp. _F4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*C. albicans*_F5	0.00[a]	0.00[a]	0.00[a]	0.00[a]	2.14[a]	7.44[b]	5.03[b]	< 0.001	**< 0.001**
*C. albicans*_F6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*C. albicans*_F7	0.14[a]	0.83[b]	0.72[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	< 0.001	**< 0.001**
*A. flavus*_F8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*C. albicans*_F9	0.00[a]	0.00[a]	1.77[b]	9.22c	6.69[bc]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
Trichosporon sp _F15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
Trichosporon sp _F17	0.14[ab]	0.13[a]	0.03[bc]	0.00[cd]	0.00[cd]	0.00[cd]	0.00[d]	< 0.001	**< 0.001**
*S. fibuligera*_F28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*A. flavus*_F13	0.00[a]	0.00[a]	0.32[b]	0.56[b]	0.35[b]	0.00[a]	0.00[a]	< 0.001	**< 0.001**
*A. amstelodami*_F16	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.06[b]	0.44[b]	0.60[b]	< 0.001	**< 0.001**
*S. fibuligera*_F32	0.00[a]	0.00[ab]	0.00[b]	0.00[ab]	0.00[ab]	0.00[ab]	0.00[ab]	0.007	**0.007**
*A. flavus*_F12	0.19[a]	0.16[a]	0.08[b]	0.00[c]	0.00[c]	0.00[c]	0.00[c]	< 0.001	**< 0.001**
Hypocreales sp. _F34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA
*A. flavus*_F14	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.04[a]	0.70[b]	0.87[b]	< 0.001	**< 0.001**
*Trichosporon sp*_F21	0.00[a]	0.00[a]	0.00[a]	0.00[a]	1.39[ab]	0.19[b]	0.23[b]	< 0.001	**< 0.001**
*A. amstelodami*_F20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NA	NA

Note: Mean relative abundance (%) of the phylum, top 20 fungal features are shown, with 10 to 12 samples per intestinal segment. Statistical significance was determined using non-parametric Kruskal Wallis test and p values were corrected for multiple comparisons using the Benjamini-Hochberg correction. For columns with an FDR ≤ 0.05, means were separated using pairwise Wilcoxon Rank Sums test. Columns not sharing a common superscript are considered significantly different ($P < 0.05$).

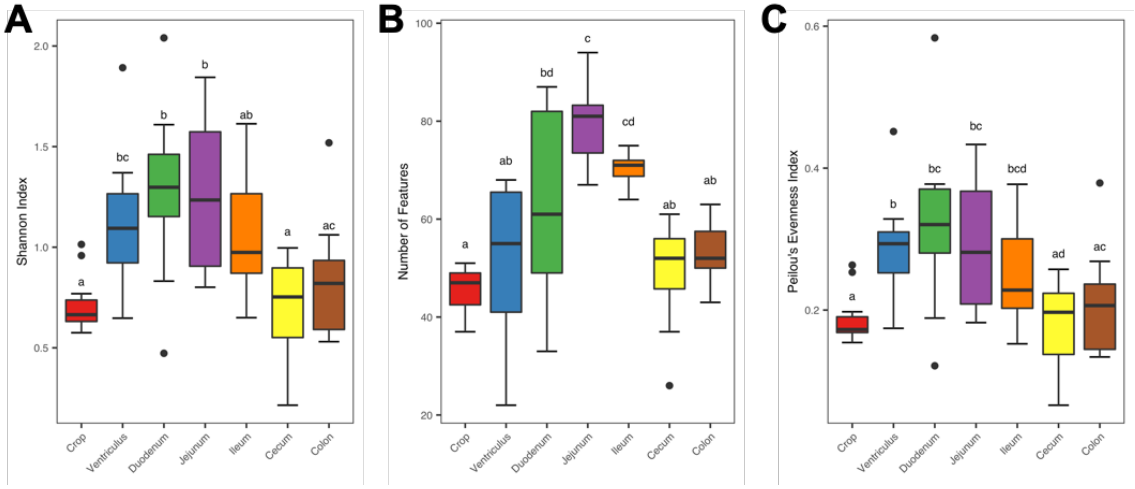

Fig. 4.1. Variation in the α-diversity of the mycobiome along the gastrointestinal tract of day-42 chickens. Overall diversity, richness, and evenness were calculated from 10-12 luminal content samples of each intestinal segment using the Shannon Index (A), Observed Features (B), and Pielou's Evenness index, respectively. Significant differences were determined using the Kruskal-Wallis test and means were separated with pairwise Wilcoxon rank-sum tests with the intestinal segments not sharing a common superscript considered significantly different ($P < 0.05$).

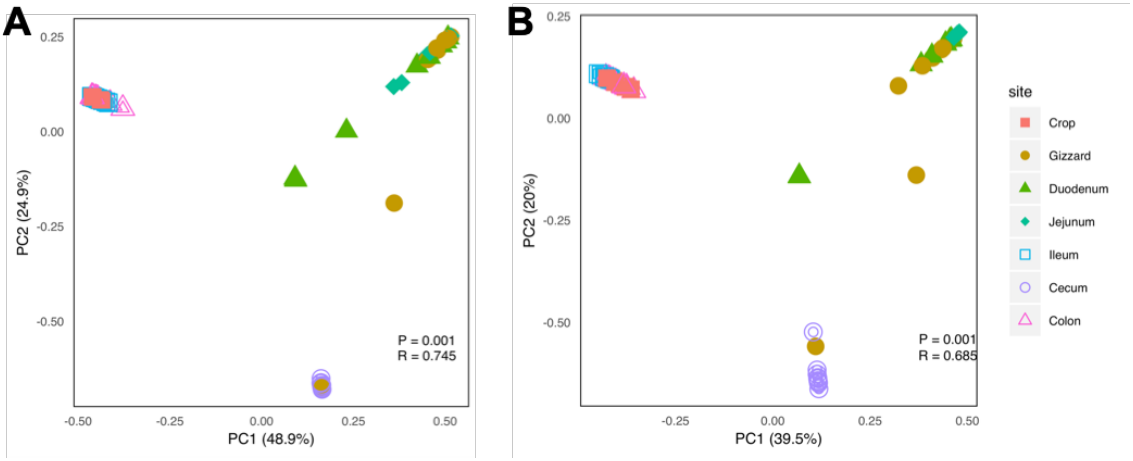

Fig. 4.2. Principal coordinate analysis (PCoA) of the mycobiome along the gastrointestinal tract of day-42 chickens. Luminal content samples were collected from each gastrointestinal segment of 10-12 chickens. PCoA was plotted using Bray-Curtis (A) and the Jaccard indices (B), respectively. Statistical significance was determined using ANOSIM and indicated in each plot.

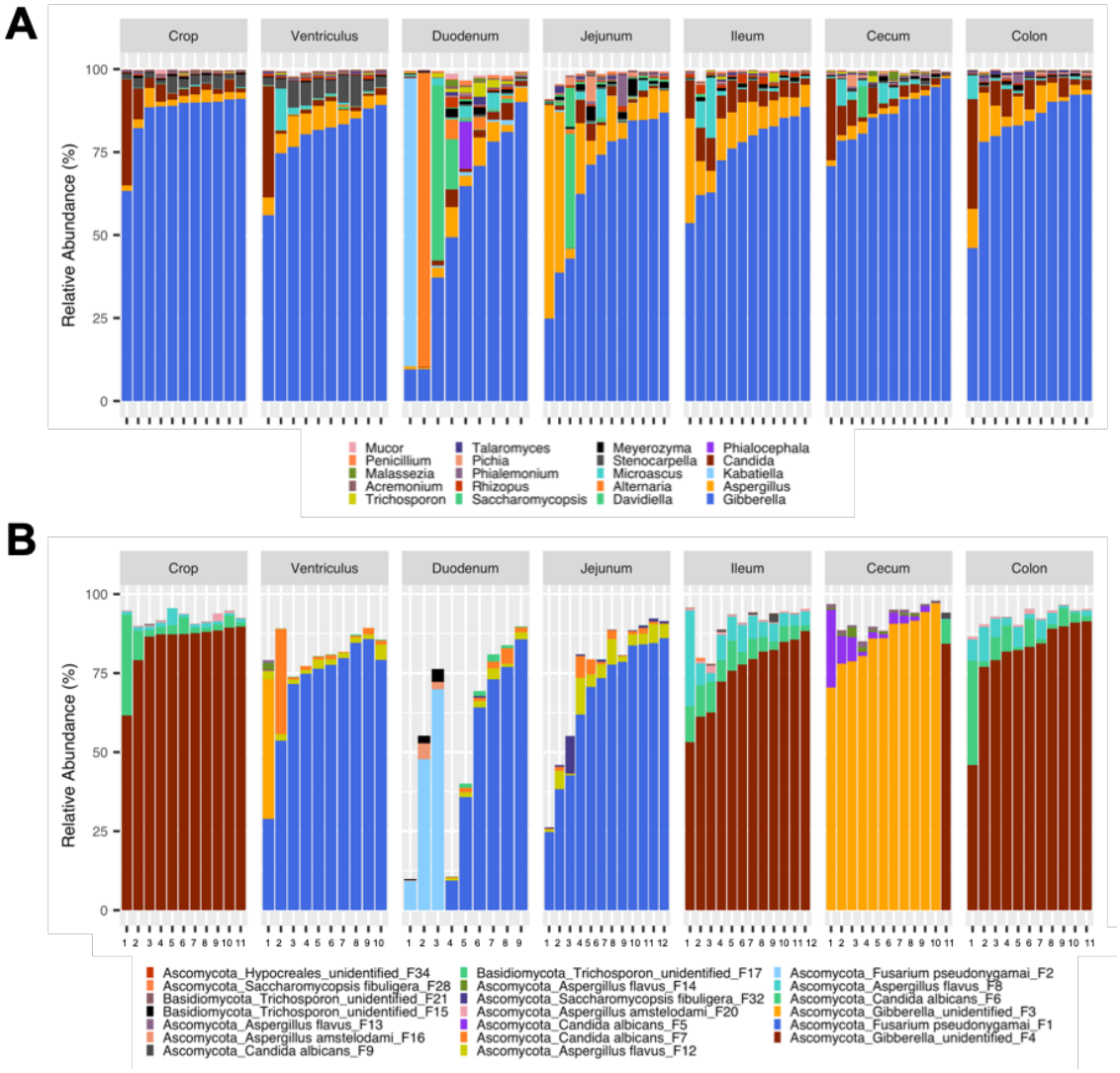

Fig. 4.3. Biogeographical variation in mycobiota composition of the gastrointestinal tract of day-42 chickens. Luminal content samples were collected from each gastrointestinal segment. Relative abundance of features was used to determine the fungal composition at the genus (A) and feature (B) levels with 10-12 samples of each segment. Only the top 20 genera and features are shown.

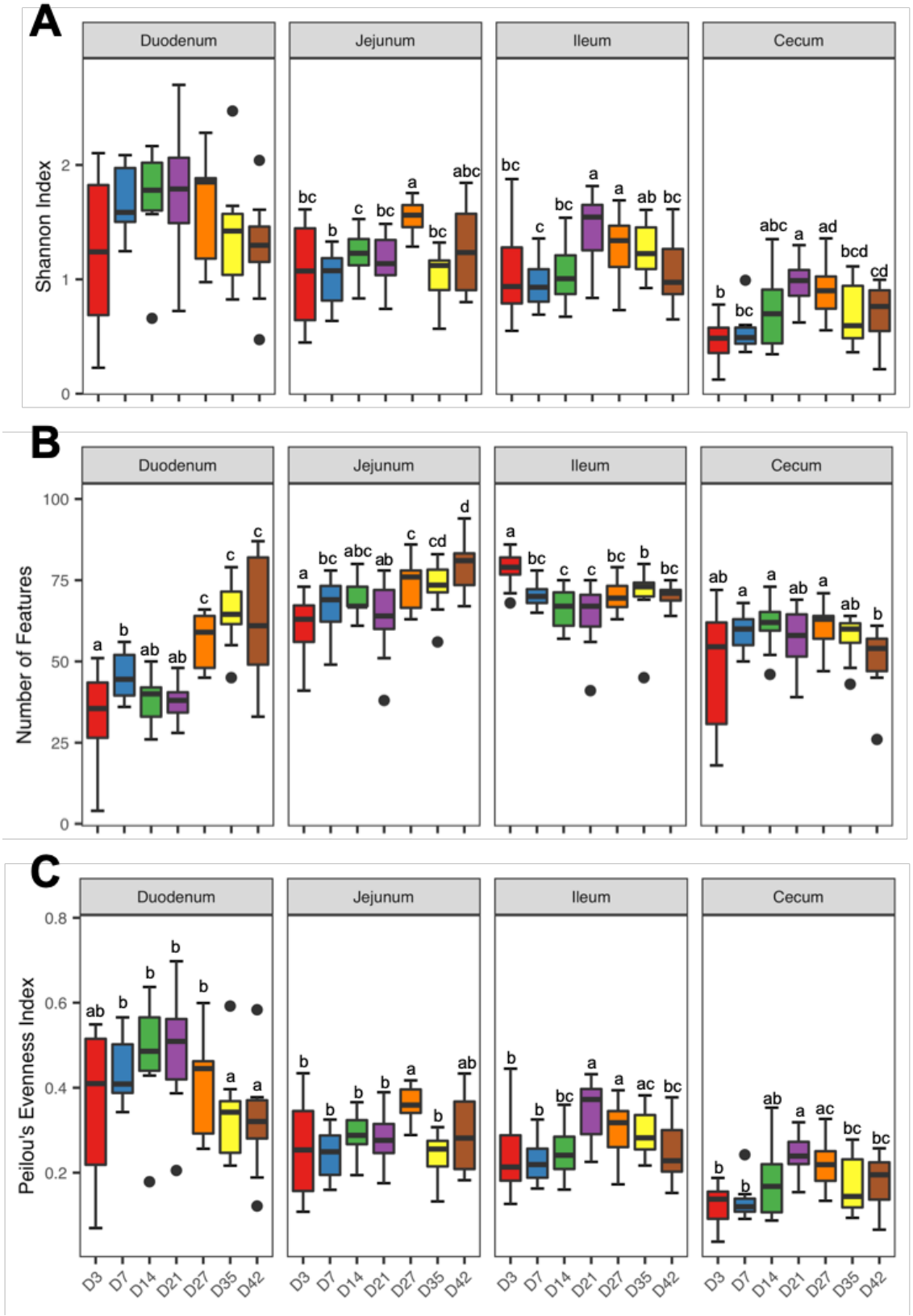
A
Duodenum
Jejunum
Ileum
Cecum
Shannon Index
0
1
2
B
Number of Features
0
25
50
75
100
C
Peilou's Evenness Index
0.2
0.4
0.6
0.8
D3
D7
D14
D21
D27
D35
D42

Fig. 4.4. Successional changes in the α-diversity chicken mycobiota. Luminal content samples were collected from the duodenum, jejunum, ileum, and cecum of chicks fed an antibiotic-free diet on days 3, 7, 14, 21, 27, 35, and 42. The α-diversity was calculated from 10 to 12 samples on each sampling day and indicated by the Shannon Index (A), Observed Features (B), and Pielou's Evenness, respectively. Significant differences were determined using the Kruskal-Wallis test and means were separated with pairwise Wilcoxon rank-sum tests with the segments not sharing a common superscript considered significantly different ($P < 0.05$).

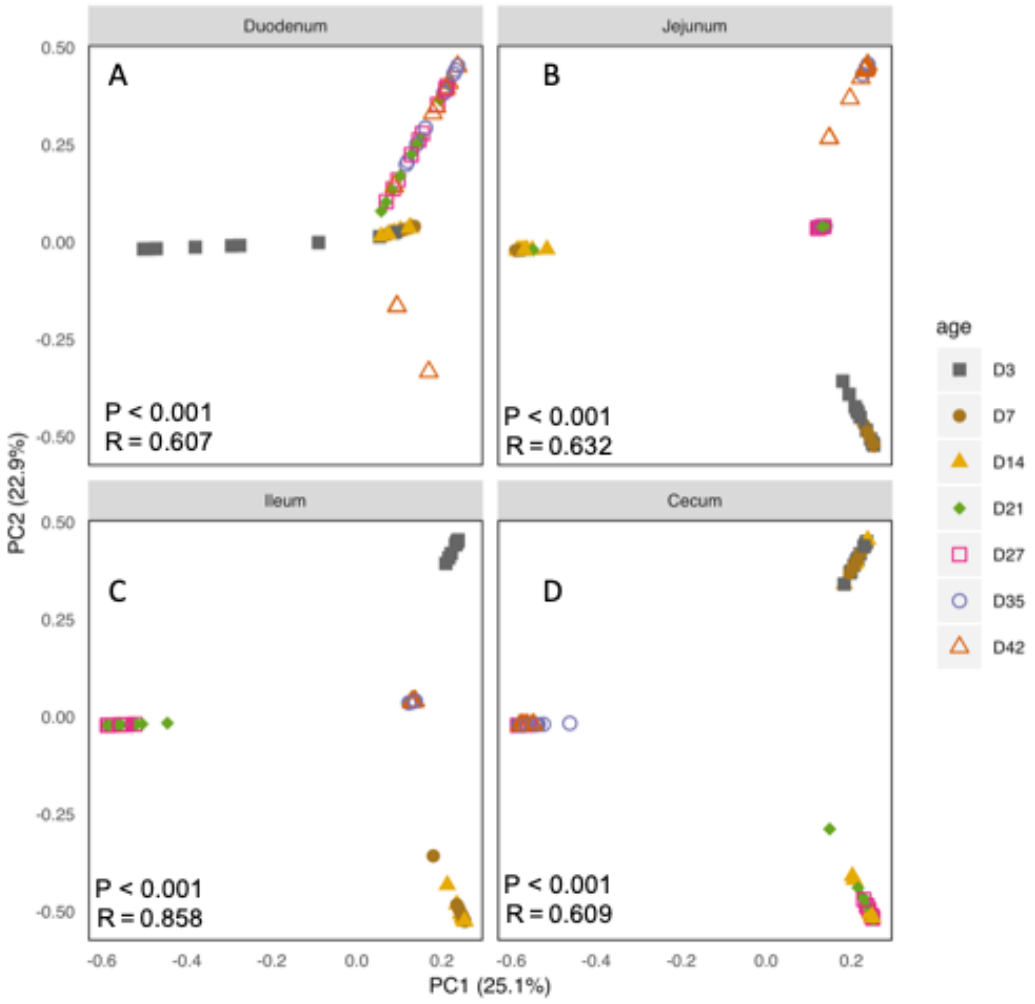

Fig. 4.5. Variation in β-diversity of the chicken intestinal mycobiota. Luminal content samples were collected from the duodenum, jejunum, ileum, and cecum of chicks fed an antibiotic-free diet on days 3, 7, 14, 21, 27, 35, and 42, respectively. Principal coordinate analysis (PCoA) was plotted from 10 to 12 samples on each sampling day using Bray-Curtis index. Statistical significance was determined using ANOSIM and indicated in each plot.

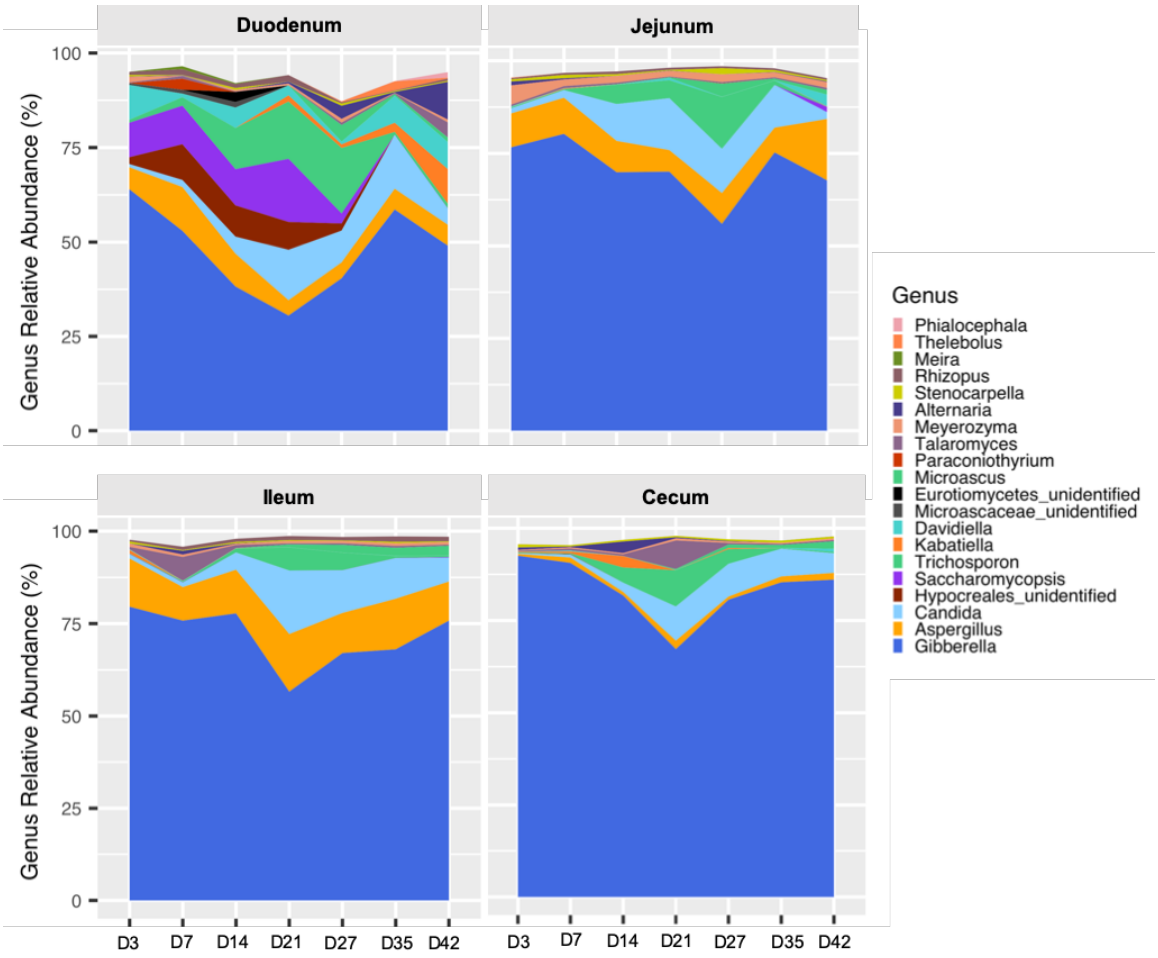

Fig. 4.6. Developmental changes in the intestinal mycobiota composition of chickens.

Luminal content samples were collected from four gastrointestinal segments of broiler chickens on seven different sampling days. Relative abundance was calculated at the genus level with 10 to 12 samples per segment per sampling day. Only the top 20 genera are shown.

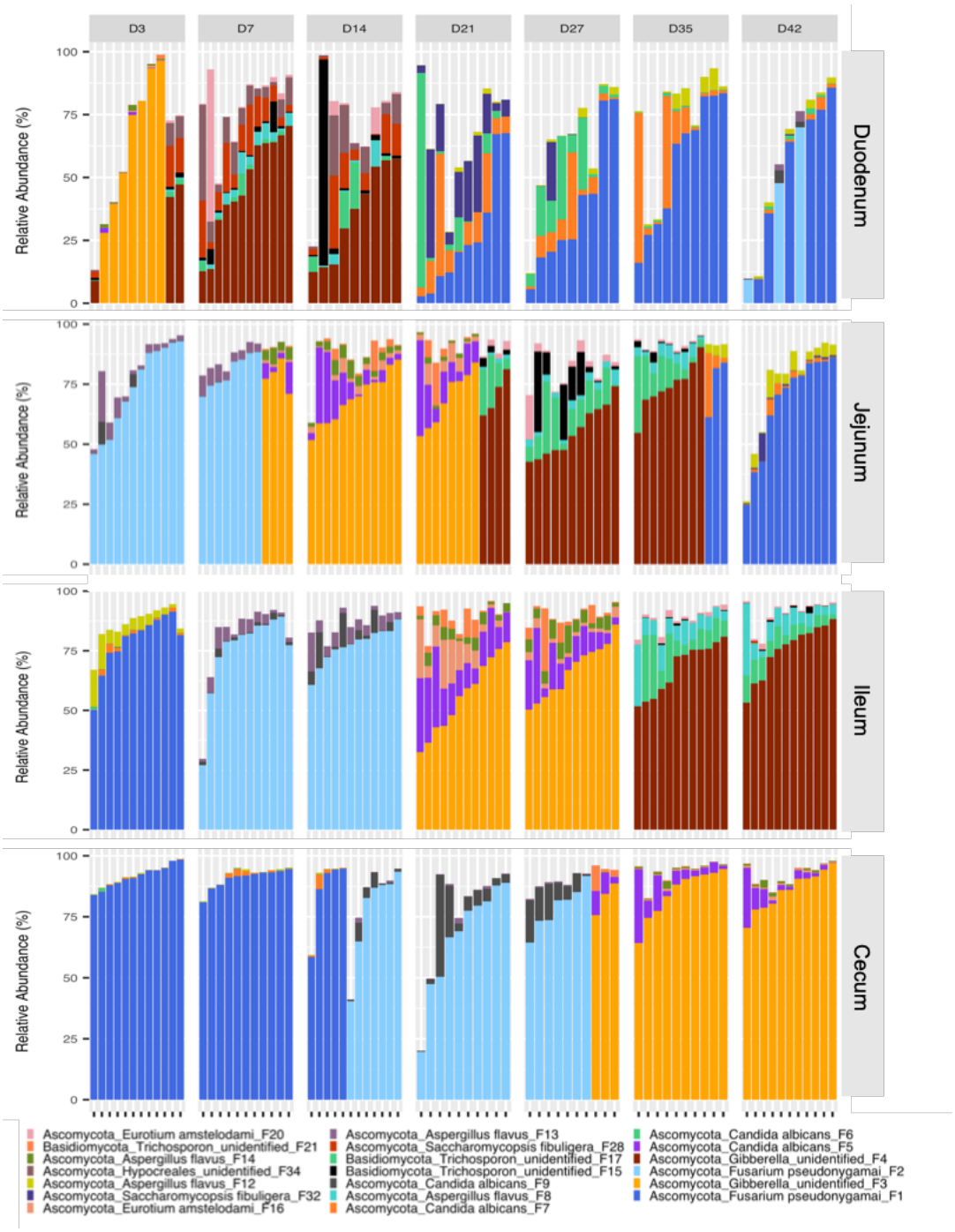

Fig. 4.7. Shift in the intestinal mycobiota composition over a production cycle of chickens. Luminal content samples were collected from four gastrointestinal segments on seven different sampling days. Relative abundance of each feature was calculated with 10 to 12 samples per segment per sampling day. Only the top 20 features are shown.

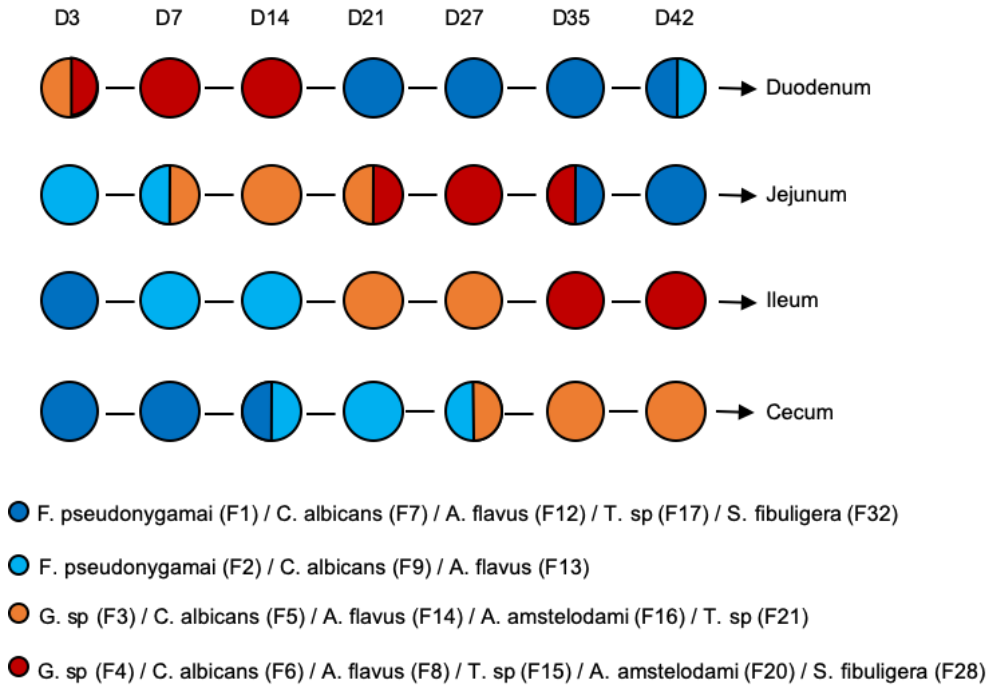

Fig. 4.8. Age-dependent transition of four specific fungal consortia along the intestinal tract. Passage of four distinct fungal consortia down the intestinal tract is summarized. Full circles indicate that only one consortium was observed for that location on that day. Half circles represent the presence of two consortia in an intestinal segment on a sampling day.

CHAPTER V

DIFFERENTIAL IMPACT OF SUBTHERAPEUTIC ANTIBIOTICS AND IONOPHORES ON CECAL MICROBIOTA OF BROILERS

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ABSTRACT

Antimicrobial growth promoters (AGPs) are commonly used in the livestock industry at subtherapeutic levels to improve production efficiency, which is achieved mainly through modulation of the intestinal microbiota. However, how different classes of AGPs, particularly ionophores, regulate the gut microbiota remains unclear. In this study, male Cobb broiler chickens were supplemented for 14 days with or without one of five commonly used AGPs including three classical antibiotics (bacitracin methylene disalicylate, tylosin, and virginiamycin) and two ionophores (monensin and salinomycin) that differ in antimicrobial spectrum and mechanisms. Deep sequencing of the V3-V4 region of the bacterial 16S rRNA gene revealed that two ionophores drastically reduced a number of rare bacteria resulting in a significant decrease in richness and a concomitant increase in evenness of the cecal microbiota, whereas three antibiotics had no obvious impact. Although each AGP modulated the gut microbiota differently, the closer the antibacterial spectrum of AGPs, the more similarly the microbiota is regulated. Importantly, all AGPs had a strong tendency to enrich butyrate- and lactic acid-producing bacteria, while reducing bile salt hydrolase-producing bacteria, suggesting enhanced metabolism and utilization of dietary carbohydrates and lipids and improved energy harvest, which is at least partially responsible for the growth-promoting effect of AGPs.

Keywords: Microbiota; antibiotics; ionophores; antimicrobial growth promoters; chickens

Introduction

Subtherapeutic antimicrobial growth promoters (AGPs) are commonly included in livestock diets to improve production performance [1,2]. This is particularly true in the poultry industry where supplementation has been shown to improve weight gain and feed efficiency, inhibit pathogen growth, and reduce mortality [2,3]. However, increased microbial resistance linked to antibiotic use in food animals has led to a ban on AGPs and a change in consumer preference towards products from animals raised without antibiotics [1,4,5]. This has, therefore, created a need to develop antibiotic alternatives to ensure animal health and growth performance.

While the exact mode of action remains elusive, AGPs are postulated to provide performance benefits through modulation of the intestinal microbiota [2,6,7,8]. Indeed, the inability of antibiotics to improve growth performance in germ-free chicks has provided compelling evidence that antibiotics work primarily by reshaping the intestinal microbiota [9], which is a unique ecosystem known to play a vital role in host health and metabolism through its effects on feed digestion, nutrient absorption, vitamin synthesis, and immune system development [10,11,12]. Consistently, a link between the intestinal microbiota and growth performance of livestock animals has been established [7,13,14]. For example, two specific fecal bacterial community structures known as enterotypes are significantly associated with body weight and average daily gain of pigs [15]. Similar studies in broiler chickens have revealed a strong correlation between certain intestinal bacterial taxa and weight gain [16]. Specific AGP-induced changes in the intestinal microbiota are beginning to be elucidated [2,7,8]. However, the wide range of AGPs used between studies, as well as differences in animal age, diet, genetics, management condition, DNA isolation, and sequencing strategies, make it difficult to draw a definitive conclusion from the current literature. It remains unknown whether different classes of AGPs such as classical antibiotics and ionophores modulate the intestinal microbiota in similar or distinct manners.

In this study, we directly compared the effects of five AGPs including bacitracin methylene disalicylate (BMD), tylosin, virginiamycin, monensin, and salinomycin on the cecal microbiota of broilers. These five AGPs were chosen because they are commonly used in the poultry industry and are known to have different antimicrobial spectra and mechanisms. BMD is a broad-spectrum cyclic peptide antibiotic that functions through inhibition of bacterial cell wall synthesis, while tylosin, a macrolide, and virginiamycin, a streptogramin, both target Gram-positive bacteria by inhibiting bacterial protein synthesis [2,17]. Monensin and salinomycin, on the other hand, are polyether ionophores that act against coccidia and Gram-positive bacteria by transporting ions and dissipating ion gradients across bacterial cell membranes [2,17]. Based on deep sequencing of the V3-V4 region of the bacterial 16S rRNA gene after 2-week subtherapeutic supplementation of five AGPs, we revealed in the current study an obvious shift in the structure of the cecal bacterial community, with two ionophores having the most striking effect. Identification of a number of bacterial taxa that are commonly and uniquely altered in response to different AGPs shed new light on their growth-promoting mechanism and may allow targeted manipulation of the cecal microbiota to improve animal health and productivity in the future.

Materials and Methods

Animal trial

All animal trials were conducted in accordance with the Institutional Animal Care and Use Committee of Oklahoma State University. A total of 576 day-of-hatch male Cobb broiler chicks were obtained from the Cobb-Vantress Hatchery (Siloam Springs, AR) and randomly assigned to one of six dietary treatments with eight birds per cage and 12 cages per treatment. Animals received either a non-medicated standard corn-soybean starter diet, formulated to meet or exceed NRC requirements, or the starter diet supplemented with one of five AGPs for 14 days. The supplemental levels were BMD (0.5 g BMD®-50/kg diet, equivalent to 55 mg BMD/kg, Zoetis,

Parsippany, NJ), tylosin (0.5 g Tylan®-40/kg, equivalent to 44 mg tylosin/kg, Elanco Animal Health, Greenfield, IN), virginiamycin (0.5 g Stafac®-20/kg, equivalent to 22 mg virginiamycin/kg, Phibro Animal Health, Teaneck, NJ), monensin (0.5 g Coban®-90/kg, equivalent to 99 mg monensin/kg, Elanco Animal Health), and salinomycin (0.5 g Bio-Cox®-60/kg, equivalent to 66 mg salinomycin/kg, Zoetis), respectively. All antimicrobial doses are recommended at subtherapeutic levels for disease prevention and growth promotion by respective manufacturers. The chickens were raised on floor cages with fresh pine wood shavings under standard management. Water and feed were provided ab libitum for the entire duration of the trial. The room temperature was started at 33˚C and decreased 3˚C every 7 days. The light to dark ratio was 24:0 for day 0, 23:1 for days 1 to 3, 18:6 for days 4 to 6, and 16:8 for days 7 to 14. Modification of room temperature and lighting was designed in accordance with industry standards to ensure optimal bird growth. On day 14, two chicks were randomly selected from each cage and euthanized via CO_2 asphyxiation. Cecal content was collected from each bird for microbiome analysis. The samples were immediately frozen in liquid nitrogen and stored at -80˚C until further processing.

DNA extraction and sequencing

Bacterial DNA was isolated from cecal contents using the ZR Fecal DNA Isolation Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The quality and quantity of DNA samples were determined using a Nanodrop ND-1000, and agarose gel electrophoresis was used to confirm the absence of degradation. High quality DNA was sequenced for the V3-V4 region of the 16S rRNA gene by Novogene (Beijing, CA) on Illumina HiSeq2000 using 341F (CTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) primers. Novogene's standard protocol using the NEB Next® Ultra™ Library Prep Kit was used for PCR amplification and library preparation.

Bioinformatic analysis and statistics

Raw sequences were processed using mothur, version 1.39.5 [18], according to the standard operating procedures. Low quality sequences and singletons were removed. Sequences were aligned using the SILVA database prior to classification using the RDP 16S rRNA training set 16. Sequences that shared no less than 97% identity were clustered into one operational taxonomic unit (OTU) and relative abundance was calculated. Differences in the microbial community structure were calculated using R version 3.5.1 [19]. The α- and β-diversities were calculated with the phyloseq package, version 1.24.2 [20], while plots were made using ggplot2 version 3.0.0 [21]. Statistical differences in α-diversity and relative abundance were determined using one-way ANOVA with *post hoc* Tukey's test in R. The α-diversity was calculated using the Shannon Evenness index and Observed OTUs as measures of evenness and richness, respectively. Results were plotted using box and whisker plots, in which the middle line denotes the median value and the lower and upper hinges represent the first and third quartiles, respectively. Whiskers extend from the hinge to the highest or lowest value no farther than 1.5× the inter-quartile range. Points outside of this range are considered outliers.

The β-diversity was calculated using Bray-Curtis and Jaccard indices and statistical difference in the microbiome composition was determined using analysis of similarity (ANOSIM) in the vegan package of R, version 2.5.2 [22]. Metastats [23] was used to determine significant differences in the relative abundance of each OTU between individual treatments and the control group. Venn diagrams were drawn using the VennDiagram package of R [24], and heatmap was generated using Heatmapper [25].

Accession number

Sequencing data for this experiment was deposited into NCBI SRA and can be found under the accession number PRJNA552082.

Results

Effect of in-feed antimicrobials on the cecal bacterial diversity

Male Cobb broiler chicks were fed a non-medicated corn-soybean basal diet supplemented with or without one of five commonly used AGPs at subtherapeutic levels for two weeks before collection of 12 cecal content samples for each treatment. Following bacterial DNA isolation and sequencing of the V3-V4 region of the 16S rRNA gene, a total of 7,767,847 raw sequence reads were obtained with an average of 107,866 ± 11,583 sequences per sample. After quality trimming and processing, 6,522,487 reads remained and were further clustered into 2,416 OTUs. Sequences were subsampled to a depth of 56,629 sequences per sample for subsequent analysis.

The α-diversity was first calculated using Shannon Evenness Index (Figure 1A) and Observed OTUs (Figure 1B). Both measurements revealed a trend toward a decrease in both evenness and richness of the cecal microbiota in response to three antibiotics (BMD, tylosin, and virginiamycin). Surprisingly, two ionophores (monensin and salinomycin) led to a significant increase ($P < 0.05$) in evenness of the microbiota, which was accompanied by a drastic decrease in richness ($P < 0.05$). To further reveal the difference in the cecal microbiota α-diversity between antibiotics and ionophores, data from three antibiotics and two ionophore groups were combined separately and α-diversity was calculated. Similar to individual treatments, ionophores caused a significant increase in evenness ($P < 0.05$) (Figure 1C) and a concomitant decrease in richness ($P < 0.05$) (Figure 1D), whereas the effect of antibiotics on cecal bacterial α-diversity was insignificant, suggesting that ionophores have a more pronounced effect than antibiotics on reshaping the cecal microbiota.

To further reveal the differences in cecal microbiota composition among individual AGPs, β-diversity was determined using the Bray-Curtis and Jaccard indices. While there was no obvious segregation of the microbiota based on the Bray-Curtis Index (Figure 2A), two ionophore

groups were clearly separated from all other treatments using the Jaccard Index (Figure 2B), reinforcing an earlier observation on α-diversity that in-feed ionophores had a stronger effect on cecal microbiota than antibiotics. Consistently, ANOSIM indicated that the differences in the Bray-Curtis index among treatments are mostly significant ($P < 0.05$), but generally minor (with a low R value ranging from 0.048 to 0.411) (Table 1). However, for the Jaccard index, all three antibiotics and the control group had very low R values of less than 0.1 among each other, whereas the highest R values were observed when comparing the two ionophore groups to any other group ($R > 0.7$ for all comparisons) (Table 1), in agreement with the α-diversity analysis in that two inophores significantly reduced richness of the cecal microbiota, while three antibiotics had relatively a mild effect (Fig. 1D).

Effect of in-feed antimicrobials on cecal bacterial composition

At the phylum level, Firmicutes was found to be the most abundant phylum accounting for over 97% of all sequences, followed by Proteobacteria and Bacteroidetes in the cecum of day-14 broilers (Figure 3A). Statistical analysis revealed no significant difference in relative abundance of Firmicutes or Proteobacteria among treatments (Supplementary Table 1). Relative abundance of Bacteroidetes and Actinobacteria varied significantly among the treatments ($P < 0.05$). Virginiamycin resulted in a significant decrease in Actinobacteria, relative to the control group, while BMD significantly increased Bacteroidetes as compared to tylosin and monensin ($P < 0.05$) (Supplementary Table 1). At the genus level, over 48% of sequences were identified as unclassifiable members of Lachnospiraceae (Figure 3A). Statistical analysis of the top 10 genera revealed differential effects of AGP supplementation on members of Ruminococcaceae, Clostridiales, and Romboutsia. For example, tylosin supplementation caused a significant increase in an unclassified genus of the Ruminococcaceae family relative to the control group ($P < 0.05$), while virginiamycin resulted in a significant diminishment of Romboutsia of the

Peptostreptococcaceae family and a concomitant increase in an unclassified genus of the Clostridia class as compared to control ($P < 0.05$) (Supplementary Table 1).

When three antibiotics and two ionophore groups were combined and compared with the control group, no significant difference in relative abundance of Firmicutes, Proteobacteria, or Bacteroidetes was observed at the phylum level, while Actinobacteria was significantly reduced by antibiotics, but not ionophores (Figure 3B and Supplementary Table 2). At the genus level, a significant increase in unclassified *Ruminococcaceae* was observed in the antibiotics group over control ($P < 0.05$), while ionophores had a minimum impact (Figure 3B and Supplementary Table 2). *Romboutsia* was significantly augmented in response to antibiotics ($P < 0.05$), but remained unaltered by ionophores (Supplementary Table 2). When all five AGP groups were pooled and compared to the control (Figure 3C), Firmicutes was slightly, but significantly decreased by AGPs, while the opposite was true with Proteobacteria (Supplementary Table 3). A significant increase in an unclassified member of both *Ruminococcaceae* and *Clostridiales* was observed ($P < 0.05$), while all other genera remained largely unchanged (Supplementary Table 3).

Differential regulation of OTUs by antimicrobial supplementation

In order to better define changes in individual OTUs, Metastats [23] was used to identify OTUs that were significantly altered ($P \leq 0.05$) by each AGP relative to the control group. Overall, 898 OTUs were significantly up- or down-regulated by at least one AGP, with 59 of those affected by all five AGPs (Figure 4). The majority of these were observed to be rare OTUs belonging to a diverse set of bacterial genera (data not shown). Apparently, each AGP also showed an obvious differential effect, with a group of OTUs being uniquely modulated by individual AGPs (Figure 4). Additionally, monensin and salinomycin supplementation resulted in a depletion of a number of lowly abundant OTUs belonging to unclassified genera of *Ruminococcaceae* and *Clostridiales*

(data not shown), consistent with a significant decrease in microbiota richness in the α-diversity analysis.

Among the top 100 most abundant OTUs, 64 were significantly affected by at least one treatment as revealed by Metastats and visualized in a heatmap (Figure 5). It is obvious that each AGP regulate different bacterial populations in the cecum. For example, a unclassified member of *Ruminococcaceae* (OTU0085) was significantly increased by BMD, but remained largely unchanged in response to other AGPs; on the other hand, another unclassified *Ruminococcaceae* (OTU0003) was significantly suppressed by monensin, but not other AGPs (Figure 5). However, several OTUs were regulated similarly in response to all five AGPs. For example, an *Escherichia/Shigella* member (OTU0021), a *Blautia* member (OTU0016), a *Clostridium XlVa* member (OTU0039), and an unclassified member of *Clostridiales* (OTU0059) were enriched by all five AGPs, whereas a *Clostridium XIVb* member (OTU0073) and a *Lactobacillus* (OTU0019) were decreased by all AGPs (Figure 5). Relative abundances of OTU0039 in the cecum of individual broilers were further illustrated in a dot plot (Figure 6A). It is not surprising that Euclidean clustering identified a clear segregation of five AGPs, with three antibiotics forming one clade and two ionophores forming the other (Figure 5). A cluster of four members of Firmicutes (OTU0047, OTU0070, OTU0080, and OTU0092) were clearly suppressed by two ionophores, but largely unaffected by three antibiotics (Figure 5 and Figure 6B). A *Streptococccus* member (OTU0052) was also significantly suppressed by two ionophores, but dramatically enriched by tylosin, while two other antibiotics had no effect (Figure 5 and Figure 6C). Conversely, an *Enterococcus* (OTU0081) was significantly enriched by two ionophores, but unaffected by antibiotics (Figure 6D). Within the antibiotics clade, tylosin and virginiamycin were shown to cluster separately from BMD, consistent with their differences in antimicrobial mechanism and spectrum [2,17]. A cluster of six OTUs (OTU0023, OTU0026, OTU0060, OTU0084, OTU0088, and OTU0098) and another cluster of two OTUs (OTU0054 and

OTU0058) were uniquely suppressed by BMD, with no obvious effect by other antibiotics (Figure 5) as exemplified by OTU0023 (Figure 6E).

Discussion

In-feed AGPs are known to modulate gut microbiota. Early culture-independent studies using molecular techniques such as terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, and Sanger sequencing of the 16S rRNA gene clone libraries revealed a consistent and obvious antibiotic-induced shift in the microbiota composition [26,27,28,29,30,31]. However, these techniques lack the depth and precision to reveal specific changes in bacterial taxa. Subsequent next-generation sequencing of the bacterial 16S rRNA gene demonstrated specific changes in certain bacterial populations, but with largely inconsistent results. Multiple studies have shown no obvious effect of antibiotics on α-diversity [32,33,34,35], while others showed a decrease [36,37] or an increase in α-diversity of the cecal microbiota [38]. Significant changes in bacterial composition, measured by β-diversity, were observed more consistently [34,37,38,39], with only a few studies not reporting a shift [36,40]. However, very few studies compared the impact of multiple AGPs, particularly antibiotics and ionophores, on the gut microbiota side-by-side.

In this study, two-week supplementation with three classical antibiotics (BMD, tylosin, and virginiamycin) had a minimum effect on α- or β-diversity of the chicken cecal microbiota. Surprisingly, a significant decrease in richness and a concurrent increase in evenness of the cecal microbiota was observed for two ionophores (monensin and salinomycin), consistent with the 16S rRNA gene sequencing results showing a significant diminishment of a large number of rare bacterial phylotypes. Previous work investigating the effects of ionophores on poultry microbiota is limited. However, Danzeisen et al. [32] reported a similar, though non-significant, decrease in α-diversity of the cecal microbiota in broilers in response to monensin supplementation.

Overall, the most dramatic effect of AGP supplementation is differential enrichment of the *Clostridiales* order, particularly the members of three most dominant bacterial families in the chicken cecum (*Ruminococcaceae*, *Lachnospiraceae,* and *Clostridiaceae*) [41]. Among the 100 most abundant bacterial taxa, a number of the *Ruminococcaceae* members were enriched in the cecum by AGPs, while many *Lachnospiraceae* species appear to be diminished (Figure 5). Previous studies have found both *Ruminococcaceae* and *Lachnospiraceae* to be increased following supplementation of broiler diets with a mixture of chlortetracycline, virginiamycin, and amoxicillin [42], but decreased by avilamycin, flavophospholipol, or zinc bacitracin [36,43]. Both *Ruminococcaceae* and *Lachnospiraceae* are known to produce butyrate [44,45]. It is noted that several *Clostridium* IV and XIVa members such as OTU0039, OTU0075, and OTU0076 were also significantly increased in abundance. Both IV and XIVa clusters of Clostridia are the two most dominant bacterial taxa in the hind gut of humans and well-known for their ability to produce butyrate from indigestible carbohydrates [46,47]. These results are consistent with earlier observations that in-feed antibiotics preferentially enriched butyrate-producing bacteria [42].

Lactic acid bacteria are widely used as probiotics to provide a myriad of beneficial effects to the host [48,49]. Only three lactic acid bacterial genera including *Enterococcus, Lactobacillus*, and *Streptococcus* were differentially regulated by AGPs among the 100 most abundant OTUs in the cecum. It is interesting to note that all three *Enterococcus* members (OTU0081, OTU0090, and OTU0094) were upregulated by AGPs, while the only *Lactobacillus* member (OTU0019) was obviously reduced in response to all but one AGPs. A reduction in the *Lactobacillus* abundance is consistent with earlier observations that AGP administration was associated with depopulation of the *Lactobacillus* species [50,51], resulting in reduced bile salt deconjugation and improved fat digestion and utilization [51]. Lactobacilli are known to be major producers of bile salt hydrolase responsible for hydrolyzing and deconjugating primary bile acids [52]. A lone *Streptococcus* member (OTU0052) was significantly reduced by two ionophores, but

significantly enriched by tylosin, while BMD and virginiamycin had a minimum impact. *Streptococcus* has been shown to be suppressed by carbadox and a mixture of three antibiotics (chlortetracycline, sulfamethazine, and penicillin) in pigs [53] or a mixture of three different antibiotics (amoxicillin, metronidazole, and bismuth) in mice [54].

These results collectively suggest that AGPs have a strong tendency to enrich butyrate- and lactic acid-producing bacteria, while reducing bile salt hydrolase-producing bacteria, in the cecum. A combination of these effects leads to enhanced metabolism and utilization of dietary carbohydrates and lipids and improved energy harvest and mucosal immune defense, all of which may be responsible for the growth-promoting effect of AGPs.

Among five AGPs selected for this study, BMD kills a broad spectrum of Gram-positive bacteria by interfering with synthesis of bacterial cell wall and peptidoglycan, while tylosin and virginiamycin act against a narrower spectrum of Gram-positive bacteria via inhibition of bacterial protein synthesis [17]. Two ionophores (monensin and salinomycin), on the other hand, kill bacteria and parasites by facilitating transportation of monovalent ions and thereby disrupting ion gradients across cell membranes, although monensin preferentially transports Na^+ and H^+, while salinomycin prefers K^+ and Na^+ [17,55]. An obvious differential effect exists among individual AGPs and among different classes of AGPs, although a number of bacteria are commonly regulated by all AGPs. It is apparent that, the larger the difference in the antibacterial spectrum and mode of action among AGPs, the more distinct the bacterial populations that they regulate. We observed that monensin and salinomycin modulate similar bacterial populations that are rather different from three antibiotics. Among three antibiotics, virginiamycin and tylosin were found to manipulate the microbiota composition in a more similar manner than BMD.

Among top 100 OTUs, 97 belong to Firmicutes. The only *Bacteroides* (OTU0037) was obviously enriched by BMD and salinomycin, but suppressed by tylosin and monensin. Such a

differential regulation pattern is interesting, but the reason is currently unknown, although *Bacteroides,* with the ability to degrade non-digestible carbohydrates to produce short-chain fatty acids [56,57], was reported to be enriched by BMD [38]. A lone Proteobacteria (*Escherichia/Shigella* OTU0021) was also enriched by all five AGPs, which is in agreement of earlier reports that a transient upregulation of *Escherichia/Shigella* in response to in-feed antibiotics [53,54].

It is important to note that closely related bacteria within a genus are not necessarily regulated in the same fashion even by the same AGP. For example, multiple clusters of the *Clostridium* genus were differentially regulated. One abundant member of *Butyricicoccus* (OTU0010) was enriched by AGPs, but a less abundant *Butyricicoccus* (OTU0080) was diminished particularly by ionophores. While *Blautia* OTU0016 is upregulated, *Blautia* OTU0017 and OTU0070 are downregulated by AGPs. It is, therefore, difficult to predict the net outcome in the abundance of certain bacterial populations. Future studies to perform absolute quantification of individual bacterial taxa will help provide a more definitive answer to the net change in each bacterial population.

In this study, we demonstrated the differential effects of five different AGPs on the cecal microbiome of broiler chickens. While each treatment displayed certain effect on cecal microbiome composition, the most drastic changes were observed with ionophores. Investigation into the regulation of specific OTUs revealed an enrichment of beneficial bacteria following antimicrobial treatment, particularly in OTUs involved in butyrate synthesis. However, we only examined the cecal microbiota changes two weeks after AGP supplementation. It will be beneficial to investigate the kinetic response of the gut microbiota in response to AGPs, revealing whether certain bacterial populations undergo temporal or persistent alterations. Furthermore, because the small intestine is the major site where most nutrients are digested and absorbed, studying the microbiota changes in the small intestine by AGPs is also warranted.

Although different AGPs appear to shift the structure of gut microbiota in distinct manners, it will be important to examine how the function of gut microbiota is altered by AGPs, which can be evaluated by using a combination of metagenomics, metabolomics, metatranscriptomics, and/or metaproteomics. It is tempting to speculate that, regardless of the antimicrobial spectrum and mode of action, AGPs improve growth performance by enhancing the functional potential of the gut microbiota resulting in more efficient digestion and utilization of dietary carbohydrates and lipids in the gastrointestinal tract.

In summary, our data indicates an ability of AGPs to modulate cecal microbiota to allow an increase in the bacteria associated with improved digestion and energy utilization. A better understanding of the mechanism by which AGPs modulate gut microbiota and enhance growth in livestock animals will lead to the development of effective antibiotic alternatives that mimic the action of AGPs.

Author Contributions: GZ conceived and designed the experiments. KR, WL, QY, and HZ performed the animal trial and isolated microbial DNA. KR, SB, YX, HY, JZ, and GZ analyzed and interpreted the data. KR, SB, and GZ drafted and revised the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Table 5.1. ANOSIM analysis of different antimicrobials on β-diversity of the cecal microbiota**.**

	Control	BMD	Tylosin	Virginiamycin	Monensin	Salinomycyin
Control		*P* = 0.043 R = 0.071	*P* = 0.052 R = 0.061	*P* = 0.036 R = 0.090	*P* < 0.001 R = 0.921	*P* < 0.001 R = 0.809
BMD	*P* = 0.013 [1] R = 0.145		*P* = 0.243 R = 0.021	*P* = 0.043 R = 0.095	*P* < 0.001 R = 0.914	*P* < 0.001 R = 0.774
Tylosin	*P* = 0.009 R = 0.149	*P* = 0.004 R = 0.187		*P* = 0.595 R = 0.015	*P* < 0.001 R = 0.855	*P* < 0.001 R = 0.714
Virginiamycin	*P* = 0.001 R = 0.226	*P* = 0.009 R = 0.164	*P* = 0.197 R = 0.048		*P* < 0.001 R = 0.957	*P* < 0.001 R = 0.832
Monensin	*P* < 0.001 R = 0.315	*P* < 0.001 R = 0.284	*P* < 0.001 R = 0.411	*P* < 0.001 R = 0.401		*P* = 0.021 R = 0.076
Salinomycin	*P* = 0.002 R = 0.163	*P* = 0.003 R = 0.205	*P* < 0.001 R = 0.294	*P* < 0.001 R = 0.292	*P* = 0.029 R = 0.105	

[1] Shaded P-values are for the Bray-Curtis Index, while non-shaded P-values values represent the Jaccard Index.

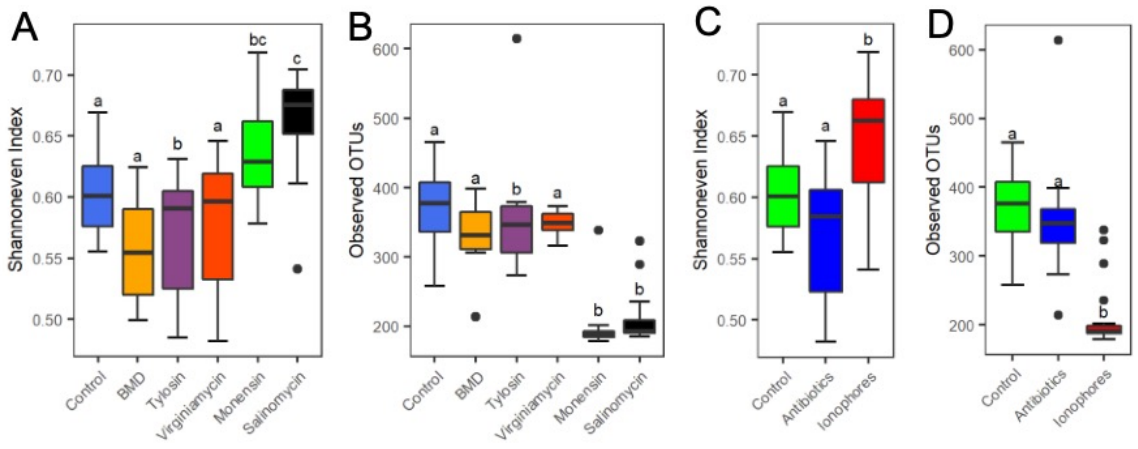

Fig. 5.1. The α-diversity of cecal microbiota of broilers following 2-week supplementation of different antimicrobials. Changes in evenness and richness were calculated from 12 samples of each treatment using the Shannon evenness index (A) and Observed OTUs (B), respectively. Data from three antibiotics and two ionophore groups were further combined separately and the Shannon evenness index (C) and Observed OTUs (D) were recalculated. One-way ANOVA with *post hoc* Tukey's test was performed, with the treatments not sharing a common superscript considered significantly different ($P < 0.05$).

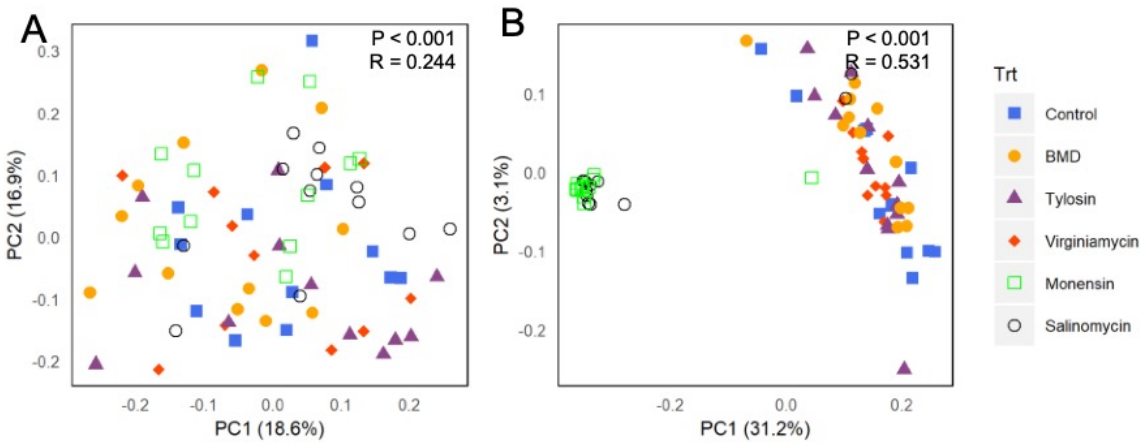

Fig. 5.2. The β-diversity of cecal microbiota of broilers following 2-week supplementation of different antimicrobials. Principal coordinate analysis (PCoA) plots were generated from 12 samples of each treatment using Bray-Curtis (A) and Jaccard indices (B), respectively. Statistical significance and R values were determined using ANOSIM and indicated in each plot.

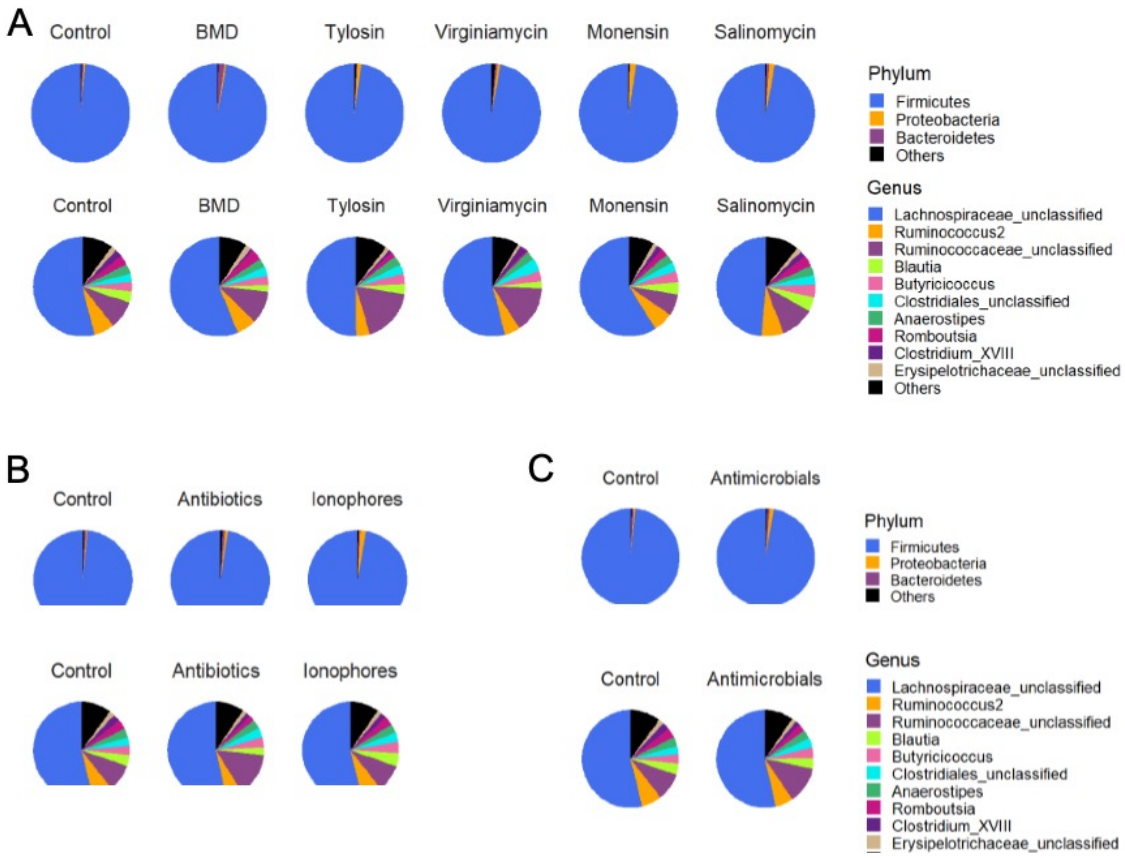

Fig. 5.3. Differences in cecal microbiota composition of broilers following 2-week supplementation of different antimicrobials. Relative abundance of OTUs were calculated and plotted at the phylum and genus (A) levels. Three antibiotics and two ionophore groups were further combined separately and relative abundance of OTUs were recalculated and plotted at the phylum and genus levels (B). All antimicrobial groups were combined and compared with the control group at the phylum and genus levels (C). Only the top three phyla and top 10 genera are shown, with unidentified and lowly abundant bacteria being collectively denoted as "Others".

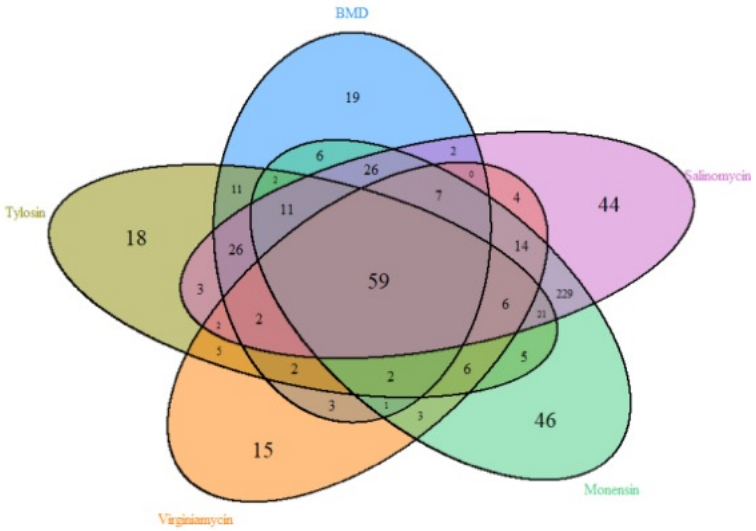

Fig. 5.4. Differential enrichment of OTUs by in-feed antimicrobials. Significant up- or down-regulation of OTUs was determined using Metastats [23], relative to the control group ($P \leq 0.05$). Venn diagram was then used to visualize the distribution of shared OTUs among individual antimicrobials.

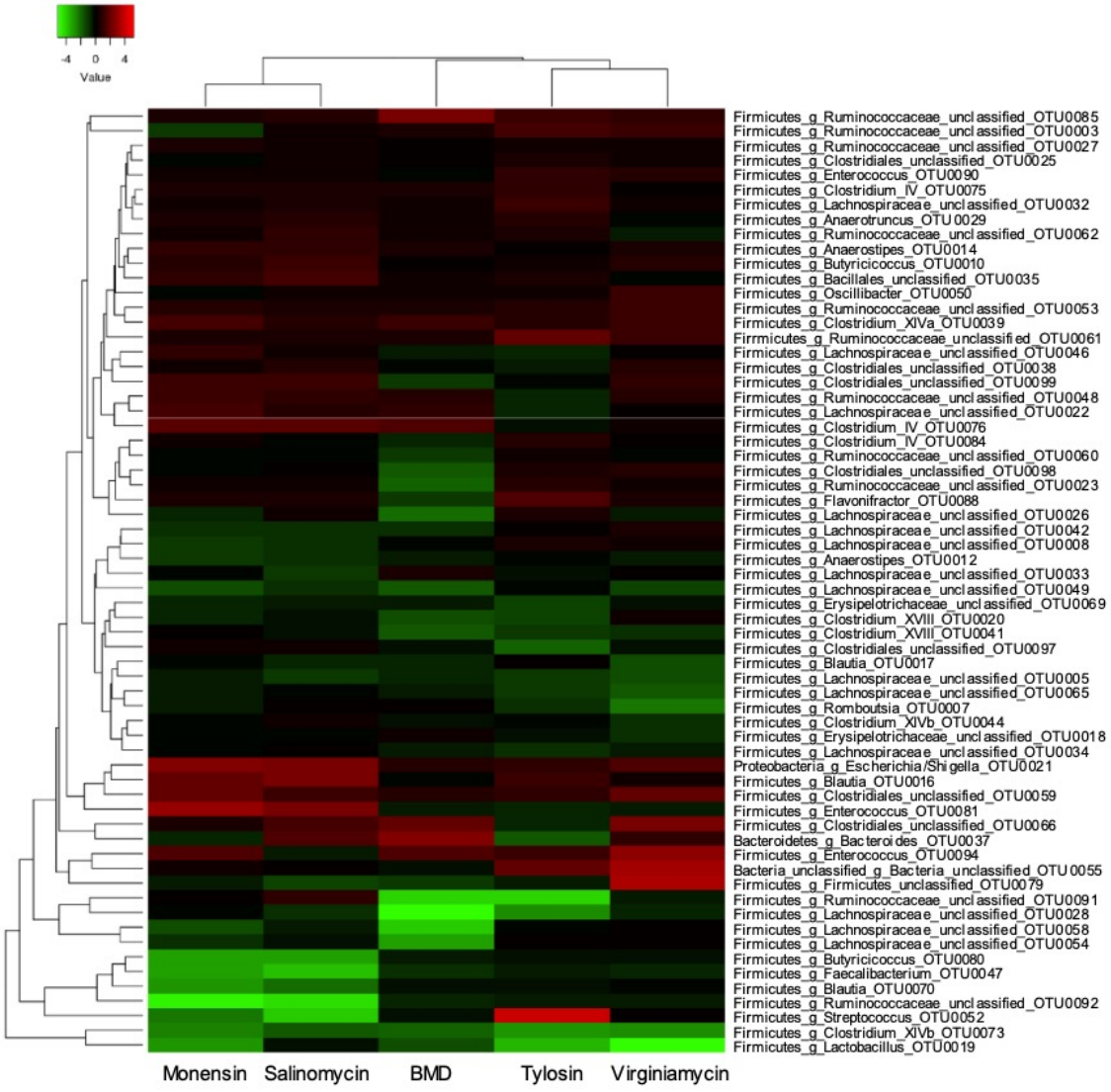

Fig. 5.5. Differential regulation of the top 100 OTUs by in-feed antimicrobials. Among top 100 OTUs, 64 were significantly affected by at least one antimicrobial and thus plotted using Heatmapper [25]. Fold change was calculated as the mean relative abundance of an OTU in an antimicrobial group relative to that in the control, followed by log2 transformation for visualization. Both rows and columns were clustered using the Euclidean distance and average linkage.

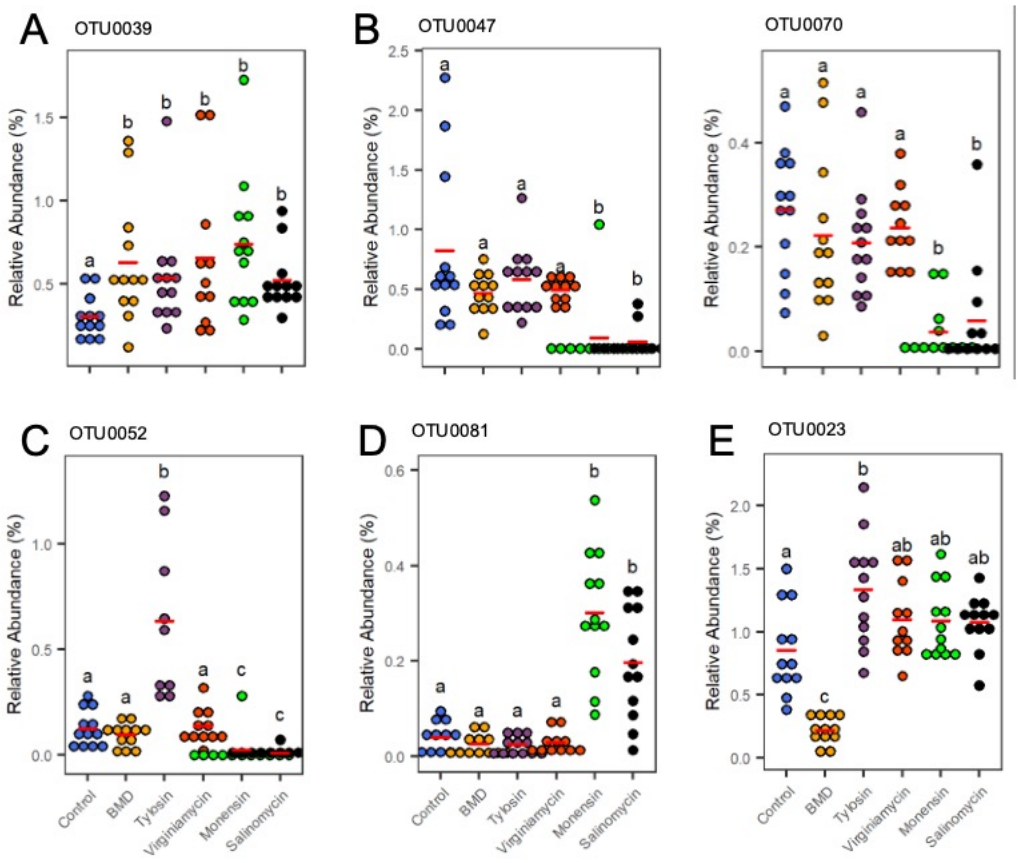

Fig. 6.6. Differential regulation of representative bacteria taxa by in-feed antimicrobials. Each group consists of 12 cecal samples. For OTU0052, three outliers (relative abundance > 3%) were omitted for better visualization of final differences among group. Statistical significance was determined using Metastats [23], with the treatments not sharing a common letter considered significantly different ($P < 0.05$).

CHAPTER VI

CONCLUSION

Biogeographical distribution and succession of the intestinal mycobiota of broiler chickens were successfully characterized throughout an entire production cycle, and the response of the intestinal mycobiota to an in-feed antibiotic was also investigated. The mycobiome was revealed to be relatively stable with the majority of sequences belonging to the phyla Ascomycota and Basidiomycota regardless of GI location or age. The most abundant genera wer *Microascus, Gibberella, Trichosporon, Aspergillus,* and *Candida.* However, the most abundant genera differed between studies. Feature-level analysis revealed a clear succession of the mycobiome through the GI tract as birds aged, while BMD supplementation produced a clear change in cecal composition at 14 days of age.

Evaluation of the effect of five subtherapeutic antibiotics on cecal microbiota composition after 14 days of supplementation revealed a clear modulation of the bacterial community with each treatment resulting in a unique bacterial profile. Of note is the observation that ionophores caused a significant decrease in the number of rare bacterial species resulting in a decrease in microbiota richness and increase in evenness. Investigation into microbes significantly affected by each treatment revealed an overall increase in bacteria associated with improved digestion and energy utilization.

This dissertation has successfully laid the foundation for future work regarding manipulation of the intestinal microbiota as a novel antibiotic-free strategy to disease control and prevention. As the main fungal genera in both studies differed significantly and are known to be associated with feed ingredients and environmental factors, it will be important to determine the source of intestinal fungi. Additionally, the role of intestinal fungi in disease can be elucidated in a chicken disease models such as necrotic enteritis and coccidiosis. It is our expectation that these studies will lead to the identification of microbial species associated with improved growth performance and disease resistance. These will then be further assessed for their potential to be developed as new probiotic supplements for use in livestock and poultry production.

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