ASSOCIATIONS BETWEEN GUT MICROBIOTA COMPOSITION AND PRODUCTION EFFICIENCY OF BROILER CHICKENS

By

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"*I know of no pursuit in which more real and important services can be rendered to any country than by improving its agriculture, its breed of useful animals, and other branches of a husbandman's care.*" – George Washington, 1779

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Abstract: Intestinal microbiota plays a key role in nutrient digestion and utilization with a profound impact on feed efficiency of livestock animals. However, the intestinal microbes that are critically involved in feed efficiency remain elusive. To identify bacteria associated with production efficiency in chickens, male Cobb broiler chicks were individually housed from day 14 to day 35. Individual RFI values were calculated for 56 chickens. Luminal contents were collected from the ileum, cecum, and cloaca of each animal on day 35. Bacterial DNA was isolated and subjected to 16S rRNA gene sequencing. Intestinal microbiota was classified to the feature level using Deblur and QIIME 2. High and low RFI groups were formed by selecting 15 and 17 chickens with the most extreme RFI values for subsequent LEfSe comparison of the difference in the microbiota. Spearman correlation analysis was further performed to identify correlations between the intestinal microbiota composition and RFI of all 56 chickens. No significant difference in evenness, richness, and overall diversity of the microbiota in the ileum, cecum, or cloaca was observed between high and low RFI groups of chickens. However, LEfSe analysis revealed a number of bacterial features being differentially enriched in either high or low RFI chickens. Spearman correlation analysis further indicated many differentially enriched bacterial features were significantly correlated with RFI ($P < 0.05$). Importantly, not all short-chain fatty acid (SCFA) producers showed a positive association with efficiency. While two novel members of *Oscillibacter* and *Butyricicoccus* were more abundant in low-RFI, high-efficiency chickens, several other SCFA producers such as *Subdoligranulum variable* and two related *Peptostreptococcaceae* members were negatively associated with feed efficiency. Moreover, a few closely-related *Lachnospiraceae* family members showed a positive correlation with feed efficiency, while others displayed an opposite relationship. Our results highlight the complexity of the intestinal microbiota and a need to differentiate bacteria to the species, subspecies, and even strain levels in order to reveal their true association with feed efficiency. Identification of RFI-associated bacteria provides possibilities to manipulate the intestinal microbiota for improving production efficiency, profitability, and sustainability of poultry production.

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CHAPTER I

REVIEW OF THE LITERATURE

1. Introduction

1.1. *Antimicrobial Growth Promotors in Animal Agriculture*

The practice of adding antibiotic growth promotors (AGPs) to livestock feed and drinking water was adopted in the late 1940s and early 1950s, following reports that sub-therapeutic concentrations of antibiotics could improve the productivity of broiler chickens (Moore et al., 1946), turkeys (McGinnis et al., 1951), and swine (Jukes et al., 1950; Brown et al., 1952). AGPs were cost-effective tools for improving weight gain, feed efficiency, herd health, and animal welfare (CAST, 1981; Kirchelle, 2018; Karavolias et al., 2018). The advent of AGPs helped facilitate conventional agriculture's transition from individual smallholder operations to the large, often vertically-integrated concentrated animal feeding operations (CAFOs) of today (Krishnasamy et al., 2015; Laxminarayan et al., 2015).

In the late 1960s and early 1970s, however, agricultural AGP use was linked to the emergence of antimicrobial resistant (AMR) pathogens. The rise of AMR infections in humans, as well as increased consumer demand for animal protein produced without antibiotics, drove many Western governments to limit or outlaw AGP use. In 2006, the European Union implemented a total ban on the non-therapeutic use of antimicrobials in food animals, restricting use for therapeutic purposes in confirmed instances of disease (European Parliament, 2005). Likewise, in 2015, the United States expanded the Veterinary Feed Directive to preclude non-

therapeutic use of "medically relevant" antibiotics (i.e., those used in to treat illness in humans) (FDA, 2015).

Advances in nutrition, genetics, and animal management have allowed producers in high-income countries to maintain the performance gains of the past 70 years while reducing AGP consumption (Sneeringer et al., 2015). Since the early 2000s, AGPs have primarily been used for disease control and prevention on CAFOs, or during particularly stressful times such as transportation or weaning (Cromwell, 2002). Nevertheless, the growing demand for affordable animal protein in lower- and middle-income countries (Van Boeckel et al., 2015), as well as ongoing concerns about livestock acting as a reservoir for AMR genes (Köck et al., 2017), illustrates the need for alternatives to AGPs for improving herd health and production efficiency.

1.2. *Intestinal Microbiome and Its Impact on Animal Physiology and Performance*

In their seminal 1946 paper, Moore et al. proposed that AGPs improve animal performance by inhibiting pathogen colonization and reducing bacterial competition with the host for nutrients in the host gut. Coates et al. (1955; 1963) lent credence to this idea by demonstrating that oral antibiotics do not have growth promoting effects in germ-free chicks. Further studies in humans (Dethlefsen et al., 2008), mice (Cho et al., 2012), swine (Kim et al., 2012), poultry (Gaucher et al., 2015), and cattle (Grønvold et al., 2011) confirmed that exposure to oral antibiotics early in life has a significant impact on the host gut microbial community and host phenotype.

The complex community of bacteria, fungi, viruses, and protists in the gastrointestinal (GI) tract is known collectively as the intestinal microbiome. The intestinal microbiome composition and diversity influence feed utilization (Stanley et al., 2012a; Stanley et al., 2013a), fat and muscle deposition (Turnbaugh et al., 2009), and disease resistance (Curtis and Sperandio, 2011). Commensal bacteria that colonize the surfaces of the rumen and intestines regulate intestinal

epithelial cell turnover rates, encouraging epithelial apoptosis, compensatory proliferation, lengthening of the microvilli to increase absorptive surface area, and induction of brush border enzyme activities for nutrient digestion (Malmuthuge et al., 2015a; Yu et al., 2012). Anaerobes in the cecum and colon ferment complex, host-indigestible carbohydrates and proteins, and produce short-chain fatty acids (SCFAs), proteogenic amino acids, vitamin K and B-complex vitamins, and other essential nutrients for the host (Portune et al., 2016; LeBlanc et al., 2013).

The three primary SCFAs in the GI tract are acetate, proprionate, and butyrate. SCFAs are the primary source of energy for intestinal and ruminal epithelial cells, meeting 20-30% of the caloric requirement for swine and up to 80% of the caloric requirement for ruminants (Bugaut et al., 1987; Bergman, 1990). Despite being the least abundant of the three, butyrate is preferentially taken up by colonic epithelial cells (Guilloteau et al., 2010). When added to feed, butyrate is associated with heightened ileal villi, higher numbers of mucin-producing goblet cells, increased antioxidant capacity, reduced inflammation, and decreased malondialdehyde (MDA, a marker of oxidative stress) levels in the intestine (Wu et al., 2018). Because it is so influential on rumen and gut physiological development in young animals, butyrate has unsurprisingly been found to increase daily gain and feed intake in poultry, weanling piglets, weanling calves, and suckling lambs (Czerwiński et al., 2012; Huang et al., 2015; McCurdy et al., 2019; Liu et al., 2019).

The presence of commensal microorganisms on the epithelial surface and in the luminal content induces production of $Foxp3^+$ regulatory T-cells (T_{regs}), which secrete anti-inflammatory cytokines and suppress pro-inflammatory T-helper-1 (Th1) and Th17 cells (Round and Mazmanian, 2010). Though the exact mechanism behind this is unclear, it is thought that blunting the pro-inflammatory response during establishment of the gut microbiome teaches the gut immune system to tolerate commensal organisms, preventing chronic inflammation and damage

to the intestines (MacDonald et al., 2011; Belkaid and Hand, 2014). Delayed exposure to commensal microbes results in permanently altered immune profiles with a bias towards the pro-inflammatory response (Hansen et al., 2012). In addition to directly modulating the host immune system, commensal organisms may minimize or prevent colonization of pathogens by competitive exclusion (Curtis and Sperandio, 2011).

Disruptions to the gut microbiome, such as sudden changes in diet (Kogut, 2019), stress (Lu et al., 2003; Yang et al., 2011), enteric infection (Stanley et al., 2012b), and long-term antibiotic use can lead to an unstable and imbalanced microbial state, known as "dysbiosis". Dysbiosis in turn is linked to poor efficiency, health, and welfare outcomes in livestock (Hermann-Bank et al., 2015).

1.3. *Biogeography and Succession of Gut and Rumen Microbes*

At birth, human and animals are virtually germ-free, having almost no contact with microorganisms in the sterile environment of the maternal uterus or egg. At birth, the neonate is immediately exposed to the microflora of the dam and/or the surrounding environment; these microbial species form the initial inoculum that colonize the gastrointestinal (GI) tract of the animal (Dominguez-Bello et al., 2010). In mammals, the introduction of easily-absorbed colostrum and milk to the GI tract, as well as continued exposure to the dam and/or environment, will introduce new microbes and result in the rapid expansion and diversification of the microbiome. During weaning and the introduction of solid feed, the microbiome composition undergoes a dramatic shift to accommodate the new substrate; once weaning is complete, a mature, stable, adult-like microbial community takes hold.

The adult gut microbiome contains an estimated 3.8×10^{13} bacterial cells (Sender et al., 2016), representing over 1,000 unique species (Qin et al., 2010). A review by Gaggìa et al. (2010)

describes the major microbial groups associated with monogastric animals as *Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Lactobacillus*, *Enterobacteriaceae, Streptococcus, Fusobacterium, Peptostreptococcus*, and *Propionibacterium*. In ruminants, these were *Fibrobacter, Ruminococcus, Butyrivibrio, Bacteroides, Prevotella, Selenomonas, Streptococcus, Lactobacillus,* and *Megasphaera.* Establishing a healthy, stable, resilient gut microbiome is paramount to ensuring that the animal performs to its genetic potential.

1.4. *Defining Production Efficiency in Meat Animals*

Production efficiency is extremely important to the modern livestock industry, as feed may account for 60 – 80% of total production costs (Willems et al., 2019). In meat animals, efficiency is described as "a function of gain in body weight and feed consumed" (Koch et al., 1963), and is generally expressed in terms of average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), and residual feed intake (RFI).

ADG is the rate of weight gain per day over a given period of time, while ADFI is the rate of feed consumption per day over a given period of time. An efficient animal should have a high ADG and low ADFI. FCR is defined by the MacLeod et al. (2013) as the rate at which "an animal converts feed into tissue, usually expressed in terms of kg of feed per kg of output", or the ratio of feed to gain, with lower FCR values indicating higher efficiency. In the US and Canada, typical FCRs range from 1.8 – 2.0 for broiler chickens, 2.73 – 3.5 for swine, 5.0 – 7.0 for finishing lambs, and 4.5 – 7.5 for finishing beef cattle (MacLeod et al., 2013; Wand, 2014; Shike, 2013).

RFI is defined by Sell-Kubiak et al. (2017) as "the difference between the measured intake and the expected feed intake of an animal [accounting] for its maintenance requirement". Expected feed intake is calculated by taking the average of ADFI and ADG of a group of animals; RFI is

then calculated by subtracting the expected intake from the actual intake. Like FCR, a lower RFI value indicates higher efficiency.

2. Analysis of the Microbiome

2.1. *16S rRNA Gene Sequencing*

Environmental microbiologists estimate that less than 1% of bacteria can be cultured in the lab (Amann et al., 1995). Molecular methods, such polymerase chain reaction (PCR) amplification of bacterial small subunit ribosomal RNA (SSU rRNA) genes, have allowed culture-independent studies of large, diverse microbial communities from environmental samples (Su et al., 2012).

Tracking changes in bacterial communities is typically done by 16s SSU rRNA gene sequencing. Woese et al. (1980) pioneered the use of the 16s rRNA gene for microbial census and phylogenetic studies. The 16s rRNA gene is approximately 1500 bp in length (Janda and Abbott, 2007) and encodes a portion of the 30S small subunit of the bacterial ribosome (Woese et al., 1980). Its slow, steady rate of evolution and ubiquity in bacteria and archaea (Gray et al., 1984; Olsen et al., 1986) make the 16s rRNA gene a reliable marker for classification and phylogeny (Woese et al., 1987). Though mostly conserved, it has nine "hypervariable" regions (V1 – V9) that can be targeted with universal primers for informatics purposes (Weisburg et al., 1991; James, 2010).

Early microbiome studies used Sanger sequencing technology to sequence the whole 16s gene. However, the process of generating and assembling reads was inefficient, expensive, and time-consuming. Sanger sequencing was ultimately superseded by high-thoroughput next-generation sequencing (NGS) methods such as Roche 454 pyrosequencing and, later, Illumina "sequencing by synthesis" flowcell technology (J. Johnson et al., 2019). NGS outputs a high volume of short (300-500 bp) reads, allowing for high resolution and deeper sampling depth at a

fraction of the cost of traditional Sanger sequencing (Liu et al., 2007; Caporaso et al., 2011). Though some high-thoroughput methods for full-length 16S sequencing exist (Burke and Darling, 2016; J. Johnson et al., 2019), most contemporary microbiome studies target one or more of the hypervariable regions for amplification and sequencing (Hamady and Knight, 2009).

The V3 (approx. 180-200 bp) and V4 (approx. 250 bp) regions are frequently used in microbiome studies. Kozich et al. (2013) amplified the V4, V3-V4, and V4-V5 regions and found that V4 had the lowest error rate (0.01%), followed by V3-V4 (0.10-0.21%) and V4-V5 (0.36-0.64%). Youssef et al. (2009) reported that species richness estimates generated by V4, V5-V6, and V6-V7 fragments were comparable to the richness estimate of the full-length fragment, while the V3 fragment underestimated species richness. Yang et al. (2016) agreed, stating that the V4, V5, and V6 regions were the most reliable for representing full-length sequences. Chakravorty et al. (2007), on the other hand, found that the V2 and V3 regions were better suited to distinguishing between closely related species than the more conserved V4, V5, and V7 regions. Muyzer et al. (1993), Huse et al. (2008), Gloor et al. (2010), and Bartram et al. (2011) have all reported that the V3 region's conserved flanking regions, high resolution, and length made it an excellent candidate for use in taxonomic studies.

2.2. *Metagenomics, Metatranscriptomics, Metaproteomics, and Metabolomics*

16S sequencing can only determine which taxa are present in a sample, and in what abundance; it cannot explain the larger role of the bacteria in the environment, or how that role changes in certain contexts. The emerging fields of metagenomics, metatranscriptomics, metaproteomics, and metabolomics attempt to fill this knowledge gap by probing the collection of bacterial genomes, RNA transcripts, proteins, and metabolites, respectively.

Metagenomics allows isolation and analysis of DNA taken directly from the environment, providing access to the whole genomic profiles of an entire populations of bacteria. Whole genome shotgun sequencing (WGSS) and computational tools for assembling, binning, classifying, and annotating DNA reveals the genetic repertoire and physiology of novel, often uncultivable bacteria (Handelsman, 2004). In livestock, metagenomics has revealed upregulation of key microbial metabolic pathways in response to AGP or phytochemical supplementation (Huang et al., 2018), as well as competitive and cooperative dynamics between gut microbes (Richards et al., 2020). Additionally, functional analysis of the metagenome can reveal differential abundance of genes for antibiotic resistance in the microbiome of AGP-treated animals (Xiong et al., 2018) or for metabolism, stress, and virulence in that of high and low-performing animals (Singh et al., 2014).

Metatranscriptomics goes a step further, analyzing the collection of RNA transcripts in a sample. The transcriptome consists of messenger RNA (mRNA), transfer RNA (tRNA), rRNA, and non-coding RNA molecules (ncRNA). The advent of microarray technology in the mid-2000s allowed researchers to study the expression profiles of thousands of genes at a time, thereby allowing direct comparisons between genomes and environments.

They did not, however, allow for discovery of novel transcripts, nor the absolute quantification of transcripts (Morozova et al., 2009). RNA sequencing (RNA-Seq) technology came about in the early 2010s, addressing the limitations of the microarray and providing improved coverage and depth for a more comprehensive picture of the transcriptome and natural variations within it (Wickramasinghe et al., 2014). Metatranscriptomics is typically used to identify the biological processes underpinning disease pathology (Bashiardes et al., 2016) or performance (Horodyska et al., 2019) of the host. However, it may also be applied to the host gut microbiota;

Johnson et al. (2017), for example, analyzed the swine fecal transcriptome to better understand the influence of the antibiotic carbadox on the gut microbiome.

Metaproteomics goes further still, using chromatography and mass spectrometry to study the collection of proteins produced and used by the host or gut microbiota (Morgan and Huttenhower, 2014). Metaproteomics allows for large-scale identification and quantification of proteins from molecular information, and provides insight into community structure and *in situ* carbon sources (Kleiner, 2019). Tröscher-Muβotter et al. (2019) published a functional analysis of the porcine GI proteome, revealing differential enrichment of proteins related to production, conversion, transport, and metabolism between luminal and mucosal microbiota and between GI segments. Proteomics may also be used to identify disease-specific biomarkers or novel therapeutic targets, as done by Erickson et al. (2012) in a study of twin pairs with Chron's disease.

Likewise, metabolomics uses chromatography and mass spectrometry to study the collection of metabolites and other low molecular weight molecules produced and utilized by the host or gut microbiota (Clish, 2015). Metabolomics has provided valuable insight into bacterial metabolism and cross-feeding behaviors (De Vuyst and Leroy, 2011), the impact of enteric infection and antibiotic treatment on host systemic energy metabolism (Le Roy et al., 2019), and the impact of subtherapeutic antibiotic and ionophore use on gut microbiota and host metabolism (T. Johnson et al., 2019).

2.3. *Bioinformatics Pipelines*

As advances in sequencing technology produced exponentially larger and more complex data sets, a need developed for computational tools that could efficiently pair, trim, align, cluster, classify, and statistically analyze sequencing reads. The two most popular tools for processing and analyzing microbial 16s data are mothur (Schloss et al., 2009; Kozich et al., 2013) and Qualitative

Insights into Microbial Ecology, or QIIME (Caporaso et al., 2010; Bolyen et al., 2019). The two differ in their default alignment, clustering, and classification algorithms; however, differences in relative abundance and diversity output between the two appear to be linked to the reference database used, rather than the software itself (López-García et al., 2018).

Clustering has traditionally been done on the basis of sequence identity, by binning sequencing reads that differ by less than a fixed, arbitrary dissimilarity threshold into "operational taxonomic units" (OTUs). Generally, sequences of 97% identity were assigned to the same species, 95% identity to the same genus, and 80% identity to the same phylum (Schloss and Handelsman, 2005). The OTU-based approach is used by the mothur program and is still widely accepted; however, new methods now allow differentiation between closely related taxa by clustering based on single nucleotide differences (Callahan et al., 2017). QIIME 2 pipelines such as DADA2 and Deblur generate these highly-resolved bins, referred to as amplicon sequence variants (ASVs) (Callahan et al., 2016; Amir et al., 2017). Taxonomic assignment is then performed on the OTUs or ASVs by comparing the experimental sequence with known sequences in a published database, such as SILVA (Quast et al., 2013), GreenGenes (DeSantis et al., 2006), and the Ribosomal Database Project (RDP) (Wang et al., 2007).

Additional databases exist for meta'omics data. Metagenomic information may be run against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and KEGG Orthology (KO) databases to reveal the physiology and function of uncultured organisms based on molecular data (Kanehisa et al., 2016). Characterization of purified transcript, protein, and metabolite samples may be done against in-house or commercial libraries; the National Center for Biotechnology Information (NCBI) also maintains open-source transcript, protein, and metabolite databases.

3. Microbiome of Ruminants

3.1. *Biogeography and Succession*

In ruminants, bacteria account for anywhere from 40% to 90% of the ruminal microbial population, at a density of 10^8-10^{11} colony forming units (CFU)/g content (Nagaraja, 2016). A meta-analysis of the RDP database by M. Kim et al. (2011) found 5,271 different bacterial and 3,516 different archaeal OTUs associated with the bovine rumen. An individual animal may host anywhere from 300 to 1000 unique OTUs in their rumen, depending on the composition of the diet (Edwards et al., 2004; Kong et al., 2010; Yeoman and White, 2014). Different microbial populations inhabit the solid particulates, liquid fraction, and epithelial lining of the rumen (Cho et al., 2006; Jewell et al., 2015).

The "bacterial baptism" hypothesis suggests that, during vaginal birth, a neonate is seeded by the maternal microbiome (Stinson et al., 2018). A study by Alipour et al. (2018) found that the intestinal microbiome of perinatal calves consists of low abundances of Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes, resembling the oral microbiome of the dam. After 24 hours, *Escherichia/Shigella* and Clostridiales emerge as the predominant taxa, but by the end of the first week, they are superseded by *Faecalibacterium, Bacteroides, Lactobacillus, Butyricicoccus*, and *Bifidobacterium*. As the calf ages, richness and evenness increases, and ruminal microbiome begins to stabilize (Klein-Jöbstl et al., 2014). A pattern of decreasing facultative anaerobes and increasing obligate anaerobes has also been observed in lambs (Fonty et al., 1987).

The "core" microbiome of the mature rumen consists of *Prevotella, Butyrivibrio*, and *Ruminococcus*, as well as a number of unclassified *Lachnospiraceae, Ruminococcaceae*, Bacteroidales, and Clostridiales. The small and large intestines, on the other hand, are dominated by Firmicutes (de Oliveira et al., 2013; Malmuthuge et al., 2014) These taxa appear in different

proportions among individuals, but are nevertheless found in the majority of ruminant species around the world (Jami et al., 2012; Henderson et al., 2015; Xue et al., 2018). In cattle, host genetics appear to have a stronger influence on gut microbiome composition and regulation than in other livestock species (Gonzalez-Recio et al., 2018). Once established, the individual rumen microbiome is highly resilient and host-specific, resisting colonization by foreign microbes and responding almost exclusively to changes in diet (Weimer, 2015).

3.2. *Role in Digestion*

The two dominant phyla in the mature bovine rumen are Firmicutes and Bacteroidetes. Firmicutes are more abundant in cattle fed a hay-based diet, whereas Bacteroidetes are more abundant in grain-based diets (Clemmons et al., 2019). As cattle are adapted from a hay-based to grain-based diet for finishing, the rumen microbial population shifts substantially. Microbes associated with fermentation of roughage (e.g. *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*) steadily decrease in abundance; likewise, microbes associated with fermentation of grain (e.g. *Megasphaera elsdenii*, *Selenomonas ruminantium, Prevotella bryantii*) rapidly increase in abundance (Tajima et al., 2001; Fernando et al., 2010). Many of the highly abundant carbohydrate-fermenting bacteria also ferment proteins (*Prevotella* spp*., B. fibrisolvens, Streptococcus bovis, S. ruminantium, M. elsdenii*) and lipids (*B. fibrisolvens, S. ruminantium*) (Nagaraja, 2016).

The proportion of grain to roughage, or ruminally-available carbohydrates to complex dietary fiber, is especially important in feedlot and dairy cattle. High grain diets are also associated with higher ruminal abundance of *Acetitomaculum, Lactobacillus, Prevotella*, and *Streptococcus*. *Lactobacillus* and *Streptococcus* produce lactic acid as a by-product of carbohydrate fermentation, and thrive in low pH, sugar-rich environments (Petri et al., 2013). Excessive lactic acid production

lowers the rumen pH and eventually causes lactic acidosis, a syndrome characterized by dehydration, damage to the ruminal and intestinal walls, decreased ruminal motility, increased ruminal osmotic pressure, decreased cardiac output, decreased blood pH, decreased renal blood flow, laminitis, liver abscesses, and, in severe cases, shock and death (Nocek, 1997; Owens et al., 1998). Supplementing grain-fed cattle with an adequate amount of roughage – or with ionophores, such as monensin and lasolocid – reduces the population of lactic acid bacteria, in turn reducing lactic acid concentrations and raising rumen pH (Nagaraja et al., 1985).

While bacteria are by far the most dominant and best-understood component of the ruminal microbiota, fungi also exert an enormous influence on rumen function and bovine performance. Mycobiome research has been hindered by the fastidious growth conditions of ruminal and intestinal fungi, as well as uncertain methods of genomic DNA isolation, variations in length of the ITS regions, and limited reference databases (Paterson et al., 2017). As a result, most fungal research has focused on opportunistic pathogens and their role in disease (Mukherjee et al., 2015; Huseyin et al., 2017).

However, recent studies have demonstrated that anaerobic ruminal fungal (phylum Neocallimastigomycota) may improve fiber digestibility and nutrient digestion while reducing methane production, thereby improving the production performance and efficiency of cattle (Puniya et al., 2015). Transcriptomics approaches have revealed that members of Neocallimastigomycota express an extensive array of transcripts coding for carbohydrate active enzymes (Gruninger et al., 2018; Comtet-Marre et al., 2018), making them exceptional plant biomass degraders and key contributors to ruminant metabolism and functional capacity (Y. Wang et al., 2019) of grass- or hay-fed cattle. In addition to their enzymatic activity, these fungi physically disrupt plant cell walls via penetrating rhizoids, facilitating bacterial metabolic activity

and improving the solubility and therefore host-accessibility of the substrate (Zebeli and Metzler-Zebeli, 2012).

3.3. *Role in Physiological and Immunological Development*

Calves are immunodeficient at birth, as no placental transfer of immunoglobulins (Igs) occurs *in utero*; they are thus reliant on passive transfer of maternal Igs, namely IgG, from colostrum until their own immune system develops (Godden, 2008). Adequate consumption of high-quality, pathogen-free colostrum is critical to the long-term health, welfare, and productivity of the calf, as it provides both maternal Igs and the initial inoculum of intestinal bacteria. Malmuthuge et al. (2015b) found that heat-treatment of colostrum before feeding increases the abundance of beneficial *Bifidobacterium* in the small intestine, and decreases the abundance of *Escherichia coli*, relative to calves receiving fresh colostrum.

Colonization by enteric pathogens is linked to lower overall microbial diversity and higher incidence of diarrhea; colonization by *Bifidobacterium* and *Faecalibacterium*, on the other hand, is linked to greater overall diversity, weight gain, and lower incidence of disease (Oikonomou et al., 2013). Colonization of the ruminal and intestinal mucosal surfaces by commensal microbiota is also believed to promote postnatal maturation of the mucosa-associated lymphoid tissues (MALT), including Peyer's patches, the site of T-cell and B-cell activation (Reynolds and Morris, 1984; Malmuthuge et al., 2015a). During weaning, pattern recognition receptors (e.g. Toll-like receptors [TLRs]) responsible for bacterial detection are downregulated as memory T-cells (e.g. $CD4^+$, $CD8^+$) increase in number, marking a shift from innate to adaptive immune responses (Malmuthuge et al., 2012; Fries et al., 2011).

Fermentation mostly occurs in the hindgut until the development of a functional rumen at 3-4 weeks of age in sheep (Oh et al., 1972) and 3-6 weeks of age in cattle (Kehoe et al., 2007).

Calves are gradually weaned from milk or milk replacer, slowly incorporating larger amounts of grain and roughage in the form of a calf starter ration or creep feed (Diao et al., 2019), though abrupt weaning does not appear to significantly alter the gut microbiome (Meale et al., 2016). Exposure to roughage and reduction of milk triggers a shift from a rumen dominated by Bacteroidetes to one dominated by Firmicutes (Meale et al., 2016). Butyrate, produced by Gram-positive Firmicutes as a by-product of polysaccharide fermentation, stimulates enzymatic activity, ruminoreticulum growth, and papillae lengthening, improving nutrient absorption and utilization by the calf (Górka et al., 2011).

4. Microbiome of Swine

4.1. *Biogeography and Succession*

In swine, microbial succession is determined by age, introduction of solid feed, and weaning, rather than breed or the dam. In the first hours and days post-partum, the GI tract of piglets is dominated by members of the families *Clostridiaceae, Enterobacteriaceae*, *Streptococcaceae*, and *Fusobacteriaceae*, presumably from exposure to colostrum and to the skin and feces of the sow. By the end of the first week, however, *Lactobacillaceae* takes over and remains the predominant family up until weaning, after which *Ruminococcaceae, Lachnospiraceae*, and *Prevotellaceae* take over (Konstantinov et al., 2006; Petri et al., 2010; Bian et al., 2016).

The gut microbiome undergoes its most rapid and expansive changes during weaning, which occurs at approximately 2-3 weeks of age. Abrupt separation from the sow and littermates, transfer to a new environment with unfamiliar pen mates, and change from liquid milk to solid feed induces stress, anorexia, malaise, intestinal inflammation, and susceptibility to enteric pathogens, consequently leading to temporary declines in feed intake and performance (Pié et al.,

2004; Lallès et al., 2007; McLamb et al., 2013). Pajarillo et al. (2014) compared fecal samples taken from piglets pre- and post-weaning, reporting a weaning trend towards increased bacterial diversity and a distinct shift from *Bacteroides* to *Prevotella* as the most abundant genus observed. A meta-analysis by Holman et al. (2017) found a distinct "core" microbiome in adult swine, consisting of *Prevotella, Clostridum, Alloprevotella*, and *Ruminococcus* in fecal samples, as well as *Clostridium, Blautia, Lactobacillus, Ruminococcus, Roseburia*, and *Subdoligranulum* in GI samples.

4.2. *Role in Digestion*

Diet composition significantly influences the gut microbiome composition of monogastric animals. Consumption of milk and creep feed encourages the proliferation of Firmicutes, especially Lactobacillales. Carbohydrate fermentation leads to the production of lactic acid, which lowers the intestinal pH and discourages the growth of *Enterobacteriaceae* (Rinttilä and Apajalahti, 2013). Fermentation also establishes a hypoxic environment favorable to the colonization of *Bifidobacterium* and *Bacteroides* (Bäckhed et al., 2015). In nursing piglets, weight gain is highly correlated with the amount of colostrum and milk consumed. Morissette et al. (2017) found that gut microbial composition of two-week old piglets was strongly associated with body weight, and is therefore likely influenced by colostrum and milk consumption. Higher body weight piglets had higher proportions of Bacteroidetes, *Bacteroides*, and *Ruminococcaceae,* and lower proportions of *Actinobacilus porcinus* and *Lactobacillus amylovorus* than lower body weight piglets.

Unlike calves, piglets are abruptly weaned and switched to solid feed. This sudden dietary shift, as well as environmental, social, and psychological stressors, causes major disruption and instability in the gut microbiome. By day 10 post-weaning, however, the gut microbiome stabilizes

and begins to resemble that of mature swine (Chen et al., 2017). On the phylum level, Fusobacteria and Proteobacteria give way to Firmicutes and Bacteroidetes; on the genus level, increases in *Blautia, Paraprevotella, Oscillibacter*, *Clostridium* XIVa, *Roseburia, Clostridium* sensu stricto, and *Prevotella* are matched by decreases in *Megasphaera, Eschierchia/Shigella, Bacteroides, Fusobacterium,* and *Lactobacillus* (Chen et al., 2017). Post-weaning pigs fed a low-fat, high-fiber diet had higher fecal abundances of lactobacilli, bifidobacteria, and *F. prausnitzii*, as well as higher SCFA concentrations; while those fed a high-fat, low-fiber diet had higher fecal abundances of *Enterobacteriaceae* (Heinritz et al., 2016).

4.3. *Role in Physiological and Immunological Development*

Like calves, piglets are born with an immature immune system, and rely on passive transfer of maternal Igs through colostrum for immune support. Bioactive compounds in porcine colostrum stimulate intestinal mucosal proliferation, gut closure, and growth and maturation of GI tissues (Xu et al., 2002; Everaert et al., 2017). *Prevotella* is positively associated with luminal secretory IgA concentrations and body weight in post-weaning pigs (García et al., 2016). Other commensal bacteria such as *Bacillus amyloliquefaciens* promote gut integrity, induce anti-inflammatory responses, and competitively exclude pathogens from the surface of the intestines (Hu et al., 2018; Liao and Nyachoti, 2017). García et al. (2016) found that weaning time (14 d vs 21 d) does not significantly impact microbiome composition or plasma IgG concentration; though earlier weaning favored earlier synthesis of intestinal IgA, later weaning favored villi length and increased numbers of goblet cells and lymphocytes.

Early colonization by pathogens can cause disease and poor performance. The colonic microbiome of suckling piglets suffering from neonatal porcine diarrhea (NPD) consists of lower proportions of Firmicutes and Actinobacteria and higher proportions of Proteobacteria and

Fusobacteria relative to healthy piglets; *E. coli* and *Enterococcus* were highly abundant, whereas normal commensals such as *Lactobacillus acidophilus* were diminished. (Hermann-Bank et al., 2008). Probiotics containing protective commensals may be fed to suckling and weanling pigs to competitively inhibit pathogens and produce butyrate during carbohydrate fermentation. While butyrate does not appear to promote growth or efficiency in piglets (Biagi et al., 2007), it has been shown to significantly increase serum IgG and jejunal IgA and significantly decrease the incidence of diarrhea, tempering the adverse effects of weaning stress by providing immune support (Fang et al., 2014).

5. Microbiome of Poultry

5.1. *Biogeography and Succession*

Conventionally-raised poultry are thought to have widely-varying colonization of their GI tracts in part due to the sanitation practices of modern commercial hatcheries. Eggs are collected and washed or fumigated prior to placement in a sanitized hatching environment, eliminating contact with pathogens but also parental microflora. Instead, newly-hatched chicks are exposed to non-avian bacterial sources, including human handlers, bedding material, transport containers, feed, and water (Stanley et al., 2013b; Stanley et al., 2014).

Nevertheless, some trends have emerged over the years. According to Apajalahti et al. (2004), bacterial densities on day of hatch reach 10^8 and 10^{10} CFU/g digesta in the ileum and cecum, respectively. By day 3 or 4, the density will plateau at 10^8-10^9 CFU/g in the ileum, and 10^{11}-10^{12} CFU/g in the cecum and cloaca (Yadav and Jha, 2019); however, the proportions at the phylum, class, family, and genus levels will continue to shift until approximately 2-3 weeks of age, after which the composition will remain relatively stable for the duration of the animal's productive life (Ballou et al., 2016; Ranjitkar et al., 2016).

At the phylum level, Firmicutes is predominant (70%) along the whole GI tract, followed by Bacteroidetes (12.3%) and Proteobacteria (9.3%) (Waite and Taylor, 2014; Choi et al., 2015; Feye et al., 2020). Lu et al. (2003) found that the class *Lactobacillaceae* dominates the ileum, followed by the classes *Clostridiaceae, Streptococcaceae*, and *Enterococcaceae;* the cecum, on the other hand, is dominated by *Clostridiaceae,* followed by *Actinobacteria, Lactobacillaceae*, and *Bacteroidaceae*. The nutrient-dense, hypoxic conditions of the jejunum and ileum facilitate the growth of facultative anaerobes, including *Lactobacillus*, *Enterococcus*, and *Streptococcus*; while the anoxic environment of the distal cecum facilitates polysaccharide fermentation and SCFA production by the obligate anaerobes of the order Clostridiales (including *Lachnospiraceae* and *Ruminococcaceae*) (Apajalahti and Vienola, 2016).

5.2. *Role in Digestion*

Feed is the most important determinant of gut microbiome composition in poultry. Starter diets for broiler chicks consist of carbohydrate-rich mash or crumble, which encourages the growth of lactic acid bacteria. The lactic acid by-product of carbohydrate fermentation lowers the pH of the duodenum and jejunum, discouraging the growth of Proteobacteria (Rinttilä and Apajalahti, 2013).

Lactobacilli are found throughout the intestines, as their β-glucanase and bile salt hydrolase (BSH) activity are important for non-starch polysaccharide (NSP) and lipid metabolism, respectively (Torok et al., 2008). Clostridiales, however, is the dominant order of the cecum and colon. Sergeant et al. (2014) analyzed the cecal metagenome and found genes encoding several carbohydrate fermentation pathways leading to the production of SCFAs, as well as genes for poly- and oligosaccharide degradation. Clostridiales members are particularly effective at degrading starch and cellulose found in plant material; *Bacteroides, Prevotella, Parabacteroides*, and

Alistipes (members of Bacteroidetes) have also been associated with carbohydrate and SCFA production (Stanley et al., 2013a).

5.3. *Role in Physiological and Immunological Development*

In chickens, the acquired immune system doesn't mature until the end of the first week of life, forcing the newly-hatched chick to rely on its innate defenses, including the gut microbiome (Bar-Shira and Friedman, 2006). The presence of commensal microbes is linked to higher goblet cell density and increased MUC2 expression, promoting secretion of protective mucus (Broom and Kogut, 2018); as well as B-cell activation and proliferation, which in turn leads to mucosal immunoglobulin A (IgA) secretion and activation of T-cells (Lex and Azizi, 2017). Commensal organisms also provide protection from pathogens via competitive exclusion (Nisbet, 2002), and appear to improve the efficacy of vaccines by through low-grade stimulation the gut immune system (Nothaft et al., 2017; Redweik et al., 2020)

6. Association of the Intestinal Microbiome with Production Performance

6.1. *Association with Average Daily Gain and Average Daily Feed Intake in Ruminants*

Body weight and fat deposition has been repeatedly linked to the Firmicutes-to-Bacteroidetes ratio. Generally, higher proportions of Bacteroidetes lead to weight loss, while higher proportions of Firmicutes lead to weight gain (Ley et al., 2006).

Myer et al. (2017) conducted a meta-analysis that split the efficiency of beef cattle into four phenotypes: greater ADG/lower ADFI (most efficient), greater ADG/greater ADFI, lower ADG/lower ADFI, and lower ADG/greater ADFI (least efficient). For ruminal populations, they found that *Butyrivibrio* and *Leucobacter* were enriched in the greater ADG/lower ADFI group. *Prevotella, Lactobacillus, Blautia*, *Coprobacillus, Dorea, Clostridium, Parabacteroides*, and *Faecalibacterium* were also generally associated with better efficiency.

The Myer et al. meta-analysis is supported by other studies performed in dairy cattle. Meale et al. (2016) found *Bacteroides* to be negatively associated with ADG and ADFI, and *Prevotella, Ruminococcus, Succinovibrio*, and *Sharpea* to be positively associated with ADG and, (in all but *Prevotella*) ADFI. Likewise, Oikonomou et al. (2013) reported that *F. prausnitzii*, a butyrate-producing member of Firmicutes, is significantly associated with ADG in suckling calves.

6.2. *Association with Feed Conversion Ratio and Residual Feed Intake in Ruminants*

The ruminal microflora has some association with FCR and RFI, but their effects appear to be heavily modulated by the diet (Carberry et al., 2012). Cattle on grain-based diets have lower bacterial and enzymatic diversity, but are generally more efficient, while cattle on roughage-based diets have higher bacterial and enzymatic diversity, but are less efficient (Li and Guan, 2017). Just as the ratio of grain to roughage alters the ratio of Firmicutes to Bacteroides, the ratio of Firmicutes and Bacteroides in the Holstein rumen significantly alters milk yield and milk composition. *Ruminococcus, Lachnospiraceae*, and *Eubacterium coprostanoligenes* are positively associated with milk yield and milk-fat, while *Prevotella*, are negatively associated with yield and milk-fat (Tong et al., 2018; Jami et al., 2014). Coriobacterales, *Mitsuokella*, and *Desulfovibrio* are positively associated with milk lactose content (Jami et al., 2014).

Jewell et al. (2015) sampled ruminal solid and fluid content, and found that increased efficiency was associated with *Coprococcus*, RF39 (of the phylum Mollicutes), and *Succinivibrionaceae*, while decreased efficiency was associated with *Anaerovibrio*, *Bacteroidales*, *Butyrivibrio*, CF231 and YRC22 (of the family *Paraprevotellaceae*), *Clostridia*, *Pseudobutyrivibrio*, and *Ruminococcaceae*. Multiple OTUs within the families Clostridiales, *Lachnospiraceae, Prevotellaceae*, and *Succinovibrionaceae* were variably associated with efficiency.

6.3. *Association with Average Daily Gain and Average Daily Feed Intake in Nonruminants*

The Firmicutes-to-Bacteroidetes ratio holds true for non-ruminants, particularly in the growing and finishing stages. In swine, ADG and ADFI are associated with a high proportion of Firmicutes and a low proportion of Bacteroidetes along the GI tract (Ban-Tokuda et al., 2017). At lower taxa levels, *Prevotella* spp. is associated with increased ADFI, while SCFA-producing bacteria (e.g. *Ruminococcaceae*) are associated with decreased ADFI (Yang et al., 2018). The effect of *Lactobacillus* varies by species and strain; though Yang et al. found *Lactobacillus* to reduce appetite and ADFI, Chiang et al. (2015) reported that freeze-dried *Lactobacillus johnsonii* and *Lactobacillus mucosae* increased ADG, ADFI, and overall feed efficiency in weaned piglets.

In growing poultry, Gammaproteobacteria such as *Acinetobacter* and *Escherichia/Shigella* are associated with higher ADFI, as is *Turicibacter* of phylum Firmicutes. *Lactobacillus,* on the other hand, is associated with lower ADFI (Siegerstetter et al., 2018a). Wen et al. (2019) found that the abundance of the archaeon genus *Methanobrevibacter* in the cecum was positively associated with abdominal fat content in broiler chickens, but did not significantly affect body weight; the bacteria *Mucispirillum schaedleri*, on the other hand, was negatively associated with both body weight and abdominal fat content. Ranjitkar et al. (2016) found that an increased abundance of *Lactobacillus salivarius* and Clostridiales in the ileum results in lower abundance of *Lactobacillus reuteri* and a higher abundance of deconjugated bile acids. Deconjugation of bile acids removes their detergent properties, preventing emulsification of fat and depressing growth (Harrow et al., 2007).

6.4. *Association with Feed Conversion Ratio and Residual Feed Intake in Nonruminants*

Tan et al. (2018) reported that FCR is associated with different microbes in different segments of the GI tract in swine. High-efficiency (low FCR) Landrace gilts had significantly

higher abundances of *Prevotella, Campylobacter*, and *Spaerochaeta* in the duodenum; *Sanguibacter* in the jejunum; *Kingella* and SMB53 (an unclassified member of Clostridiaceae) in the ileum; *Campylobacter* and *Butyricoccus* in the cecum; and *Coprobacillus* and *Lactococcus* in the colon. Likewise, low-efficiency (high FCR) gilts had higher abundances of *Anaeroplasma*, *Arthrobacter*, and *Megasphaera* in the ileum; *Rhodoplanes, Megasphaera*, and *Mitsuokella* in the cecum; and *Peptococcus* in the colon. In contrast, an earlier study by the same group found *Lactobacillus* to be associated with low FCR, and *Prevotella* with high FCR (Tan et al., 2015).

Higher abundance of *Lactobacillus* spp. has been linked to both lower RFI and lower gene expression of pro-inflammatory cytokines in the colon of swine following LPS challenge (Vigors et al., 2016). Yang et al. (2017) found that OTUs related to dietary polysaccharide metabolism, such as *Ruminococcaceae, Christensenellaceae, Lachnospiraceae*, and *Akkermansia*, showed a positive tendency towards association with feed efficiency (low RFI). *Prevotella* and *Faecalibacterium*, on the other hand, were associated with high RFI.

In broilers, Clostridiales is linked to lower FCR while lactobacilli – especially *L. salivarius* – is linked to higher ADFI and, consequently, higher FCR. (Torok et al., 2011; Stanley et al., 2016). Stanley et al. also reported the families *Lachnospiraceae, Ruminococcaceae*, and *Erysipelotrichaceae* and genera *Ruminococcus* and *Faecalibacterium* were highly abundant in highly efficient birds.

7. Modulation of the Gut Microbiome

7.1. *Theory*

AGPs are believed to promote animal health and production efficiency by modulating the gut microflora. AGPs treat subclinical infections and reduce pathogen load (Allen and Stanton, 2014), reduce competition between the host and microbiota for nutrients (Apajalahti and Vienola,

2016), and reduce production of harmful metabolic by-products (Grant et al., 2018). AGPs also select for commensals that stimulate the host immune system (Belkaid and Hand, 2014); competitively exclude pathogens (Ma et al., 2018); efficiently break down nutrients to a form that can be used by the host (Liao and Nyachoti, 2017); or produce metabolites that are known to improve performance by meeting a nutritional need or enhancing gut immunity and absorptive capacity (Robinson et al., 2019; Gadde et. al, 2018).

However, AGPs no longer a legal or viable option for many producers, and some veterinary antibiotics (e.g. tylosin) have demonstrated potential to select for resistance to related medically-important antibiotics (e.g. vancomycin) (Aarestrup, 2000; Allen et al., 2013). Attention has therefore turned to other methods by which to establish a healthy, robust, production-enhancing gut microbiome.

7.2. *Current Practices*

Beneficial bacteria are encouraged to grow and flourish in the GI tract by supplementing animal feed and drinking water with probiotics, prebiotics, and phytochemicals. The Food and Agriculture Organization (FAO) and World Health Organization (WHO) (2002) define direct-fed microbials (DFMs) or "probiotics" as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Probiotics often include *Lactobacillus*, *Bifidobacterium*, *Bacillus,* and yeasts such as *Saccharomyces* (Bajaj, 2014; Angelakis, 2017). Commercial probiotic mixes are particularly popular among poultry and swine producers, and especially for use in younger, more vulnerable animals (Nisbet, 2002; Hu et al., 2018). They've also been shown to improve gain, efficiency, and disease resistance in veal calves (Timmerman et al., 2005), but are mostly ineffective in adult ruminants due to the resilience and host-specificity of the established rumen microbiome (Weimar, 2015).

Probiotics can effectively overcome production declines due to heat stress in layer hens (Deng et al., 2012), but not in broilers (Song et al., 2014); nevertheless, a meta-analysis by Faria Filho et al. (2006) found that supplementing drinking water with probiotics improved body weight gain and reduced FCR in broilers. Other broiler nutrition studies demonstrate the efficacy of probiotics at improving weight gain and feed efficiency (Jin et al., 1998; Salim et al., 2013), fat digestibility and nitrogen retention (Tortuero, 1973), increasing ileal villus height (Salim et al., 2013), resistance to and resilience against *Salmonella* infections (Higgins et al., 2007; Redweik et al., 2020), and decreased cecal loads of foodborne pathogens such as *E. coli* and *Salmonella* (Tortuero, 1973; Knap et al., 2011; Salim et al., 2013).

Prebiotics are defined as "non-digestible food ingredients that can be utilized by intestinal microflora, which beneficially affects the host" (Gibson and Roberfroid, 1995). They generally include inulin, lactulose, and fructo-, galacto-, manno-, xylo-, soya-, and isomalto-oligosaccharides (Huyghebaert et al., 2011; Sugiharto, 2016), which provide a substrate for favorable commensals (Ramirez-Farias et al., 2009) and an alternative binding site for *E. coli* and *Salmonella* that facilitate their elimination in digesta flow (Parks et al., 2001; Fernandez et al., 2002). Like probiotics, prebiotics have been shown to improve weight gain, efficiency, absorptive capacity, and immune function while decreasing stress and cecal pathogen loads in broilers (G. Kim et. al, 2011; Sohail et al., 2012). Alizadeh et. al (2015) found that adding galacto-oligosaccharides to formula milk increased fecal lactobacilli, bifidobacteria, and butyrate concentration; improved intestinal architecture; and reduced the incidence of diarrhea in piglets.

Phytochemicals are compounds produced by plants for protection against predators, pathogens, and competitors (Molyneux et al., 2007). Phytochemicals found in herbs, spices, and other edible plants are a staple of traditional human medicine and are being explored as feed

additives for livestock. Samanta et al. (2015) found that dried, powdered ginger root (*Zingiber officinale*) both increased the abundance of beneficial organisms and while decreasing the abundance of pathogens in fecal samples from grower pigs.

Essential oils are gaining popularity among swine and poultry producers for their antimicrobial, anti-inflammatory, anti-oxidative, and coccidostatic effects (Omonijo et al., 2018). Components of essential oils – especially carvacrol (found in oregano, thyme, and bergamot) and cinnemaldehyde (found in cinnamon) – are reported to inhibit *Salmonella, E. coli, S. aureus*, and *Listeria monocytogenes* growth *in vitro* (Hulánková and Bořilová, 2011) and *in vivo* for lactating sows (Tan et al., 2015) and broilers (Amerah et al., 2012). Tan et al. also noted improvements in ADFI for carvacrol-supplemented sows and ADG for their piglets, which they attribute to reduced oxidative stress. This is supported by Hashemipour et al. (2013), who reported reduced lipid oxidation in the muscles of broilers. For poultry, essential oil blends may also act as coccidiostats; they've been shown to destroy *Eimeria* oocysts *in vitro* (Remmal et al., 2011), improve immune response against *Eimeria* following avian coccidiosis vaccination (Lee et al., 2011), and prevent dramatic microbiome shifts after *Eimeria* challenge *in vivo* (Oviedo-Rondón et al., 2006).

7.3. *Future Directions*

In 2013, van Nood et al. announced the success of their fecal microbiota transplant (FMT) study, with 81% of human patients seeing resolution of their *Clostridum difficile* infection following a single FMT infusion. FMT is now a widely-accepted treatment for human *C. difficile* infections, and is being explored as a therapeutic for other nosocomial infections, ulcerative colitis (UC), Crohn's disease, irritable bowel syndrome (IBS), and neuro-psychiatric disorders (Pettigrew et al., 2016; Smits et al., 2013; Moayyedi et al., 2015; Wang et al., 2014).

Human and mice studies demonstrate that the proportion of Firmicutes to Bacteroidetes in the gut is linked to obese and lean phenotypes (Turnbaugh et al., 2009), and mice studies further demonstrate that FMT from an obese or lean donor will induce the donor phenotype in the germ-free recipient (Cox et al., 2014; Lai et al., 2018). Although FMT from high-performing adult donors has been shown to improve gut health and immune responses in piglets (Niederwerder et al., 2018; Teng et al., 2020), and promotes establishment of protective commensal microorganisms *in ovo* for poultry (Pedrosa et al., 2015), it does not appear to influence growth or production efficiency in broilers (Siegerstetter et al., 2018b). Both Le Roy et al. (2018) and Rodriguez et al. (2019) found that diet and environment have a stronger influence on performance than exposure to donor microbes.

Nevertheless, monitoring and targeted modulation of the gut microbiome still holds promise for improving animal productivity. Maltecca et al. (2019) recently demonstrated that machine learning algorithms such as Random Forest can predict growth and carcass traits of growing and finishing swine from microbiome data. In cattle, deep nasopharyngeal swabs accurately predict infection of the lower respiratory tract by *Mannheimia haemolytica* and *Mycoplasma bovis*, providing a quicker, safer, less stressful method of testing and monitoring for bovine respiratory disease (BVD) (Godinho et al., 2007). Though cecal and fecal microbial evenness differ significantly in poultry, they have similar richness, suggesting that fecal sampling may be used to monitor for some shifts in the cecal microbiota (Stanley et al., 2015)

8. Conclusion

The gut and ruminal microbiomes are complex, dynamic communities with tremendous influence on health, welfare, and performance outcomes in livestock. We now understand that modulation of the gut microbiome in early life can, for better or for worse, induce long-lasting

changes to the core microbiome and to host phenotype. Early establishment of a healthy, diverse commensal population ensures proper development of the host gut and gut immunity, reduces the incidence and severity of enteric infection, aids in efficient digestion and absorption of nutrients, and minimizes performance declines during weaning and transportation.

Advances in sequencing and computational tools have expanded our understanding of the gut microbiome composition, function, and relationship with the host. Not only does long-term antibiotic use provide selective pressure that induce the emergence of AMR, it may also cause irreparable injury to the host gut microbiome, killing off beneficial commensals and allowing potentially dangerous pathogens to proliferate unchecked. We know that feed and environment are strong determinants of microbiome composition, and realize that early intervention with prebiotics, probiotics, and phytochemicals encourage the establishment of a healthy gut microbiome, as well as promote its resilience and stability – thereby fostering animal health and productivity while reducing reliance on antibiotics.

However, further research is needed to identify specific bacterial species and strains associated with performance in poultry, and the mechanisms by which they do so. Previous correlation studies in poultry have yielded inconsistent results, even under controlled conditions (Stanley et al., 2016). In addition, we've only recently developed computational tools that can resolve differences in 16s rRNA sequences down to a single nucleotide difference. Whereas previous microbial censuses have relied on OTUs, this study of the mature broiler gut microbiome uses ASVs to resolve differences between closely-related species, giving us a more comprehensive picture of which taxa are present and in what proportions. We hope that, by successfully identifying taxa associated with ADG, ADFI, FCR, and RFI, we may come one step closer to developing newer, more potent products for promoting animal health and production efficiency.

CHAPTER II

ASSOCIATION BETWEEN THE INTESTINAL MICROBIOTA AND PRODUCTION EFFICIENCY OF BROILER CHICKENS

1. Introduction

Feed accounts for up to 70% of costs in broiler production (Willems et al., 2019). Maximizing feed efficiency is paramount to ensuring the sustainability of the industry. In livestock production, feed efficiency is generally measured by feed conversion ratio (FCR) or residual feed intake (RFI). FCR is defined as the ratio of feed intake to weight gain, with lower FCR values indicating high efficiency. On the other hand, RFI is defined as the difference between the actual measured feed intake and the expected feed intake of an animal accounting for its maintenance requirement, where expected feed intake is calculated based on average feed intake and weight gain of a group of animals (Herd and Arthur, 2009; Aggrey and Rekaya, 2013). Similar to FCR, a lower RFI value indicates higher efficiency. However, unlike FCR, RFI is independent of body weight, mature size, and growth rate and thus has become a method of choice for measuring feed efficiency (Aggrey and Rekaya, 2013; Aggrey et al., 2010).

The intestinal microbiota is known to play a key role in feed digestion, nutrient absorption, vitamin synthesis, and immune development (Lallès, 2016; Durack and Lynch, 2019; Wang et al., 2019; Yadav and Jha, 2019). Manipulation of the intestinal microbiota could potentially enhance animal health and feed efficiency (Yadav and Jha, 2019). Relative to that of other livestock species, the chicken intestinal microbiota has a much higher proportion of Firmicutes to Bacteroidetes (Wei

et al., 2013). Lactobacilli are predominant in the small intestine, while clostridia are abundantly colonized in the cecum of chickens (Yadav and Jah, 2019; Wei et al., 2013). As major producers of short-chain fatty acids (SCFAs), clostridia are represented by a large, diverse group of obligate anaerobic Firmicutes (Guo et al., 2020; Lopetuso et al., 2013). Several clostridial families such as Clostridiaceae, Ruminococcaceae, and Lachnospiraceae are generally regarded to improve feed efficiency through SCFA production (Yang et al., 2017).

Microbiome studies of broiler chickens have thus far revealed a high degree of inter-flock variation, with diet, environment, management, breed, age, and sex exerting a significant influence on the composition and function of the intestinal microbiome (Stanley et al., 2013b; Stanley et al., 2014; Diaz Carrasco et al., 2019). Several studies have attempted to identify the intestinal microbes associated with RFI in both broiler and layer chickens (Yan et al., 2017; Siegerstetter et al., 2017; Siegerstetter et al., 2018a; Siegerstetter et al., 2018b; Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b). However, the findings thus far have been inconsistent and sometimes contradictory. No specific bacterial taxa have been reproducibly identified across multiple studies. Even the same dietary composition and experimental design being duplicated at two different locations have resulted in different outcomes (Siegerstetter et al., 2017).

Moreover, all aforementioned studies have classified bacteria to the level of genus of operational taxonomic unit (OTU), which arbitrarily combines all sequencing reads that share ≥ 97% identity as a single OTU (Blaxter et al., 2005). In those studies, only differentially enriched bacterial genera or OTUs were identified (Yan et al., 2017; Siegerstetter et al., 2017; Siegerstetter et al., 2018a; Siegerstetter et al., 2018b; Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b). A need, therefore, exists to clarify the discrepancies among these studies and potentially further identify specific RFI-associated microbes to the species or subspecies level. Deblur, a newly-

developed bioinformatic tool, separates rather than combines closely-related bacterial taxa even with a single nucleotide difference (Amir et al., 2017). Each unique sequence is referred to as an amplicon sequence variant (ASV) or a 'feature'. It is, therefore, now possible to accurately define the microbiota composition and compare them among studies (Amir et al., 2017; Callahan et al., 2017).

In this study, to identify bacterial features that are associated with feed efficiency, we housed broilers individually, calculated their RFI values, analyzed the compositions of the microbiome in the ileum, cecum, and cloaca separately using Deblur, and further compared them between the broilers with extremely high and low RFI values. As a result, a number of bacterial features were found to be differentially enriched between high and low RFI broilers in each of the three intestinal locations. We were able to separate closely-related bacteria from each other and we found, in several cases, both positive and negative associations with feed efficiency among them, highlighting a need to differentiate phylogenetically related bacteria from each other to reveal their true involvement in nutrient digestion and nutrient utilization and possibly other physiological processes.

2. Materials and Methods

2.1. Animal Trial and Sample Collection

All animal procedures were approved by the Institutional Animal Care and Use Committee of Oklahoma State University under protocol number AG-17-3. Day-of-hatch male Cobb broiler chicks were obtained from Cobb-Vantress Hatchery (Siloam Springs, AR). Upon arrival, chicks were individually weighed and apparently healthy animals of similar body weight (BW) (40 ± 2 g) were retained. Animals were tagged with wing bands and group-housed on an open floor with fresh pinewood shavings for bedding. Chickens were provided *ad libitum* access to tap water and

non-medicated, three-stage, corn-soybean diets formulated to meet the NRC requirements (**Table 1**). Animals were housed in an environmentally-controlled room with temperatures starting at 33°C and decreasing 3°C every 7 days. The light-to-dark ratio (h) 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. On day 14, chickens were individually weighed, and 72 healthy animals were transferred to individual floor cages with fresh pinewood bedding. From day 14 to day 35, individual body weight and feed intake were recorded weekly.

On day 35, 56 apparently healthy chickens were euthanized by CO_2 asphyxiation, followed by cervical dislocation. Approximately 0.5 g of luminal contents from the ileum, cecum, and cloaca of each bird were collected and flash frozen in liquid nitrogen. The samples were stored at -80ºC until further processing. Feed efficiency of individual animals was calculated as RFI = TFI – (a_1 + b_1 * MMW + b_2 * TBWG), where TFI is total feed intake, a_1 is the intercept, b_1 and b_2 are partial regression coefficients of mid-test metabolic weight (MMW), and TBWG is total body weight gain. MMW was calculated as $[(D7\ BW + D35\ BW)/2]^{0.75}$ as described by Metzler-Zebeli et al. (2016).

2.2. Bacterial DNA Extraction and Sequencing

Intestinal bacterial DNA was isolated from each luminal content sample using ZR Fecal 96-well DNA Isolation Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. DNA quality and quantity were determined using Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and the absence of degradation was confirmed using agarose gel electrophoresis. High quality DNA was shipped on dry ice to Novogene (Beijing, China) for PE250 deep sequencing of the 16S rRNA gene using the V3-V4 primers (341F: CCTAYGGGRBGCASCAG and 806R: GGACTACNNGGGTATCTAAT) on an Illumina HiSeq

platform. PCR amplification and library preparation were performed by Novogene (Beijing, China) using NEBNext® Ultra™ Library Prep kit (New England Biolabs, Ipswich, MA).

2.3. Bioinformatic and Statistical Analyses

Illumina paired-end reads were analyzed in QIIME 2 2019.7 (Boylen et al., 2019). Briefly, primers were removed from each sequencing read with the cut-adapt plugin (v. 2.10) (Martin, 2011). After quality filtering, reads were denoised with the Deblur algorithm (v. 2020.2.0). The resulting ASVs were then classified into bacterial features using the RDP 16S rRNA training set (v. 16) and Bayesian classifier (Wang et al., 2007). 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". Features appearing in less than 5% of samples were removed from downstream analysis. Data were normalized using cumulative sum scaling (CSS) in the metagenomeSeq package of R (v. 3.6.3) (Paulson et al., 2013).

Analysis and visualization of the microbiota composition were conducted in R (v. 3.6.3) (R Core Team, 2020). The α- and β-diversity analyses were calculated with the phyloseq package (v. 1.28.0), while plots were made using ggplot2 (v. 3.3.0).. The α-diversity was calculated using number of features, Shannon Index, and Pielou's Evenness Index. The β-diversity was calculated using Bray-Curtis and Jaccard indices. Significance in β-diversity was determined using non-parametric permutational multivariate analysis of variance (PERMANOVA) via the adonis function in the vegan package (v. 2.5-6) (Oksanen et al., 2019).

Differential enrichment of bacterial features between high and low RFI chickens was determined using linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011), with the all-against-all multiclass analysis and a logarithmic LDA threshold of 2.0. Spearman correlation analysis was performed on all bacterial features in the ileum, cecum, and cloaca of all

56 chickens using the psych package (v. 1.9.12.31). Associations were considered significant if $P \leq 0.05$ and $|R| \geq 0.3$. To minimize type I error, rare bacterial features with average relative abundances of < 0.01% in an intestinal segment were excluded in both LEfSe and Spearman correlation analyses.

BLAST search of the GenBank database was further conducted to reveal the identified of those features showing differential enrichment or significant correlations with RFI. Multiple sequence alignment was conducted and sequence percent identities were revealed using Clustal Omega at https://www.ebi.ac.uk/Tools/msa/clustalo/.

2.4 Data Deposition

The raw sequencing reads of this study have been deposited in the NCBI GenBank SRA database under the accession number PRJNA647670.

3. Results

3.1. Production Parameters

A total of 56 apparently healthy chickens were retained on day 35 for calculation of RFI and collection of the intestinal contents. These chickens displayed a large variation of feed efficiency with the RFI values ranging from -379.9 to 483.1 (**Fig. 1A**). We selected 15 and 17 chickens with the most extreme RFI values were selected to form 'high' and 'low' RFI groups, respectively, for comparison (**Fig. 1A**). These two groups of chickens indeed showed a significant difference in RFI ($P < 0.001$) with an average RFI value of 166.5 and -141.7 for high and low groups, respectively (**Fig. 1B**). As expected, there was no difference in day-35 BW (**Fig. 1C**) or average daily gain (**Fig. 1D**). Significant differences in average daily feed intake (ADFI) ($P = 0.047$) (**Fig. 1E)** and feed conversation ratio (FCR) ($P < 0.001$) (**Fig. 1F)** were observed between the high and low RFI groups.

3.2. Diversity of the Intestinal Microbiome

A total of 166 luminal content samples of the day-35 ileum, cecum, and cloaca were subjected to 16S rRNA gene sequencing. Following quality control, 11,027,919 high-quality sequence reads were obtained with an average of 66,433 sequences per sample. Sequences were further denoised by Deblur, and the reads present in less than 5% of samples were removed, resulting in the identification of a total of 551 bacterial features. The α-diversity of the intestinal microbiota was calculated using observed features, Pielou's Evenness Index, and Shannon Index as indications of richness, evenness, and overall diversity, respectively. No significant difference was observed in the ileum, cecum, or cloaca with any of these three indices between high- and low RFI chickens (**Fig. 2**). The β-diversity was further calculated using the Bray-Curtis and Jaccard indices as indications of dissimilarity in overall diversity and richness, respectively. However, no significant separation between high and low RFI groups was observed in any of the intestinal segments (**Fig. 3**).

3.3. Composition of the Intestinal Microbiome

The compositions of the microbiota were apparently different among the ileum, cecum, and cloaca. At the genus level, the ileal microbiota was dominated by *Lactobacillus, Romboutsia, Enterococcus*, and *Turicibacter* (**Fig. 4A**), while *Lactobacillus, Faecalibacterium,* and an unidentified genus in each of the *Lachnospiraceae* and *Ruminococcaceae* families dominated the cecal microbiota (**Fig. 4B**). On the other hand, the four most predominant genera of the cloacal microbiota included *Lactobacillus, Romboutsia, Enterococcus*, and an unidentified genus of the *Lachnospiraceae* family (**Fig. 4C**). However, statistical analysis of the top 15 most abundant genera in each of the ileum, cecum and cloaca revealed no significant difference (FDR > 0.05), although *Subdoligranulum* (P = 0.020), *Anaerostipes* (P = 0.027), an unidentified genus of

Peptostreptococcaceae (P = 0.013), and an unidentified genus of *Ruminococcaceae* (P = 0.030) tended to be more abundant in the ileum of high RFI chickens (**Table 2**).

At the feature level, four bacterial features including a member of *Lactobacillus* F1*, Romboutsia* F2*, Enterococcus* F3, and *Turicibacter* F4 accounted for more than 80% of the bacterial population in the ileum **(Fig. 4D**), while the microbiota was much more diverse in the cecum with top 30 features totaling less than 70% of the bacteria (**Fig. 4E**). In the cloaca, the four most abundant features, i.e., *Lactobacillus* F1*, Romboutsia* F2*, Enterococcus* F3, and *Turicibacter* F4, accounted for less than 45% bacteria, with top 30 features totaling more than 80% (**Fig. 4F**). Statistical analysis of the top 30 features revealed no significant difference (FDR > 0.05) in any of the ileum, cecum, or cloaca between high- and low-RFI chickens, while *Subdoligranulum* F6 (P = 0.003), *Anaerostipes* F17 (P = 0.015), and two unidentified features of *Peptostreptococcaceae* F15 (P = 0.018) and F43 (P = 0.009) tended to be more abundant in the ileum of high RFI chickens (**Table 3**). While there was no tendency for any feature to show differential enrichment in the cecum of high RFI chickens, an unidentified *Lachnospiraceae* feature F33 tended to be more abundant (P = 0.002) in the cloaca of high RFI chickens. No feature in the cecum showed no difference (P > 0.05) between high and low RFI chickens (**Table 3**).

3.4. Differential Enrichment of the Intestinal Microbiome

LEfSe analysis was employed to identify differential enrichment of bacterial features between high and low RFI chickens using a LDA score of 2.0 as the threshold. In the ileum, two unidentified *Peptostreptococcaceae* features F15 and F43 as well as S*ubdoligranulum* F6 were enriched in the high RFI group, although no bacteria were found to be enriched in low RFI chickens (**Fig. 5A)**. Kruskal-Wallis test confirmed statistical significances (P < 0.05) with all three features in the ileum (**Fig. 5B**). It is noted that F15 and F43 are highly related, differing by only one

nucleotide along 403 nucleotides in the V3-V4 region of the 16S rRNA gene. A BLAST search of the GenBank database revealed that F15 and F43 shared an approximately 98% identity to *Clostridium difficile*, *Intestinibacter bartlettii* (formerly known as *Clostridium bartlettii*), and *Romboutsia ilealis*, all of which belong to *Clostridium* cluster XI (Gerritsen et al., 2017). A BLAST search of *Subdoligranulum* F6 confirmed it to be 100% identical to *S. variabile*, a *Ruminococcaceae* family member (*Clostridium* cluster IV) initially reported in human feces (Holmstrøm et al., 2004).

In the cecum, an unidentified *Lachnospiraceae* F116 (96.5% identical to *Blautia hominis* or *B. marasmi*) and *Oscillibacter* F220 (97.3% identical to *O. valericigenes*) were enriched in the low RFI group, while an unidentified *Faecalicoccus* F195 (93.6% identical to *F. acidoformans*) and an unidentified *Lachnospiraceae* F92 were enriched in the high RFI group (**Fig. 6A and 6B**). In the cloaca, among a total of 12 differentially enriched features, an unidentified *Lachnospiraceae* F76 and *Butyricicoccus* F149 (97.5% identical to *B. faecihominis*) were more abundant in the low RFI group, while an unidentified *Peptostreptococcaceae* F15, unidentified *Lachnospiraceae* F33, and *Blautia* F42 (98.3% identical to *B. obeum*) were preferentially present in the high RFI group (**Fig. 7A and 7B**).

3.5. Correlation between the Intestinal Microbiome and RFI

To further reveal the correlations between RFI and relative abundances of all bacterial features in the ileal, cecal, and cloacal samples of all 54 chickens, Spearman correlation analysis was performed. A total of 6 features showed a significant positive correlation ($P < 0.05$) with RFI in the ileum (**Fig. 8A**), with a R value ranging from 0.3 to 0.42 (**Fig. 8B**), albeit with no bacteria showing a negative correlation. In the cecum, two features were significantly negatively correlated with RFI ($P < 0.05$), with another four showing significant positive correlation ($P < 0.05$) (**Fig.**

9A). The |R| values of these features ranged from 0.31 to 0.45 (**Fig. 9B**). In the cloaca, a total of six features were found to be significantly associated with RFI ($P < 0.05$), with one displaying a negative correlation and five showing a positive correlation (**Fig. 10A**). Among the most strongly associated features were an unidentified *Peptostreptococcaceae* F15 ($P = 0.009$, $R = 0.34$) and *Subdoligranulum* F6 ($P = 0.002$, $R = 0.40$) in the ileum (**Fig. 8B**); an identified Firmicutes F254 ($P < 0.001$, $R = 0.45$) and an unidentified *Lachnospiraceae* F92 ($P = 0.008$, $R = 0.35$) in the cecum (**Fig. 9B**); and *Blautia* F42 ($P < 0.001$, $R = 0.45$) and two unidentified *Lachnospiraceae* F33 ($P = 0.001$, $R = 0.43$) and F203 ($P = 0.007$, $R = -0.36$) in the cloaca (**Fig. 10B**).

It is noteworthy that Lachnospiraceae F33, F76, and F92 are closely related and a BLAST search revealed that all three are 100% identical to a number of uncultured and uncharacterized poultry intestinal microbes in GenBank (data not shown). They showed 96-97% identity to *M. faecis, M. lactaris,* or *M. torques* in the *Mediterraneibacter* genus (Togo et al., 2018).

Because of the availability of individual FCR, ADG, and ADFI values, we also performed Spearman correlation analysis between each phenotype and the intestinal microbiota profiles. As expected, most of the bacterial features that were correlated with RFI were also similarly correlated with FCR, but not with ADG or ADFI, in the ileum, cecum, and cloaca (**Fig. 8A, 9A, 10A**). Apparently, a different group of bacteria were commonly associated with ADG and ADFI in each of the three intestinal locations.

4. Discussion

Identifying feed efficiency-related intestinal microbes are critically important in reducing feed costs and thus maintaining the profitability and sustainability of livestock production. Metzler-Zebeli's group has explored a possible association between the intestinal microbiota and RFI in broiler chickens and attempted to identify RFI-associated bacteria; however, the outcomes

have been inconsistent among their studies, and the bacteria were only classified to the genus or OTU level (Siegerstetter et al., 2017; Siegerstetter et al., 2018a; Siegerstetter et al., 2018b; Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b). There is a need to address the discrepancies and further classify RFI-associated bacteria to the species and subspecies levels. In this study, we analyzed the ileal, cecal, and cloacal microbiotas, simultaneously, for their relationships with feed efficiency of broilers and explored for the first time QIIME 2's Deblur method of denoising, allowing for single-nucleotide resolution in differentiating bacterial features. As a result, we have identified a number of bacterial features in the ileum, cecum, and cloaca that are strongly-linked to RFI. All seven bacterial features that are differentially enriched in the ileal and cecal microbiota of high or low RFI chickens based on LEfSE analysis are significantly correlated with RFI. Three differentially enriched cloacal bacterial features also show a significant correlation with RFI.

Most of the bacteria that are strongly associated with high or low RFI belong to Clostridiales, a highly diverse order of obligate anaerobes that ferment host-indigestible plant polysaccharides into SCFAs (Parks et al., 2018; Boutard et al., 2014). Clostridia are abundant in soil and also in the gastrointestinal (GI) tract, representing up to 20% of uncultured genomes in the human GI tract (Almeida et al., 2019). Among the families of Clostridiales are *Clostridiaceae* (*Clostridium* Cluster I),), *Ruminococcaceae* (Cluster IV), *Peptostreptococcaceae* (Cluster XI), and *Lachnospiraceae* (Cluster XIVa) (Galperin et al., 2016). *Lachnospiraceae* and *Ruminococcaceae* are highly abundant in poultry and are particularly effective at degrading cellulose and other host-indigestible polysaccharides (Biddle et al., 2013). Consistently, we found that *Oscillibacter* F220 (family *Oscillospiraceae*) in the cecum and *Butyricicoccus* F149 (family

Clostridiaceae) in the cloaca are enriched in low RFI chickens and positively associated with feed efficiency.

However, to our surprise, a majority of other SCFA-producing clostridia are more abundant in high RFI chickens and negatively correlated with feed efficiency. For example, two unidentified and closely-related *Peptostreptococcaceae* F15 and F43 show differential enrichment in high RFI chickens and significant positive correlations with RFI in the ileum, meaning that both are negatively correlated with feed efficiency. F15 is also negatively correlated with feed efficiency in the cloaca. F15 and F43 are closely related to *Clostridium* cluster XI bacteria such as *C. difficile*, *I. bartlettii,* and *R. ilealis*. Although *C. difficile* is a well-known enteric pathogen, little is known about *I. bartlettii* or *R. ilealis*. Both *I. bartlettii* and *R. ilealis* were recently found to be enriched in children with neurodevelopmental disorders (Bojović et al., 2020). Analysis of the *R. ilealis* genome revealed its limited capacity to synthesize amino acids and vitamins with the ability to utilize different relatively simple carbohydrates such as glucose, L-fructose and fructo-oligosaccharides (Gerritsen et al., 2017). The reason why *Peptostreptococcaceae* F15 and F43 reduce feed efficiency remains to be investigated, although *I. bartlettii* appears to be more abundant in the ileum of turkeys with heavier BW (Danzeisen et al., 2013).

Subdoligranulum F6 (identical to *S. variabile*, family *Ruminococcaceae*) in the ileum is also enriched in the ileum of high-RFI, low-feed efficiency chickens with a significant negative correlation with feed efficiency. *S. variable* is known to be differentially enriched in children with food sensitization (Chen et al., 2016) and correlated positively with lipid metabolic dysfunction and inflammatory responses in the ileum of pigs (Huang et al., 2020). Perhaps it is not surprising why *S. variable* is correlated negatively with feed efficiency. Several other clostridial bacteria including two unidentified members of *Ruminococcaceae* F27 and F97 in the ileum, *Faecalicoccus*

F195 (family *Erysipelotrichaceae*) in the cecum, and an unidentified Clostridiales F72 in the cloaca also show a significant positive correlation with RFI. The reason for a negative correlation between these SCFA-producing bacteria and feed efficiency remains to be further investigated. Apparently, these bacteria influence feed efficiency beyond mere fermentation of plant polysaccharides.

Our results have also clearly revealed that multiple closely-related members of the *Lachnospiraceae* family are significantly associated with RFI. To our surprise, among those differentially enriched *Lachnospiraceae* that also show a strong correlation with RFI, several features (e.g. F76, F116, and F203) are negatively correlated with RFI while others (e.g. F33, F42, and F92) show a positive correlation. Alignment of these six sequences reveals a minimum 91.3% identity among them (**Fig. 11**). In particular, F33, F76, and F92 are highly related, showing 96.5%-97.8% identities among each other. Although F33, F76, and F92 could otherwise be grouped as a single OTU, their impact on feed efficiency is totally opposite. While F33 in the cloaca and F92 in the cecum are negatively correlated with feed efficiency, F76 is highly enriched in the cloaca of low RFI chickens with a strong tendency to be positively correlated with feed efficiency.

Similarly, two related *Blautia* F42 and F116 of the *Lachnospiraceae* family also show an opposite association with feed efficiency. Albeit with 94.8% identity to each other **(Fig. 11**), F42 is negatively correlated with feed efficiency in the cloaca, while F116 has an opposite association in the cecum. These results clearly demonstrate the advantage of differentiating the intestinal microbes to the single-nucleotide resolution at the species, subspecies, or strain level. Otherwise, the physiological effect of some of the bacteria could be masked. In fact, our findings are consistent with the well-known fact that functional variations exist among different species of a bacterial family or even different strains of the same bacterial species. However, because of limitation of

the current nonredundant (NR) and 16S rRNA gene databases in GenBank, most of the newly identified RFI-associated bacterial features are found to be 100% associated with uncultured and unclassified 16S rRNA sequences. Only *Subdoligranulum* F6 can be unequivocally be assigned to *S. variabile*, and we failed to assign a specific bacterial species or strain name to the remaining features.

Notably, the RFI-associated bacteria revealed in this study are mostly different from several earlier attempts by Metzler-Zebeli's group (Siegerstetter et al., 2017; Siegerstetter et al., 2018a; Siegerstetter et al., 2018b; Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b). In fact, their results vary from study to study as well. For example, among three different studies, *Dorea* (family *Lachnospiraceae*) (Siegerstetter et al., 2017), an *Anaerobacterium* OTU (family *Ruminococcaceae*), two *Lactobacillus* OTUs (Siegerstetter et al., 2018b), and the *Christensenellaceae* family (Mezler-Zebeli et al., 2019b) have been found to be significantly associated with low RFI chickens, while two *Gracilibacter* OTUs and a *Clostridium* OTU are associated with high RFI chickens (Siegerstetter et al., 2018b). The results are even different between two animal experiments being duplicated at two different locations (Siegerstetter et al., 2017).

These apparently inconsistent outcomes may potentially be due to dietary and environmental differences among different flocks in the studies. Microorganisms present in the hatchery, diet, bedding, and caging materials are known to contribute significantly to the intestinal microbiota. Secondly, the discrepancies among different studies may be due to relatively low stringency of selection for high and low RFI birds. In our study, 32 out of 56 chickens or 57% of the chickens were selected to form high and low RFI groups, whereas approximately 50% of chickens were chosen in most earlier studies (Siegerstetter et al., 2018a; Siegerstetter et al., 2018b;

Metzler-Zebeli et al., 2019a; Metzler-Zebeli et al., 2019b) and 20% of chickens were selected in a fifth study (Siegerstetter et al., 2017). It is possible that a higher stringency of selection for extreme high and low RFI chickens may lead to more reproducible results. Thirdly, the discrepancies among the studies may also be because it is the function, not the composition, of the intestinal microbiota that matters in feed efficiency. Although different studies have revealed an association of relative abundance of different bacteria with RFI, it is plausible that consistent functional alternations might occur between high and low RFI animals. Techniques such as metagenomics, metabolomics, metatranscriptomics, and metaproteomics (Heintz-Buschart et al., 2018; Zhang et al., 2019) will be useful to reveal the differences in the functional capacity of the intestinal microbiota between high- and low-performing chickens.

5. Conclusion

The intestinal microbiota is a complex community of microorganisms and variations in the structure and function of individual animals have a profound impact on the health and performance outcomes of host animals. In this study, we have identified a number of bacteria that are strongly associated with feed efficiency in three different GI locations of broiler chickens. Among those newly identified RFI-associated bacteria, most belong to the order Clostridiales. Importantly, we revealed the complexity of the intestinal microbiota. While a few *Lachnospiraceae* family members are positively correlated with feed efficiency, other closely related bacteria have an opposite impact, highlighting a need to differentiate the bacteria to the species, subspecies, and even strain levels. Apparently, this work enhances our understanding of the link between the intestinal microbiome and feed efficiency in broilers. Identification of performance-associated bacterial taxa marks a first step towards developing efficacious, cost-effective pre- and probiotic formulations to replace antibiotics as feed additives for growth promotion and disease prevention.

It will be beneficial to further increase the selection pressure for high- and low-performing animals with a larger difference in RFI values in future studies. It is also important to investigate not only the composition, but also the functional potential of the intestinal microbiota and evaluation their functional correlations with feed efficiency in the future.

Table 1. **Composition of the experimental diets**

Ingredients	**Starter (D0 – D8)**	**Grower (D9 – D18)**	**Finisher (D19 – D35)**
Yellow Corn (%)	52.8	57.1	60.4
Soybean Meal (%)	39.7	34.9	30.7
Soybean Oil (%)	3.5	4.2	5.2
Dicalcium Phosphate, 18.5% (%)	2.04	1.81	1.68
Limestone (%)	1.06	0.97	0.95
Salt (%)	0.48	0.48	0.43
DL-Methionine (%)	0.16	0.22	0.20
Threonine (%)	0.05	0.07	0.11
Poultry Premix, NB 3000[1] (%)	0.03	0.25	0.25
Total ME (kcal/kg)	2987.60	3082.20	3176.80
Total CP (%)	21.50	19.61	18.00

[1] Supplied per kilogram of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; thiamine, 2.20 mg; manadione, 0.83 mg; vitamin B12, 0.01 mg; vitamin E, 16.53 IU; vitamin D3, 2,133 ICU; vitamin A, 7,716 IU.

Table 2. **Relative abundance (%) of the intestinal bacterial genera in day-35 high and low RFI chickens**

Genera	High RFI	Low RFI	P-Value	FDR
Ileum				
Lactobacillus	35.69	32.03	0.355	0.568
Romboutsia	21.06	29.68	0.417	0.568
Enterococcus	18.72	19.39	0.396	0.568
Turicibacter	12.98	8.22	0.748	0.748
Streptococcus	0.49	7.16	0.299	0.560
*Peptostreptococcaceae*_unidentified	6.14	0.16	0.013 *	0.112
Escherichia/Shigella	3.03	2.18	0.584	0.626
*Lachnospiraceae*_unidentified	0.45	0.36	0.117	0.351
Subdoligranulum	0.26	0.14	0.020 *	0.112
Terrisporobacter	0.26	0.05	0.199	0.427
Faecalibacterium	0.18	0.14	0.157	0.392
*Ruminococcaceae*_unidentified	0.17	0.10	0.030 *	0.112
Anaerostipes	0.06	0.04	0.027 *	0.112
Bacteroides	0.06	0.05	0.558	0.626
Streptophyta	0.03	0.07	0.466	0.583
Cecum				
*Lachnospiraceae*_unidentified	22.18	23.15	0.462	0.865
Lactobacillus	14.26	11.77	0.664	0.897
Faecalibacterium	13.23	10.68	0.355	0.865
*Ruminococcaceae*_unidentified	10.54	10.67	0.985	0.985
Subdoligranulum	8.12	12.84	0.193	0.722
Bacteroides	4.67	5.34	0.748	0.897
*Clostridiales*_unidentified	3.64	2.93	0.193	0.722
Romboutsia	3.14	3.40	0.777	0.897
Enterococccus	2.44	3.54	0.462	0.865
Blautia	2.67	3.53	0.720	0.897
Anaerostipes	1.88	1.95	0.955	0.985
Butyricicoccus	1.82	1.55	0.375	0.865
*Lachnospiraceae*_incertae_sedis	1.42	1.48	0.610	0.897
Ruminococcus2	1.65	1.13	0.168	0.722
*Clostridium*_IV	1.08	0.90	0.168	0.722
Cloaca				
Lactobacillus	17.05	17.62	0.895	0.895
Lachnospiraceae_unidentified	14.40	14.81	0.895	0.895
Romboutsia	13.50	11.63	0.865	0.895
Enterococcus	7.72	13.16	0.584	0.895
Subdoligranulum	5.12	7.39	0.692	0.895
Bacteroides	7.42	4.67	0.146	0.895
Turicibacter	9.23	2.57	0.317	0.895
Faecalibacterium	6.10	4.55	0.299	0.895
Ruminococcaceae_unidentified	4.81	4.60	0.806	0.895
Streptococcus	0.56	6.36	0.533	0.895
Clostridiales_unidentified	2.78	2.31	0.417	0.895
Anaerostipes	1.50	1.93	0.865	0.895
Escherichia/Shigella	1.26	1.82	0.835	0.895
Blautia	1.46	1.19	0.417	0.895
*Lachnospiraceae*_incertae_sedis	0.85	0.81	0.835	0.895

Note: Mean relative abundance (%) of the top 15 genera are shown, with 15 high and 17 low RFI samples per intestinal segment. Statistical significance was determined using the Kruskal-Wallis test with Benjamini-Hochberg correction. False discovery rate (FDR) is shown.

Table 3. Relative abundance (%) of the intestinal bacterial features in day-35 high and low RFI chickens

Features	High RFI	Low RFI	P-Value	FDR
Ileum				
Firmicutes_Lactobacillus_F1	29.18	27.26	0.462	0.674
Firmicutes_Romboutsia_F2	21.05	29.68	0.417	0.674
Firmicutes_Enterococcus_F3	18.70	19.38	0.396	0.674
Firmicutes_Turicibacter_F4	12.97	8.21	0.748	0.876
Firmicutes_Streptococcus_F8	0.49	7.16	0.335	0.674
Firmicutes_Lactobacillus_F10	3.19	2.89	0.865	0.927
Firmicutes_Peptostreptococcaceae_unidentified_F15	5.16	0.14	0.018 *	0.137
Proteobacteria_Escherichia/Shigella_F12	3.03	2.18	0.584	0.730
Firmicutes_Lactobacillus_F24	1.13	0.92	0.788	0.876
Firmicutes_Lactobacillus_F26	1.25	0.25	0.433	0.674
Firmicutes_Lactobacillus_F22	0.73	0.68	0.472	0.674
Firmicutes_Peptostreptococcaceae_unidentified_F43	0.98	0.02	0.009 *	0.137
Firmicutes_Terrisporobacter_F75	0.26	0.05	0.199	0.597
Firmicutes_Subdoligranulum_F6	0.18	0.09	0.003 *	0.115
Firmicutes_Lactobacillus_F102	0.16	0.02	0.252	0.674
Firmicutes_Ruminococcaecea_unidentified_F11	0.09	0.06	0.155	0.597
Firmicutes_Faecalibacterium_F9	0.07	0.07	0.985	0.985
Firmicutes_Faecalibacterium_F5	0.06	0.04	0.570	0.730
Firmicutes_Lachnospiraceae_unidentified_F14	0.05	0.04	0.375	0.674
Firmicutes_Lachnospiraceae_unidentified_F16	0.05	0.03	0.060	0.362
Firmicutes_Anaerostipes_F17	0.03	0.03	0.015 *	0.137
Firmicutes_Lachnospiraceae_unidentified_F19	0.03	0.06	0.772	0.876
Firmicutes_Lachnospiraceae_unidentified_F20	0.05	0.03	0.121	0.597
Cyanobacteria_Chloroplast_Streptophyta_F192	0.02	0.05	0.466	0.674
Firmicutes_Lachnospiraceae_unidentified_F18	0.04	0.03	0.416	0.674
Firmicutes_Faecalibacterium_F21	0.05	0.02	0.174	0.597
Firmicutes_Bacteroides_F7	0.03	0.02	0.450	0.674
Firmicutes_Subdoligranulum_F30	0.03	0.02	0.195	0.597
Firmicutes_Lachnospiraceae_unidentified_F23	0.03	0.03	0.544	0.730
Firmicutes_Clostridium_sensus_stricto_F227	0.04	0.00	0.960	0.985
Cecum				
Firmicutes_Lactobacillus_F1	11.78	10.28	0.985	0.985
Firmicutes_Subdoligranulum_F6	4.68	8.89	0.439	0.894
Firmicutes_Faecalibacterium_F5	6.76	5.45	0.748	0.894
Firmicutes_Faecalibacterium_F9	5.10	4.25	0.637	0.894
Firmicutes_Ruminococcaceae_unidentied_F11	4.27	4.60	0.610	0.894
Firmicutes_Romboutsia_F2	3.14	3.40	0.777	0.894
Firmicutes_Enterococcus_F3	2.43	3.52	0.462	0.894
Bacteroidetes_Bacteroides_F7	2.42	2.81	0.748	0.894
Firmicutes_Lachnospiraceae_unidentified_F20	1.40	2.59	0.558	0.894
Firmicutes_Lachnospiraceae_unidentified_F18	1.85	1.98	0.439	0.894
Bacteroidetes_Bacteroides_F13	1.68	1.94	0.664	0.894
Firmicutes_Lachnospiraceae_unidentified_F16	2.30	1.18	0.558	0.894
Firmicutes_Lachnospiraceae_unidentified_F19	0.60	2.96	0.116	0.894
Firmicutes_Lachnospiraceae_unidentified_F14	1.76	1.64	0.439	0.894
Firmicutes_Subdoligranulum_F25	1.66	1.37	0.834	0.894
Firmicutes_Anaerostipes_F17	1.46	1.57	0.777	0.894
Firmicutes_Lachnospiraceae_unidentified_F23	1.49	1.30	0.610	0.894
Firmicutes_Ruminococcaceae_unidentified_F27	1.14	1.35	0.664	0.894
Firmicutes_Subdoligranulum_F31	1.24	0.91	0.954	0.985

Firmicutes_Subdoligranulum_F30	0.54	1.67	0.739	0.894
Firmicutes_Faecalibacterium_F21	1.12	0.82	0.625	0.894
Firmicutes_Turicibacter_F4	1.18	0.43	0.533	0.894
Firmicutes_Ruminococcaceae_unidentified_F34	0.91	0.74	0.485	0.894
Firmicutes_Lachnospiraceae_unidentified_F33	0.89	0.70	0.146	0.894
Firmicutes_Lachnospiraceae_incertae_sedis_F32	0.93	0.58	0.213	0.894
Firmicutes_Lachnospiraceae_unidentified_F35	0.83	0.68	0.157	0.894
Firmicutes_Ruminococcus2_F28	0.92	0.58	0.156	0.894
Firmicutes_Butyricoccus_F36	0.77	0.71	0.610	0.894
Firmicutes_Ruminococcaceae_unidentified_F37	0.65	0.82	0.806	0.894
Firmicutes_Lachnospiraceae_incertae_sedis_F40	0.48	0.88	0.089	0.894
Cloaca				
Firmicutes_Lactobacillus_F1	14.17	15.05	0.835	0.985
Firmicutes_Romboutsia_F2	13.50	11.62	0.865	0.985
Firmicutes_Enterococcus_F3	7.71	13.16	0.584	0.985
Firmicutes_Turicibacter_F4	9.23	2.57	0.317	0.985
Firmicutes_Subdoligranulum_F6	3.16	5.40	0.835	0.985
Firmicutes_Streptococcus_F8	0.56	6.36	0.509	0.985
Bacteroidetes_Bacteroides_F7	3.76	2.48	0.146	0.985
Firmicutes_Faecalibacterium_F5	3.44	2.48	0.193	0.985
Bacteroidetes_Bacteroides_F13	2.72	1.65	0.109	0.985
Firmicutes_Ruminococcaceae_unidentified_F11	2.18	1.99	0.865	0.985
Firmicutes_Faecalibacterium_F9	2.24	1.76	0.290	0.985
Firmicutes_Lachnospiraceae_unidentified_F19	0.59	3.00	0.290	0.985
Proteobacteria_Escherichia/Shigella_F12	1.26	1.82	0.835	0.985
Firmicutes_Anaerostipes_F17	1.25	1.68	0.835	0.985
Firmicutes_Lachnospiraceae_unidentified_F20	0.85	1.91	0.720	0.985
Firmicutes_Lachnospiraceae_unidentified_F14	1.39	1.29	0.336	0.985
Firmicutes_Lactobacillus_F10	1.19	1.40	0.508	0.985
Firmicutes_Lachnospiraceae_unidentified_F23	1.30	1.02	0.985	0.985
Firmicutes_Lachnospiraceae_unidentified_F16	1.55	0.69	0.508	0.985
Firmicutes_Lachnospiraceae_unidentified_F18	0.91	0.97	0.835	0.985
Firmicutes_Subdoligranulum_F30	0.48	1.30	0.984	0.985
Bacteroidetes_Bacteroides_F29	0.94	0.55	0.145	0.985
Firmicutes_Subdoligranulum_F25	0.78	0.42	0.832	0.985
Firmicutes_Lactobacillus_F26	0.82	0.34	0.554	0.985
Firmicutes_Ruminococcaceae_unidentified_F27	0.47	0.57	0.865	0.985
Firmicutes_Subdoligranulum_F31	0.69	0.26	0.984	0.985
Firmicutes_Clostridiales_unidentified_F41	0.50	0.46	0.417	0.985
Firmicutes_Lachnospiraceae_unidentified_F33	0.64	0.26	0.002 *	0.985
Firmicutes_Lactobacillus_F22	0.46	0.40	0.891	0.985
Firmicutes_Lachnospiraceae_incertae_sedis_F40	0.32	0.54	0.336	0.985

Note: Mean relative abundance (%) of the top 30 features are shown, with 15 high and 17 low RFI samples per intestinal segment. Statistical significance was determined using the Kruskal-Wallis test with Benjamini-Hochberg correction. False discovery rate (FDR) is shown.

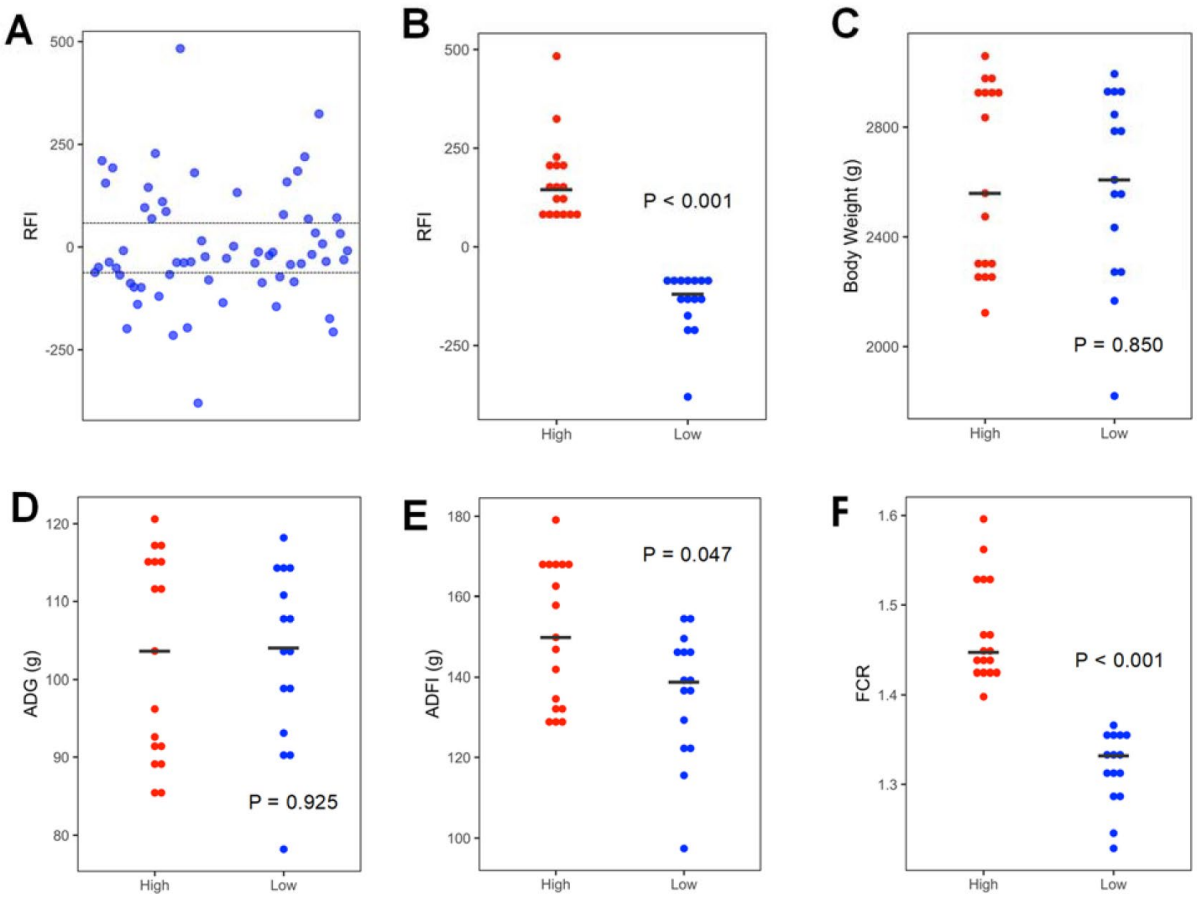

Fig. 1. Production performance of the chickens with extremely high and low RFI values. Male Cobb chickens were individually housed from day 14 to 35 with free access to non-medicated feed. Residual feed intake (RFI) was calculated individually for 56 apparently healthy chickens, from which 15 and 17 chickens with extremely high and low RFI values, respectively, were selected (as shown by dashed lines) (**A**). RFI (**B**), body weight (**C**), average daily gain (ADG) (**D**), average daily feed intake (ADFI) (**E**), and feed conversion ratio (FCR) (**F**) were calculated for two groups of selected chickens. Statistical significance was determined using Student's t-test.

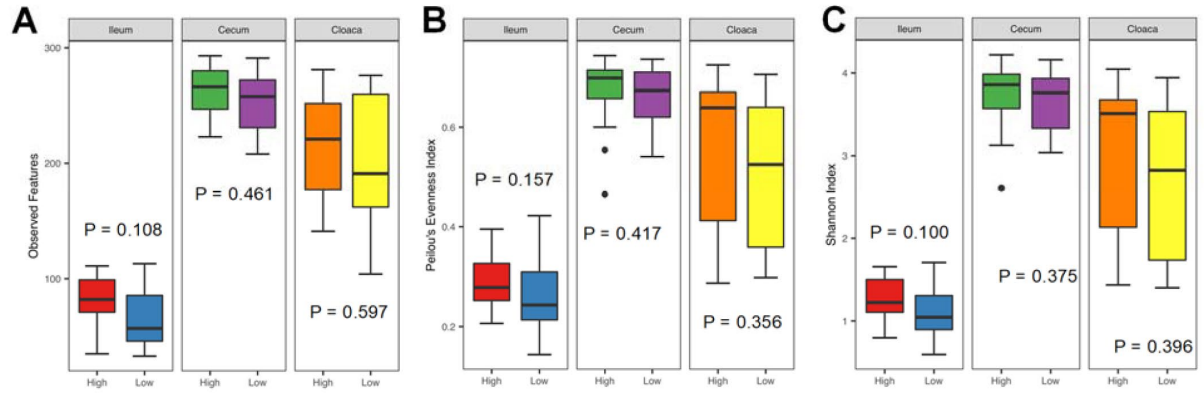

Fig. 2. Alpha diversity of the ileal, cecal, and cloacal microbiota on day 35 between high and low RFI chickens. Differences in richness, overall diversity, and evenness were calculated using observed features (**A**), Pielou's Evenness Index (**B**), and Shannon Index (**C**), respectively. Results were plotted using box and whisker plots, in which the middle line denoted the median value and the lower and upper hinges represented the first and third quartiles, respectively. Whiskers extended from the hinge to the highest or lowest value no farther than 1.5 × the interquartile range. Points outside of this range are considered outliers. Statistical significance was determined using the Kruskal-Wallis test.

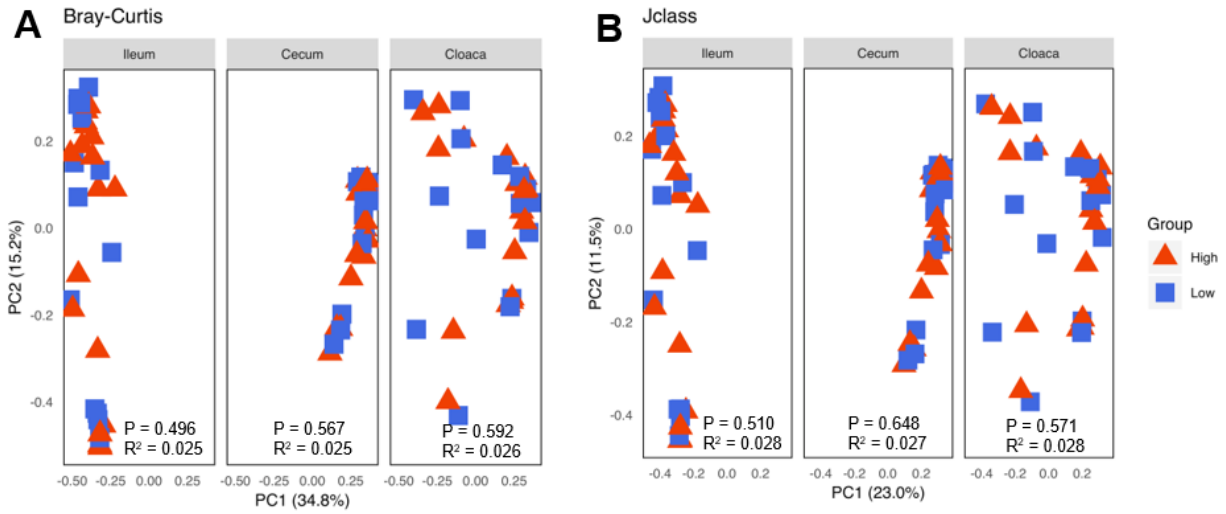

Fig. 3. **Beta diversity of the ileal, cecal, and cloacal microbiota on day 35 between high and low RFI chickens.** Principle coordinate analysis (PCoA) plots were generated using Bray-Curtis (**A**) and Jaccard indices (**B**), respectively. Statistical significance and R-values were determined using permutational multivariate analysis of variance (PERMANOVA).

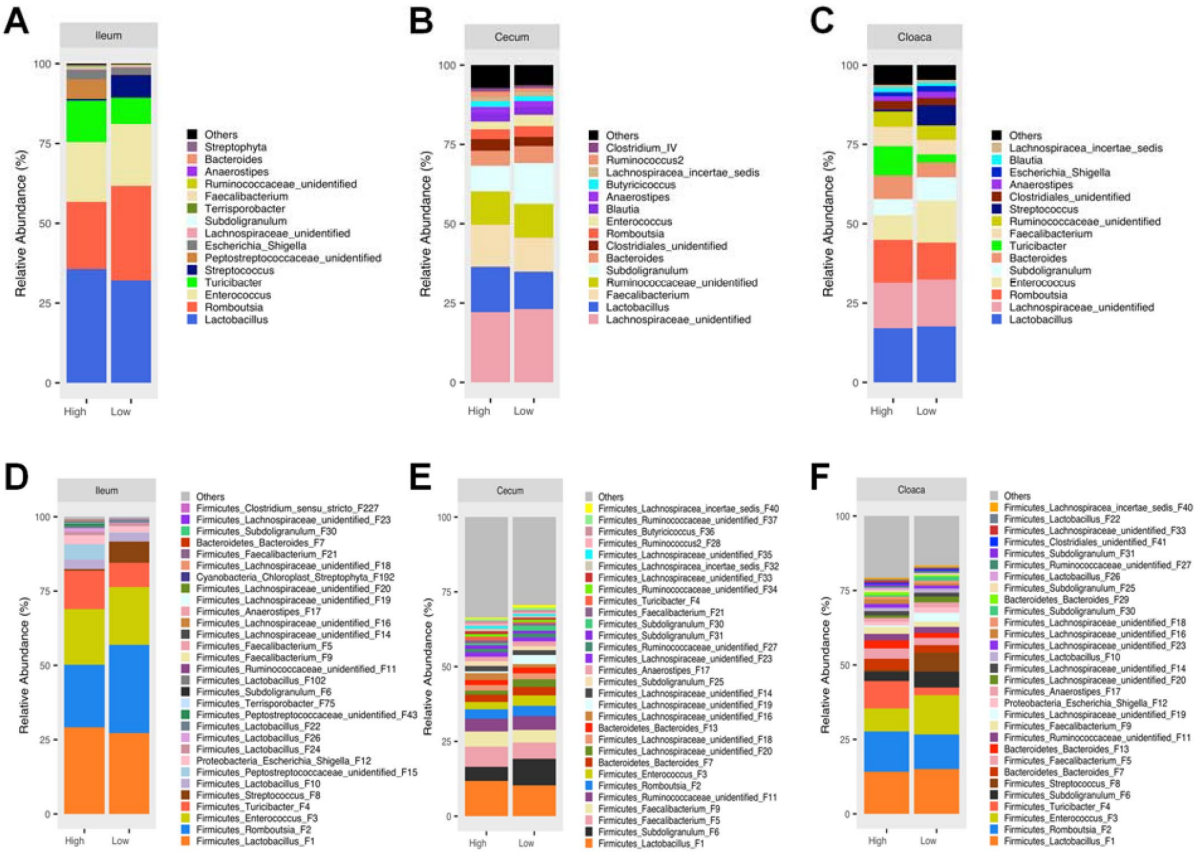

Fig. 4. **Composition of the ileal, cecal, and cloacal microbiota on day 35 between high and low RFI chickens.** Relative abundance of the top 15 genera (**A, B,** and **C**) and top 30 features (**D, E,** and **F**) were shown at each intestinal location.

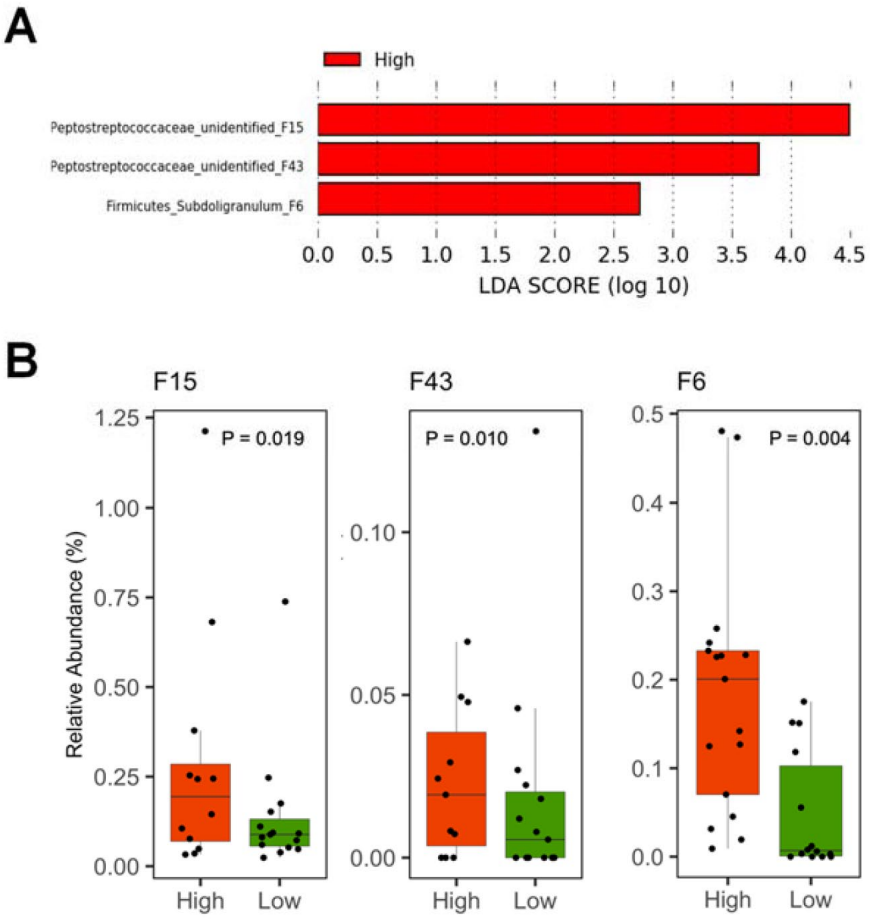

Fig. 5. **LEfSe analysis of the day-35 ileal microbiota of high and low RFI chickens.** (**A**) Differential enrichment of the bacterial features was determined using LEfSe with a logarithmic LDA threshold of 2.0. Note that only three bacterial features were enriched in the high RFI group, while no preferential enrichment was detected in the low RFI group. (**B**) Relative abundance of three differentially enriched bacterial features. Results were plotted using box and whisker plots, in which the middle line denoted the median value and the lower and upper hinges represented the first and third quartiles, respectively. Whiskers extended from the hinge to the highest or lowest value no farther than 1.5 × the interquartile range. Points outside of this range are considered outliers. Significance was calculated using the Kruskal-Wallis test.

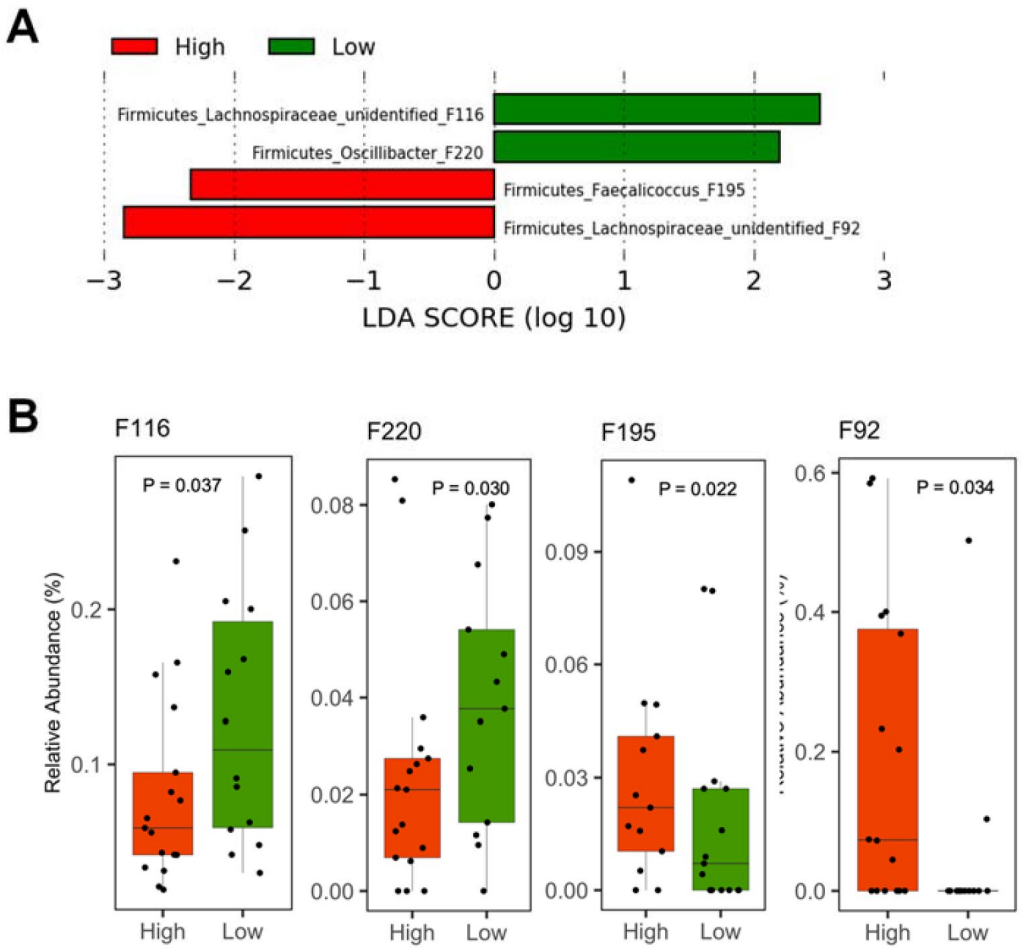

Fig. 6. LEfSe analysis of the day-35 cecal microbiota of high and low RFI chickens. (**A**) Differential enrichment of the bacterial features was determined using LEfSe with a logarithmic LDA threshold of 2.0. (**B**) Relative abundance of five differentially enriched bacterial features. Results were plotted using box and whisker plots, in which the middle line denoted the median value and the lower and upper hinges represented the first and third quartiles, respectively. Whiskers extended from the hinge to the highest or lowest value no farther than 1.5 × the interquartile range. Points outside of this range are considered outliers. Significance was calculated using the Kruskal-Wallis test.

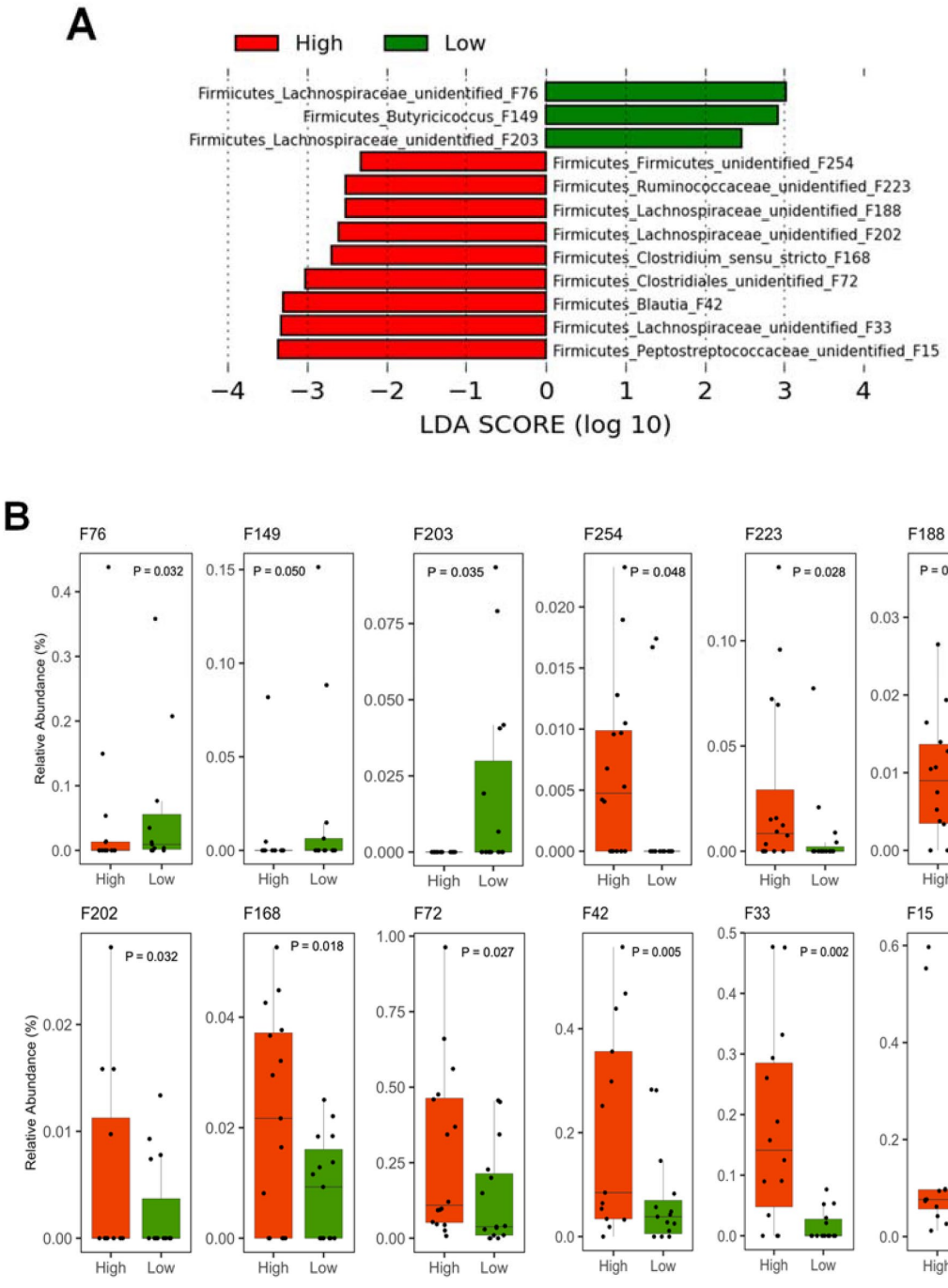

Fig. 7. LEfSe analysis of the day-35 cloacal microbiota of high and low RFI chickens. A. Differential enrichment of the bacterial features was determined using LEfSe with a logarithmic LDA threshold of 2.0. **B.** Relative abundance of differentially enriched bacterial features. Results were plotted using box and whisker plots, in which the middle line denoted the median value and the lower and upper hinges represented the first and third quartiles, respectively. Whiskers extended from the hinge to the highest or lowest value no farther than 1.5 × the interquartile range. Points outside of this range are considered outliers. Significance was calculated using the Kruskal-Wallis test.

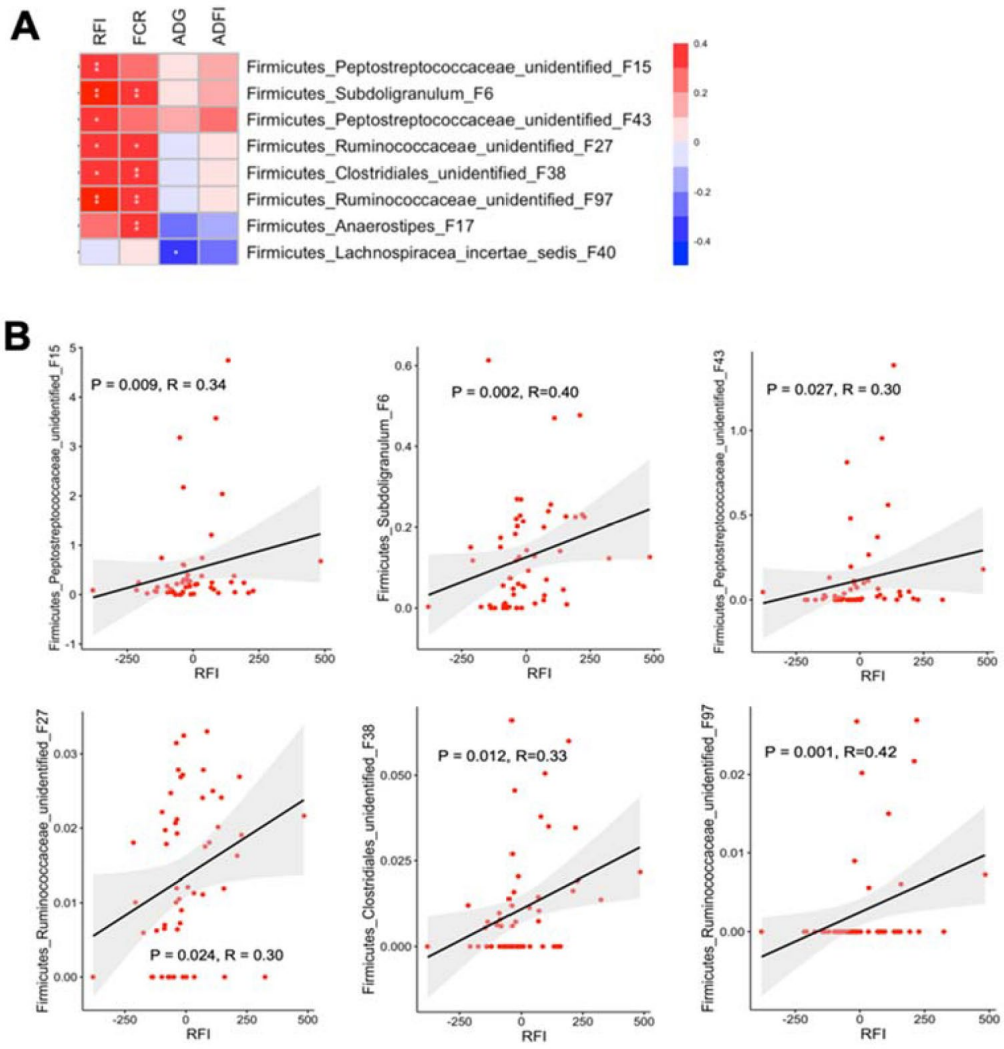

Fig. 8. Spearman correlation between RFI and relative abundance of bacterial features in the ileum of day-35 chickens. (**A**) All 56 ileal samples were used in Spearman's rank correlation analysis and only those features with $P < 0.05$ and $|R| \geq 0.30$ were shown. Note that there were no features showing a negative correlation with RFI. (**B**) Scatterplots of individual ileal bacterial features showing a significant correlation with RFI. P and R values were indicated for each feature. The solid line in the graph represented the line of best fit, while gray shading around the line indicated the 95% confidence interval. In a few cases, 1-3 extremely outlier samples were omitted for the sake of better visualization.

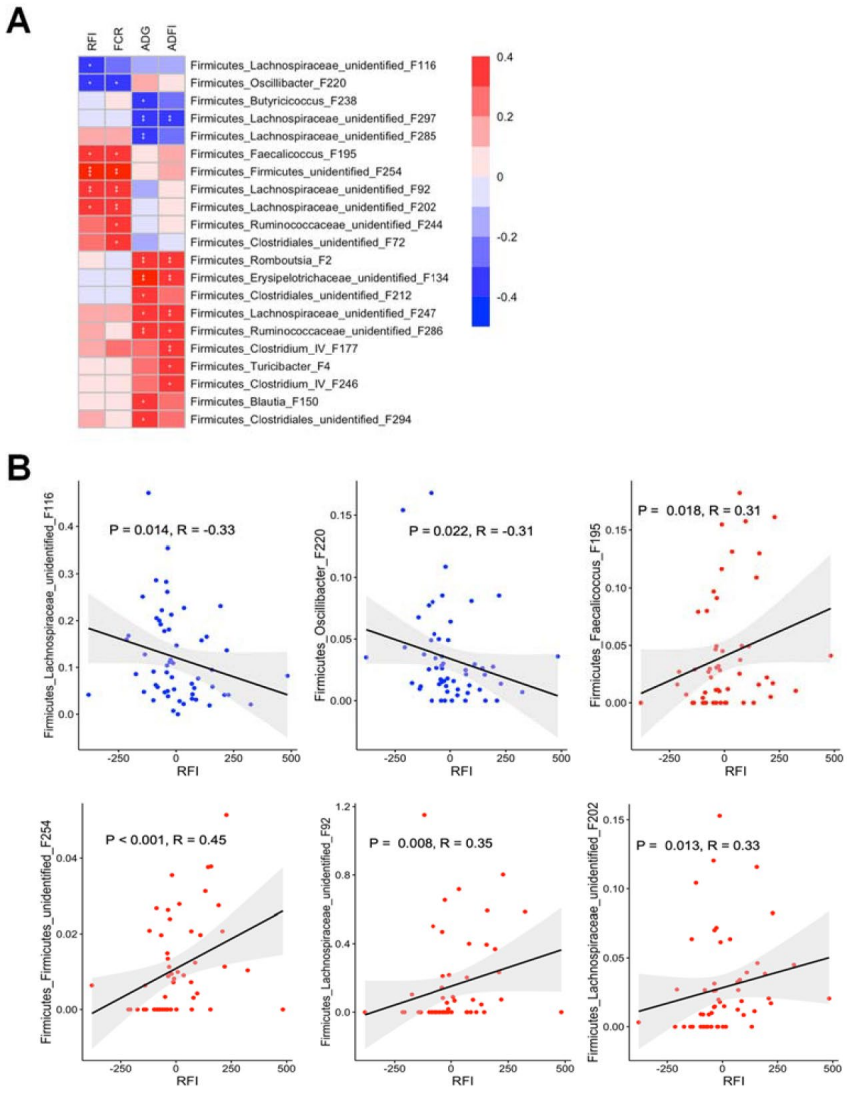

Fig. 9. Spearman correlation between RFI and relative abundance of bacterial features in the cecum of day-35 chickens. (**A**) All 56 cecal samples were used in Spearman's rank correlation analysis and only those features with $P < 0.05$ and $|R| \geq 0.30$ were shown. (**B**) Scatterplots of individual cecal bacterial features showing a significant correlation with RFI. P and R values were indicated for each feature. The solid line in the graph represented the line of best fit, while gray shading around the line indicated the 95% confidence interval.

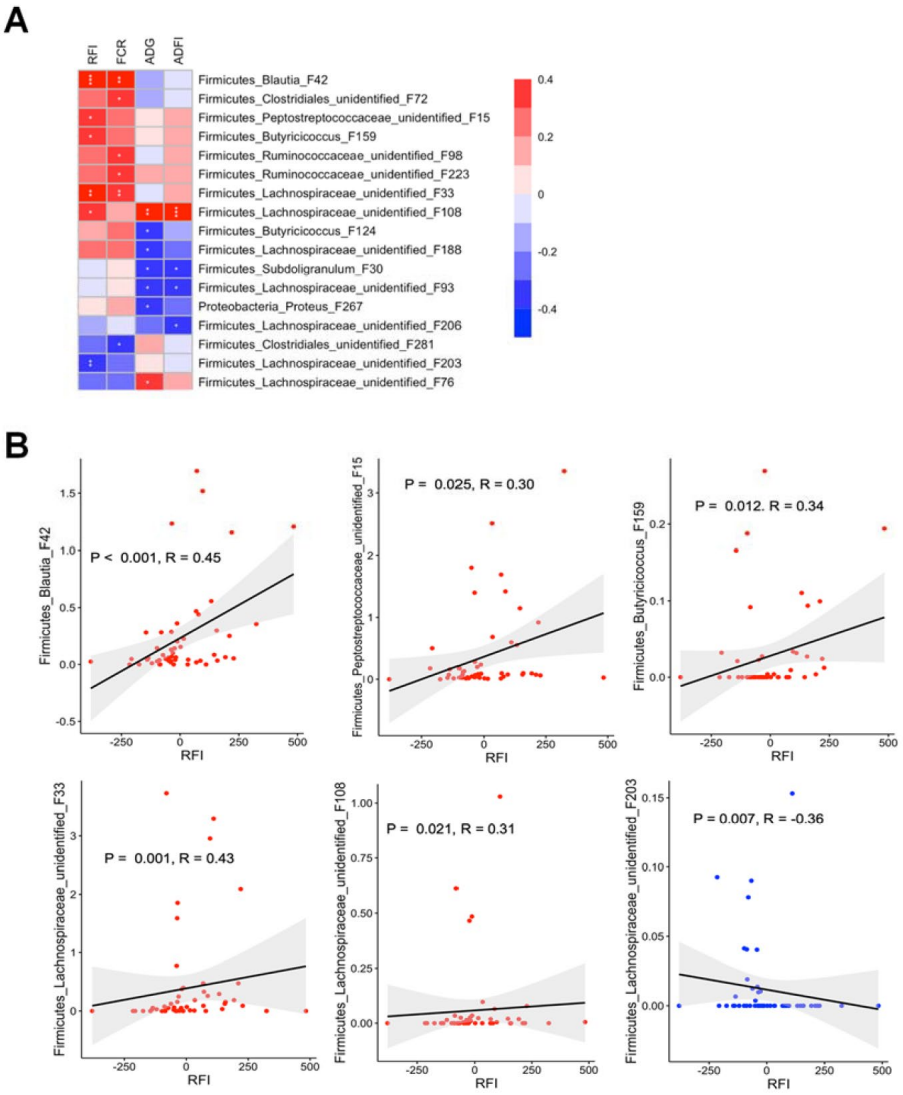

Fig. 10. Spearman correlation between RFI and relative abundance of bacterial features in the cloaca of day-35 chickens. (**A**) All 56 cloacal samples were used in Spearman's rank correlation analysis and only those features with $P < 0.05$ and $|R| \geq 0.30$ were shown. (**B**) Scatterplots of individual cloacal bacterial features showing a significant correlation with RFI. P and R values were indicated for each feature. The solid line in the graph represented the line of best fit, while gray shading around the line indicated the 95% confidence interval. In a few cases, 1-3 extremely outlier samples were omitted for the sake of better visualization.

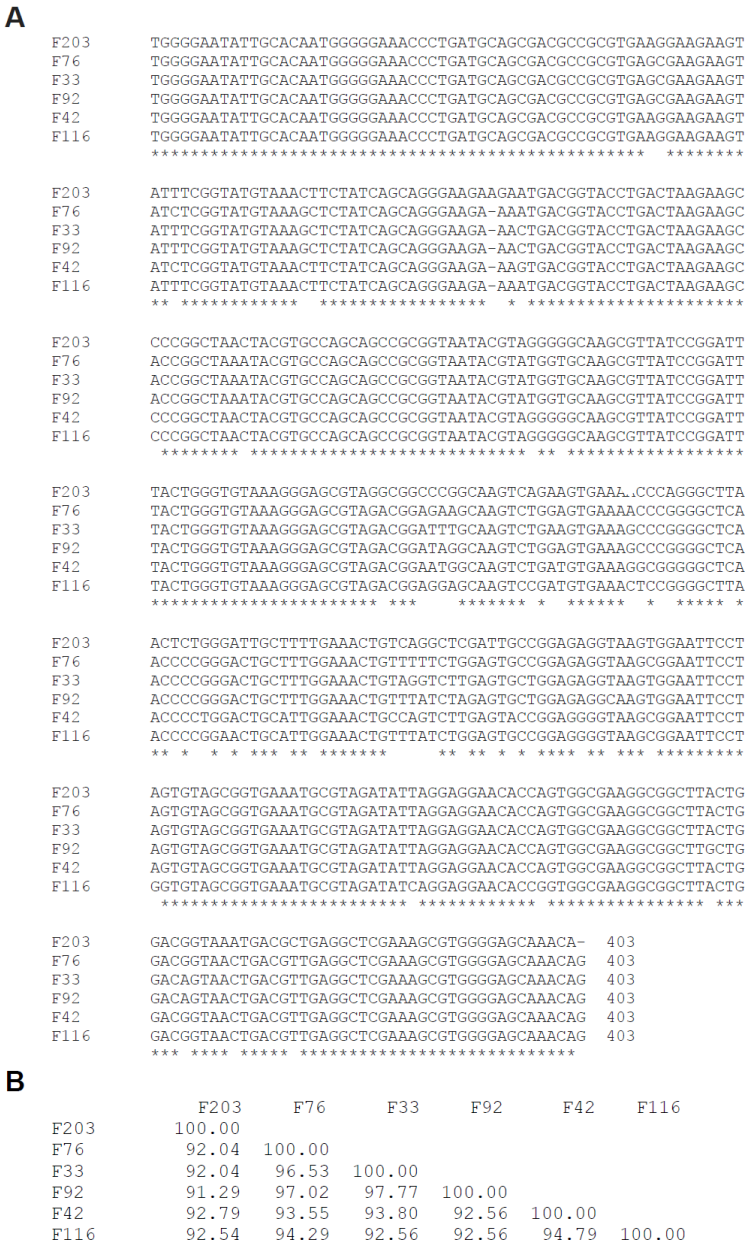

Figure 11. Multiple sequence alignment (A) and percent identity matrix (B) among six closely related *Lachnospiraceae* members that are strongly associated with residual feed intake.

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Biographical:

Education:

Completed the requirements for the Master of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma in July, 2020.

Completed the requirements for the Bachelor of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma in 2017.

Professional Memberships:

Phi Beta Delta Honors Society for International Scholars, 2019 – 2020

Poultry Science Association, 2019 – 2020

The Fulbright Association, 2018 – 2020

Awards:

Stilington Enriched Graduate Fellowship, 2018 – 2020

Fulbright U.S. Student Grant (Germany), 2017 – 2018

Wentz Undergraduate Research Scholarship, 2015 – 2016 and 2016 – 2017

Animal Science Undergraduate Research Scholarship, 2014 – 2015