# GENETIC, ENVIRONMENTAL AND MANAGEMENT INFLUENCES ON SELECTED GUT BACTERIAL POPULATIONS IN CHICKENS

A Dissertation

by

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# ABSTRACT

The indigenous gut bacteria of chicken influences both the health and disease. Many factors affect the gut microbiome of commercial chickens. Host-biology and the environment are two key factors recognized as important drivers of the community structure of the gut bacteria. However, compared to mammalian literature, the host genetic and environment have been little explored in productive chickens. Three experiments were designed to study the effects of host-biology and environmental conditions on the gut bacterial composition: 1) two genetically divergent chicken lines (meat-type and egg-type) sharing the same rearing area and diet; 2) individually caged broiler breeder hens maintained with feed-restricted or fed-satiated regimen; and 3) a naturally occurring necrotic enteritis chicken model. In each study the intestinal bacteria population was sequenced with Illumina technology targeting the V4 regions of 16S rRNA bacterial gene to annotate the ecological diversity of the bacterial communities. The rarefaction sequencing depth of the three experiments were: 1) 70,750; 2) 31,000, and 3)18,500 reads/sample.

In the first experiment, genetically divergent chickens (broiler- and egg-type) hatched and reared in a common area and fed the same diet developed similar fecal microbial populations but showed significant variations in the abundance of some groups of bacteria as the chickens grew older. Among the observed taxonomic differences, the order *Lactobacillales* was consistently enriched in broiler-type chickens. In experiment 2, it was found that doubling the feed ration of previously feed-restricted mature broiler hens decreased the proportion of *Lactobacillus* spp., and mediated few alterations in low abundant groups of bacteria. These alterations suggest a modification in the bacterial functionality. In the third experiment, chickens with necrotic enteritis had reduced species richness and diversity of the ileal and cecal bacterial communities compared to healthy pen-mates. Bacterial alterations in the sick chickens included reduced numbers of the

butyrate producer bacteria *Ruminococcaceae* and increased *Clostridiaceae*. Within the ileum of "healthy" chickens the proportion of *Lactobacillus* species was reduced compared to those reported in the literature. This might suggest the environment used in that study was limiting in acquirable *Lactobacillus* species and so developed an aberrant gut microbiota.

Overall these three different types of study designs found that the environmental factors were more influential than the host-genetic background and feed intake on the community structure of the gut bacteria in chickens.

# DEDICATION

To my Heavenly Father, my beloved husband and daughter, and to my family members from Honduras and Mexico.

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The upstream stage of QIIME and the analysis of similarities ANOSIM test for the microbiota data of Chapter II, III, and IV were conducted by the Bioinformatics Student Worker of the Gastrointestinal Laboratory. The bacteria sequencing data of Chapter IV is from a research sponsored by Huvepharma with Dr. Audrey McElroy as principal investigator and Dr. Rosemary Walzem and Dr. Tri Duong as co-investigators. The productive parameters and its Statistic from Chapter IV were provided by Dr. Audrey McElroy and the enumeration of *Clostridium perfringens* and its statistical analysis by Dr. Tri Duong; both are members of the POSC Department. All other work conducted for the dissertation was completed by the student independently.

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## NOMENCLATURE

| | |
|---|---|
| cfu | Colony-Forming Units |
| OTUs | Operational Taxonomic Units |
| BSH | Bile Salt Hydrolase |
| LPS | Lipopolysaccharide |
| DNA | Deoxyribonucleic acid |
| 16S rRNA | 16S ribosomal Ribonucleic acid |
| T-RFLP | Terminal Restriction Fragment Length Polymorphisms |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| PCR | Polymerase Chain Reaction |
| NGS | Next Generation Sequencing |
| QIIME | Quantitative Insights Into Microbial Ecology |
| LEfSe | Linear Discriminant Analysis Effect Size |
| ANOSIM | Analysis of Similarity |
| PICRUSt | Phylogenetic Investigation of Communities by Reconstruction of Unobserved STates |
| PCoA | Principal Coordinate Analysis |
| UniFrac | Unique Fraction Metric |
| NSTI | Nearest Sequence Taxon Index |
| wk | week |
| d | day |
| IGF | Insulin like Growth Factor |

| | |
|---|---|
| FR | Feed Restricted |
| UR | Unrestricted |
| SEM | Standard Error of the Mean |
| NE | Necrotic Enteritis |
| *Cp* | *Clostridium perfringens* |
| BW | Body Weight |
| FI | Feed Intake |
| FCR | Feed Conversion Ratio |

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# CHAPTER I

# INTRODUCTION

*The indigenous gut bacteria of commercial chickens*

The chicken intestinal microbiota—like in other animals—is composed of thousands of symbiotic microorganisms in the gastrointestinal tract. The avian microbiome is composed by 95% of bacteria species and the remaining profile is occupied by eukaryotes, archaea, and viruses (Singh et al., 2014a). There is more genetic material in the gut microbiome than in the host, but both, microbes and host co-evolved together (Ley et al., 2008). A better insight into the composition and function of the gut microbiota from different animal species have been achieved with the emerge of the "omics" technologies. Most of our current understanding on host-microbial interaction derives from mammalian literature. Studies conducted in avian species recognize that chickens depend on the gut microbes for nutritional, physiological, and health aspects (Drew et al., 2004; Oakley and Kogut, 2016; Yu et al., 2012). Therefore, more studies should be undertaken to improve existing understanding of the avian gut microbiota in various research disciplines, especially in those areas that include avian species destined to feed the growing word wide population.

The relationship between enteric microorganisms and the host is of mutual benefits. While the host provides optimal environmental conditions and a constant supply of nutrients, there are some microbial by-product with nutritional implications that can be utilized by the host such as the synthesis of vitamin K (Walther et al., 2013), microbial enzymes that aid in the digestion of carbohydrate such as carbohydrate-active enzymes (Lombard et al., 2014), and the bacteria short-chain fatty acids, **SCFAs**. Volatile short-chain fatty acids are the energy substrates of the bacteria,

and in avian species they are produced by the activity of dietary fiber fermentation in the ceca and then are absorbed by the cecal epithelial membranes and transformed into energy currency in the liver (Peng, 2007). The major volatile fatty acids with a nutritional relevance in ruminants are acetate, butyrate, and propionate because the host metabolize them into substrates of the citric cycle (Cheeke and Dierenfeld, 2014). Others microbial metabolites with relevance to the host are antibiotic molecules, such as bacteriocins, produced by some probiotic bacteria that can inhibit the growth of potential pathogenic species (Kamada et al., 2013).

*Intestinal bacteria and the immune system*

The gut microbial community assist in the development of the immune system of the host. It is attributed commensal microorganisms can exclude potential pathogens by competing for nutrients and space. This competitive exclusion action of the gut microbiota is of relevance in post-natal stage of the host since it becomes the first line of defense when the intestinal immunity is immature (Kerr et al., 2015). Postnatal period comprises a series of events that lead to the development of the own host defense mechanisms concomitant with progress of the enteric microorganisms. The host mechanism developed to tolerate commensalisms while eliminate pathogenic species comprise the innate and adaptive immunity.

The intestinal innate immunity creates the conditions that limit the contact of microbes with the epithelial cells, it also responds to pathogens while inducing tolerance to commensal microorganisms. The innate immunity of the gastrointestinal tract uptake and process antigens that can trigger and lead to an adaptive immune response. One innate mechanism that separate microbes from the epithelial membrane is the production of mucin. Mucins are glycosylated proteins constantly secreted by the goblet cells (Ermund et al., 2013) that create a viscous barrier

and have antibacterial properties. It was reported that the mucins of chickens can attenuate the virulence of relevant pathogenic bacteria such as *Campylobacter jejuni* (Byrne et al., 2007). The constant mucins flow and production can assist in the renewal and growth of epithelial cells (Liévin-Le Moal and Servin, 2006). Differential biosynthesis and degradation rate of mucins have been reported when chickens are feed-supplemented with probiotic and antibiotic (Smirnov et al., 2005), this suggest the composition of the indigenous gut bacteria can regulate the functionality of the mucins. In fact, commensal and pathogenic microorganisms can digest the glycan of mucins, since both microorganisms have genes involved in the digestion of carbohydrates (Koropatkin et al., 2012).The epithelial cells, contribute to the innate immunity by secreting peptides active against a wide range of microorganisms, such as defensins, also known as gallinacins and cathelicidins in chickens (Xiao et al., 2006; Xiao et al., 2004; Zhao et al., 2001). The intestinal epithelial cells participate in the process of inflammation by generating proinflammatory signals (chemokine and cytokines) when stimulated by bacteria antigens (Jobin and Sartor, 2000), a process that recruits macrophages, dendritic cells from the lamina propria (Jobin, 2003). Cells of the innate immunity (lymphocytes embedded in the mucosa epithelial and epithelial cells) express pattern recognition receptors that identify pathogen-associated molecular patterns. The extracellular toll-like receptors and the cytoplasmic node-like receptors are the major molecules that recognize pathogen-associated molecular patterns and trigger an immune response for invasive pathogens. Some bacterial pathogen-associated molecular patterns able to induce an immune reaction are the lipopolysaccharide (**LPS**), found in the outer membrane of gram-negative bacteria, peptidoglycan, a constituent of the plasma membrane of most bacteria, and flagellin, a constituent of the flagellin bacteria. Toll-like receptor molecules are expressed in all segments of the small and large intestine of the chickens (Iqbal et al., 2005) and some innate mechanisms are

already active at hatch (Bar-Shira and Friedman, 2006) which enable the newly hatched chickens to tolerate an adult-like diet.

The gut-associated lymphoid tissue (**GALT**) are the major site of adaptive immunity distributed alongside the intestinal mucosa. The GALT contains specialized tissues, such as the ceca tonsils, Peyer's Patches, and the bursa of Fabricius, that are organized sites with multiple lymphoid follicles to assist the antigen sampling and presentation, and the maturation and differentiation of naïve B and T lymphocytes. The adaptive immunity of chickens includes the humoral or antibody mediate response for extracellular antigens, and the cell-mediated immunity for intracellular antigens. The humoral responses are mediated by the production of IgA antibody that is stimulated by the intestinal microbes and prevents their entry and neutralize their toxins. It has been recognized different antigen mechanism for the secretion of IgA by commensal and pathogenic bacteria. Commensal bacteria induce the production of IgA with broader specificity and lower affinity by stimulating T regulatory cells (Andrew et al., 2000), while IgA secretion by pathogenic microbes is mediated by the effector T cell and have a high affinity and specificity (Bachmann et al., 1997). The adaptive immune system in commercial chickens may be fully functional at 10-days post-hatch, during this maturation period it is attributed that maternal antibodies transferred in the albumin and egg yolk confer immune protection to young chickens (Friedman et al., 2012; Hamal et al., 2006).

The high bacterial cells per gram of digesta that chickens have in the small intestine and the ceca, $10^8$ and $10^{11}$ respectively (Gong et al., 2002a), demands an effective immune mechanism to preserve the intestinal homeostasis; nevertheless, the microbe-host interaction is needed for the host to develop the gut associated immune organs. This statement is based on chicken-independent studies that showed the intestinal immune organs in germ-free models underdeveloped (Hooper et

al., 1998; Macpherson and Harris, 2004). Gnotobiotic mice had Peyer's patched with fewer germinal centers, reduced production of IgA (Macpherson and Harris, 2004; Macpherson et al., 2001), and the spleen and lymph nodes without a defined B and T cells compartmentation (Bauer et al., 1963). However, these conditions can be reversed when exposing the mice to intestinal bacteria. According to Klaasen et al. (1993) gnotobiotic mice deliberated colonized with segmented filamentous bacteria (**SFB**) restored the number of lymphoid cells in the lamina propria of the ileum and cecum and increased the secretion of IgA. The SFB are spore forming gram-positive bacteria that cluster within the genus *Clostridium* and have been reported to be commensal bacteria of humans and various animal species (Snel et al., 1995). The ability of SFB to attach to the epithelial cells may enable this group of bacteria to exclude pathogenic microorganisms (Garland et al., 1982), at the same time, this interaction appears to be beneficial for the gut homeostasis of the host and activates both: humoral and mucosa immune systems (Cebra, 1999). The depletion of SFB population from the gut of chickens with necrotic enteritis have been documented as a gut microbial alteration taking place during the onset of this disease (Antonissen et al., 2016). Another example of bacteria species assisting in the development of immune organs is the gram-negative anaerobe *Bacteroides fragilis* which monocolonization corrected the portion of $CD4^+$ T-cells in the spleen and defined the lymphocyte zones in the gnotobiotic mice model (Mazmanian et al., 2005).

Through the production of bacterial by-products SCFAs (primary acetate, propionate and butyrate), commensal bacteria can mediate adaptive immune responses (Smith et al., 2013). In particular, butyrate, which is the main energy source of colonocytes (Mingming et al., 2016), can induce the generation, trafficking, and activation of innate and adaptive immune cells (Goncalves et al., 2018). Gathered literature has demonstrated the anti-inflammatory effect of butyrate short-

chain fatty acid by inhibiting the pro-inflammatory activity of neutrophils, macrophages, dendritic cells and effector T cells (Goncalves et al., 2018). Although the exact anti-inflammatory mechanisms of butyrate remain unclear, a study that explored the effect of butyrate in the monocytes of humans and mice with colitis showed butyrate increased the level of peripheral blood T reg cells and the cytokines IL-10 and IL-12 which suppressed the tissue inflammation and destruction induced by T-helper cell 17 (Mingming et al., 2016). Another inflammatory pathway reported to be inhibited by the administration of butyrate is the IL-1β-mediated nuclear factor-kappa B activation (Lũhrs et al., 2001), that if it is activated induce the expression of multiple genes participating in the inflammatory reaction. Additionally, butyrate appears to reduce the oxidative stress of cells by down-regulating the enzyme NADPH oxidase and iNOS that reduce the migration of macrophages to the site of the lesion (Aguilar et al., 2016). An in vitro study with chicken macrophages stimulated with lipopolysaccharide and then treated with butyrate reported a reduction in the production of pro-inflammatory cytokines (Zhou et al., 2014). Chickens with intestinal infection, such as necrotic enteritis, tend to reduce the gut bacteria population that produce butyrate (Antonissen et al., 2016), but the oral supplementation of butyrate was reported to downregulate genes in pathogenic species, such as *Salmonella* spp., that participate in the invasion mechanisms of the bacterium (Van Immerseel et al., 2006). Furthermore, butyrate derived supplements have successfully reduced the population of infectious bacteria (*Salmonella* spp.) in the flock, thereby, they have been proposed as a potential antibiotic substitute (Cox et al., 1994; Fernández-Rubio et al., 2009). The stimulation of butyrate producer bacteria is a recommended strategy to maintain the intestinal health in the flock, and one way to favor butyrate producers is by the provision of non-digestible prebiotic supplements (Louis and Flint, 2009). For instance, the prebiotic xylo-oligosaccharide and mannan-oligosaccharide enhanced the growth of

*Bifidobacteria* and *Lactobacillus*, which increased the levels of acetate and butyrate fatty acids in the intestinal environment and stimulated the mucosal secretion of IgA in chickens species (De Maesschalck et al., 2015; Ding et al., 2018; Gómez-Verduzco et al., 2009; Pourabedin et al., 2015). The probiotic *Lactobacillus plantarum* and the prebiotic fructo-oligosaccharide were active against *Escherichia coli* by increasing the total SCFA, especially butyric acid (Ding et al., 2019). All these evidences showed the multiple benefits of the gut microbial metabolite SCFA at maintaining the intestinal health, therefore, there is a need to modulate the gut microbial population toward one that produces the ideal SCFA concentrations that confer protection to the host.

*The spatial bacterial variations of chickens*

The different gut sections of the chickens are dominated by different bacteria communities, and the mucosa-associated bacteria vary in species richness and in diversity from the profile of the luminal microbiota. Zhang et al. (2018) reported that the species richness estimated with Chao1 and the diversity index reported with Shannon were lower in the mucosa-associated community of the chicken compared to the lumen profile. The ceca of the chicken are the gut sections with greater bacteria richness and diversity compared to the less diverse community of the crop (Ranjitkar et al., 2016). Variations in the bacteria taxonomy and community structure of each gut segments are likely driven by the various niches supported by the different processes that each gut sections own to support nutrients digestion and absorption (Van der Wielen et al., 2002). The spatial bacterial variations in commercial chickens were initially demonstrated utilizing culture-dependent techniques, and recently confirmed with the 16S rRNA bacteria gene amplicon and taxonomic annotation with bacterial fingerprinting and massive parallel sequencing methods (Barnes et al., 1972; Choi et al., 2014; Gong et al., 2002a; Sekelja et al., 2012). The upper gastrointestinal tract,

duodenum and jejunum, contain lower number of bacteria species compared to the ceca, and are mostly dominated by facultative anaerobes from the bacteria genus *Lactobacillus*, *Enterococcus*, *Bacteroides*, and *Corynebacterium* (Choi et al., 2014). A different study reported the phyla *Proteobacteria* dominating the profile of the duodenum and *Actinobacteria* in the ileum of the chicken (Xiao et al., 2017b). Contrarily, the distal gastrointestinal tract, composed by the ceca and colon, are dominated by strict anaerobes especially from the *Clostridium* group (Wise and Siragusa, 2007; Xiao et al., 2017b). The two most dominant bacteria phyla of the ceca microbiota are *Bacteroidetes* (Xiao et al., 2017b) and *Firmicutes* (Wei et al., 2013). The cecum microbial profile contains more unique bacteria members and its community structure can be markedly distinguished from other gut sections (Xiao et al., 2017b) implying this gut segment delivers a different microenvironment and suggest a different microbial functionality.

The ceca are the gut regions with higher counts of bacteria ($10^{11}$ CFU/g) and species diversity in the gastrointestinal tract of the chicken (Barnes et al., 1972; Maglio, 1978). This feature is supported by the shape of the ceca, two blind finger sacs, that provide ideal conditions for the bacteria fermentation and further feed digestion. It was identified the ceca are the sites for the utilization and absorption of water and nitrogen (Clench and Mathias, 1995; Obst and Diamond, 1989). Such evolutionary functionalities are opportune in wild birds during stress periods when food is scarce and of low quality (Clench and Mathias, 1995). While the ceca functions may be retained in wild avian species, it is unclear if domesticated chickens preserve the same function since cecectomiced chickens had the same growth performance as chickens with intact ceca (Thornburn and Willcox, 1965). Notwithstanding, the massive aggregation of lymphocytes found in the ceca of domesticated chickens suppose this organ may function as a lymphatic organ (Hamoda and Farag, 2018). Of note, the proximal ceca regions contain the ceca tonsil that share

morphological similarities (germinal center) with the Peyer's patch in mammals (Yasuda et al., 2002). Based on this evidence, there is an immunological relevance on the microbial population of the ceca, therefore, the ceca have been the target of investigation when exploring the indigenous bacteria population of commercial chickens.

Insightful information can be attained exploring the microbial population from fecal samples, especially, when conducting longitudinal studies and the change of the microbial population is part of the research question. Studies conducted in mammal models have associate the community of the fecal bacteria with the age and the health conditions of the host (Hopkins and Macfarlane, 2002; John and Mullin, 2016; Kalliomaki et al., 2008). In fact, fecal samples are the popular sampling technique performed to investigate the gut microbial population in humans and high value animals. It was determined that the fecal bacteria contain representative microorganisms from all the gut sections of the digestive system (Sekelja et al., 2012). The fecal microbiota of chickens is composed by four groups of bacteria, two of them belong to the group of *Clostridia,* which correlate with the communities of the cecum, colon, and small intestine; and a group of *Lactobacillus* that is positive associated with the microbial population of the crop and gizzard (Sekelja et al., 2012). A comparative microbial analysis utilizing high throughput sequencing approach was conducted to compare the bacteria population between the fecal and ceca of broiler chickens (Stanley et al., 2015). That study found the communities of the ceca and feces shared a operational taxonomic units (**OTUs**) percentage, 88.55% of the 99.25% of the ceca sequences, but they were different in the abundance (Stanley et al., 2015). This suggests the fecal samples can be used as a proxy to conduct qualitative questions about the indigenous bacteria of chickens.

*Temporal bacteria variations in commercial chickens*

The gut microbial community is highly dynamic and marked bacterial changes have been accounted throughout the lifespan of the host. It was identified the dominant bacteria species vary from neonate to elder people (Agans et al., 2011; Claesson et al., 2011; Hopkins and Macfarlane, 2002; Palmer et al., 2007). Various authors have identified a gut microbial shift associated with the age of the chickens (Perumbakkam et al., 2014; Sekelja et al., 2012) that appears to be independent to the nutritional content of the diet (Oakley et al., 2014) and the genetic background of the chickens (Lumpkins et al., 2010; Van der Wielen et al., 2002). According to Oakley and Kogut (2016) the initial bacteria that colonize the chicken intestine expand and rapidly increase in taxonomic richness and diversity following hatch. It was reported the ceca bacterial community of chickens increased from five bacteria at day of hatch to 42 species at day 19 (Crhanova et al., 2011). Lumpkins et al. (2010) reported the gut microbiota development was concomitant to the development of the intestinal morphology parameters, suggesting a cooperation that can reach stability around 21 days of age. Van der Wielen et al. (2002) measured the gut bacteria changes of broiler chickens with bacterial fingerprint approach and found the number of bands did not change when chickens were older than 11 days, suggesting the enteric bacteria of chickens reach stability at 11 days. Another study measured the gut microbial population with bacterial pyrosequencing technology and revealed the community structure of the crop, gizzard, ileum, and cecum of broiler chickens stabilized around 22 days of age (Ranjitkar et al., 2016). This discrepancy found in the literature regarding the maturation period of the gut microbiota of chickens may be because of the variations on the technique to measure the bacterial community of the samples. Different gut segments may reach stability at a different time point, according to Torok et al. (2011) the ceca bacterial community mature sooner than other segment of the digestive system of commercial

chickens. Additionally, the maturation of the gut microbiota is host dependent since a fingerprinting analysis of the ileal population of egg Lohman Brown chickens (egg-type chicken) from hatch to 10 weeks reported the stabilization of enteric bacteria occurred around 42 days (Den Hartog et al., 2016). All these observations imply the age of the chicken an important factor driving the composition of the gut microbial population; therefore, more longitudinal investigations should be conducted to define the taxonomic changes occurring at different ages of commercial chicken species.

Clear phylogenetic changes have been identified in the human gut microbiome. The gut microbiota of neonate stabilize and evolve towards an adult-like structure around 3 years of age (Yatsunenko et al., 2012) and different microbial patterns have been reported among adolescence, adulthood and old-age (Agans et al., 2011; Claesson et al., 2011; Hopkins and Macfarlane, 2002; Palmer et al., 2007). The bacteria succession in the whole lifespan of the chickens was evaluated in egg-type hens including relevant microbial information from the growing period of broiler chickens (Videnska et al., 2014b). That study identified the bacteria community structure of chickens clustering in four groups and varying in the phylum of the dominant bacteria, or what the author described as stages (Figure 1).Those stages were defined as follows: Stage I from hatch to week two, was characterized by a high prevalence of representatives of the phylum *Proteobacteria*. Stage II, from week two to eight, bacteria from *Firmicutes* phylum dominated the gut microbial profile. Stage III from two to six months of age, the dominant *Firmicutes* phyla was replaced by *Bacteroidetes*. And stage IV, from seven months onward, was identified to have a constant ratio with equal numbers of bacteria representatives from the phylum *Bacteroidetes* and *Firmicutes* (Videnska et al., 2014b). However, these bacterial changes may not reflect those occurring in same age meat-type chickens since adult broiler chickens are for breeding purposes, they are feed-

restricted to avoid overweight. Such differences in farming practicing between adult chickens for meat and egg purposes may derive variations in the gut bacteria succession between these two genetically divergent breeds of chickens.

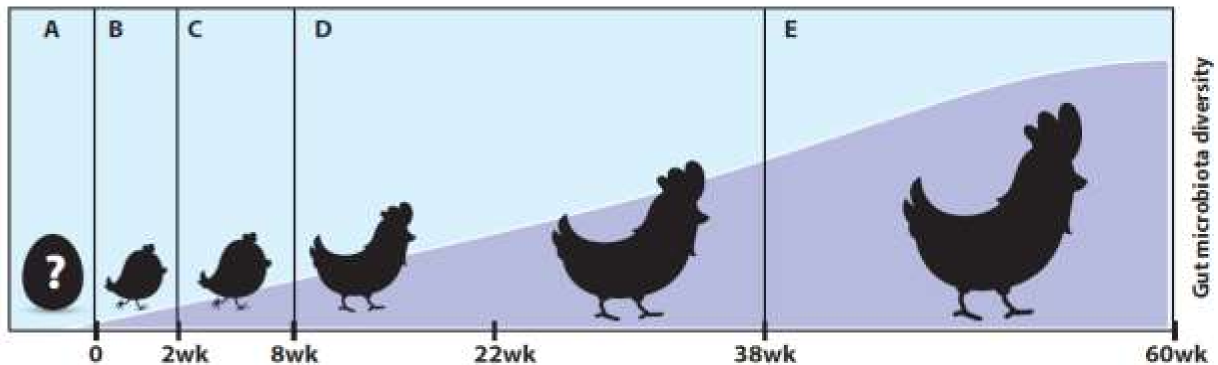


**Figure 1. Growth curve of the species richness and major change of the indigenous gut bacteria throughout the lifespan of egg-type chickens. (A) Bacteria species can be vertically and horizontally transmitted to the egg, in such a way that newly-hatched chickens own a complex bacterial community. (B) Two weeks post-hatch members of the phylum *Proteobacteria* increases and dominate the profile of the gut microbiota. (C) From 2 to 8 weeks of age, bacterial representatives of the phylum *Firmicutes* dominate the bacterial profile of chickens. (D) From 8 to 38 weeks of age members of the phylum *Firmicutes* are replaced by representatives of *Bacteroidetes*. (E) The gut microbial profile in this period is a constant ratio of equal numbers of *Bacteroidetes* and *Firmicutes*. Adapted from Videnska et al. (2014).**

*Acquisition of the gut bacteria in chickens*

It was believed chickens hatch with a sterile gastrointestinal tract, but the improvement of molecular technologies revealed low concentrations of bacteria in the intestine of the chicken embryo that grow in complexity before hatch (Kizerwetter-Swida and Binek, 2008; Pedroso et al., 2005). It remains unclear whether the colonization of bacteria begins at incubation or is a process that is initiated in the breeder flocks. Evidences have referred to the vertical bacteria transmission can take place during egg formation period since the reproductive tract of hens is not fully sterile (Buhr et al., 2002; Hiett et al., 2002) and bacteria species can be isolated from the semen and be a potential source of contamination for both: hens and eggs (Cox et al., 2002b). In fact, the

transmission of food-borne pathogens motivates a great deal of investigations to understand the transovarian bacteria transmission, because pathogenic bacteria *Campylobacter* (Cox et al., 2002a) and *Salmonella* (Liljebjelke et al., 2005) were identified to be vertically transmitted. Considering that both feces and eggs pass through the cloaca, this enables the possibility that some fecal bacteria from hens may attach to the eggshell surface. Neira et al. (2017) used massive parallel sequencing and identified that 50% of the bacteria in the eggshell surface are members of the phylum *Firmicutes* and bacteria representatives of the phylum *Firmicutes* are identified as commensal bacteria in chickens, such as *Clostridiaceae*, *Ruminococcaceae* and *Lachnospiraceae* (Ranjitkar et al., 2016). However, current farming practices minimize the exposition of the egg with maternal bacteria by frequently collecting eggs and performing disinfection actions. But the permeable nature of the eggshell may allow the horizontal bacteria transmissions during egg handling, storage, and incubation since bacteria from the hatchery environment have been associated as a source of commensal (Rodgers et al., 1999) and pathogenic bacteria (Craven et al., 2001). Based on these observations, the population of the indigenous bacteria of newly hatched chickens may be influenced by the parental and hatchery environmental conditions, thus, microbiome experiments are encouraged to homogenize the conditions to avoid variations from these sources.

*Factors affecting the composition of the gut bacteria in commercial chickens*

The composition of the avian microbiome is influenced by several extrinsic and intrinsic factors such as diet and feed supplementations, environmental conditions, and host genetic background. Of these, dietary factor has been explored with greater intensity because of the availability of various types of ingredients and feed additives to promote the development of a

balanced gut microbiota, especially now that the supplementation with antibiotic growth promoter is prohibited.

**Nutritional factor and the indigenous bacteria of chickens**

Bacterial shifts in avian species have been identified when diets alter the proportion of the macronutrients (carbohydrates, protein, and fat composition) (Duncan et al., 2007; Russell et al., 2011; Scott et al., 2013). The sources of fat and the particles size were also accounted as gut modulators of the enteric microorganisms in productive animals (Knarreborg et al., 2002; Singh et al., 2014b). Diet-induced modifications of the indigenous bacteria occur in all gut sections of the chickens (Torok et al., 2008). Two broiler diets varying in the percentage of wheat middling altered the abundance of *Lactobacillus* inhabiting the crop of the chickens (Hammons et al., 2010). There are dietary components that when metabolized by enteric microorganisms can be deleterious to the host health. For instance, the bacteria by-product of choline can cause the production of the compound trimethylamine which is absorbed and is oxidized in the liver and converted into trimethylamine N-oxide. Elevated circulation of this oxidized product were reported to impair glucose tolerance, to cause adipose inflammation (Gao et al., 2014) and induce atherosclerosis in mammals (Chen et al., 2016). In broiler species, elevated crude protein from animal and soy bean sources stimulate the growth of the pathogenic bacterium *Clostridium perfringens* which induces necrotic enteritis disease (Drew et al., 2004; Wu et al., 2014). Additionally, diet can deliver anti-nutrients such as non-starch polysaccharide (**NSP**), mostly found in diets based on wheat and rye, that increases the viscosity of the digesta and reduces the passage rate such conditions favor the overgrowth of anaerobic bacteria (Kermanshahi et al., 2018; Teirlynck et al., 2009). Nevertheless, the diet is the most effective mean can be delivered to modulate the intestinal microbiota towards

one that confers protection. To date, most of the gut microbial studies in productive animals assess the bacteria changes resulting from the addition of feed supplements with antimicrobial properties to improve productive performance, welfare, and health. Driving much of this area of research is the need to find alternatives to the use of antibiotic growth promoters. There are various feed supplements with different modes of action, but they all have in common targeting and stimulating the growth of beneficial intestinal bacteria. Among the feed additives available in the poultry industry to maintain the intestinal health and ameliorate side effect of pathogenic challenges are organic acids, direct-fed microbials, medium-chain fatty acids, and phytochemical whose proposed mode of action are discussed below.

Organic acids additives, also known as short-chain fatty acids, volatile fatty acids or weak carboxylic acids, are organic compounds that contain one or more carboxyl groups with the molecular structure R-COOH, where R is the monovalent functional group (Ng and Koh, 2017). The antimicrobial properties of organic acids rely in their ability to diffuse the bacterial cell membrane and dissociate inside the cell impairing the synthesis of proteins in the pH sensitive bacteria such as *E coli*, *Salmonella* spp., *Clostridium perfringens,* and *Campylobacter* spp. (Long et al., 2018; Mroz et al., 2006). Multiples trials under various dietary and environmental conditions have been conducted in order to assess organic acids efficacy in commercial chicken species. For instance, potassium diformate reduced the mortality and improved the concentration of butyric acid in the ceca of chickens with necrotic enteritis (Mikkelsen et al., 2009). A recent metanalysis reported diets supplemented with organic acids fed to challenged and non-challenged birds improved the feed conversion ratio, but the magnitude of improvement in the challenged birds was relative low compared to chickens treated with a therapeutic antibiotic (Polycarpo et al., 2017).

Direct-fed antimicrobials (**DFM**) or probiotic is a naturally occurring microorganisms, bacteria or yeast, able to shape the gut microbial balance when provided in adequate doses (Seo et al., 2010). Probiotic products can be combined with prebiotic, a food ingredient that is utilized by the gut microbial instead of the host (Gibson et al., 2004). Starter diets supplemented with DFM have given positive outcome, such as the DFM *Enterococcus faecium* that promoted the development of immune organs and improved the intestinal morphology of young broiler chickens (Luo et al., 2013; Samli et al., 2007). It was documented that broilers provided a starter diet supplemented with probiotic bacteria and dried whey also improved the number of lactic acid bacteria in the ileum microbial population (Samli et al., 2007) which constrain the colonization of pathogenic bacteria. Another probiotic bacterium showed to have a regulatory effect against *Campylobacteria* species is *Lactobacillus salivarius* (Saint-Cyr et al., 2017). The benefits of in-feed supplementation with DFM have been perceived at different chicken ages. Probiotic species delivered in ovo enhanced the development of intestinal microbiota at day of hatch by modulating the establishment of beneficial bacteria before placement (Pedroso et al., 2016). The supplementation with *Lactobacillus plantarum* in growing period and *Bacillus subtilis* two weeks previous to slaughtering improved growth performance and feed conversion ratio in broiler chickens (Li et al., 2016; Peng et al., 2016). Egg-laying hens supplemented with DFM products, *Enterococcus faecium,* increased egg production, improved eggshell quality, and reduced the fecal coliforms (Park et al., 2016). Molting by starvation, is a husbandry practice in egg-laying hens that improve egg production, reduces gut bacteria diversity and affect the intestinal immunity (Callaway et al., 2009), such side effects were improved when hens were supplemented with DFM products (Dastar et al., 2016).

Others feed supplements for the intestinal health are the medium-chain fatty (**MCFA**) acids and phytochemical. Medium-chain fatty acids are saturated fatty acids with 6 to 12 carbons and their proposed antimicrobial mechanisms are the penetration of the cell membrane, destabilization of the phospholipid bi-layer, dissociation, and reduction of the intracellular bacteria pH (Hanczakowska, 2017). Diet supplemented with MCFA products decreased the susceptibility to *Campylobacter jejuni* colonization in broiler chickens (Van Gerwe et al., 2010). Caprylic acid, an MCFA from coconut and palm kernel oil, reduced *Salmonella* population in the cecum and liver of egg-laying hens (Upadhyaya et al., 2015). An in-vitro study showed lauric acids reduced the number of cfu of *Streptococci* A and B and capric acids very active against *Staphylococcus aureus* (Bergsson et al., 2001). Phytochemical products are volatile and aromatic plant derived compounds with antimicrobial, antifungal, and repel insects actions (Tiihonen et al., 2010). Phytochemical may inhibit the bacterial growth by disrupting the cell wall, the cytoplasmic membrane, and produce a $K^+$ leakage (Lopez-Romero et al., 2015). Another proposed mode of action of phytochemical products is the disruption of the communications of pathogenic bacteria or quorum sensing, which inhibit the bacterial responses to produce toxins and form biofilm (Simões et al., 2009). Within the phytochemical compounds are essential oils, phenolics, terpenoids, alkaloids, lectins and polypeptides, and polyacetylenes (Simões et al., 2009). A blend of essential oils (thymol and cinnamaldehyde) were found to modified the microbial metabolite of the ceca by increasing the proportion of butyrate SCFA (Tiihonen et al., 2010). In-feed supplementation of essential oils also promoted chickens to be resilient against coccidiosis (Hume et al., 2006; Oviedo-Rondon et al., 2006) and controlled the colonization and proliferation of *Clostridium perfringens* in broiler chickens (Mitsch et al., 2004). Synergisms have been found when various feed supplements are combined and sometimes an enhanced antimicrobial action is achieved. For

instance, a blend of organic acids and phytochemical compounds were found to have better productive outcomes than the supplementation of the organic acid by itself in piglets (Bosi et al., 1999). The combination of MCFA and organic acids showed similar protective level as the pharmacological doses of zinc oxide in nursery diets in piglets (Kuang et al., 2015). All these feed supplements, either in combination or by itself, offer several options to the poultry industry to raise antibiotic-free chickens by modulating the composition of the indigenous bacteria, but their efficacy may vary according to individual husbandry and farm conditions.

**Environmental factor and the indigenous bacteria of chickens**

Our understanding of the human microbiome display the indigenous gut bacteria of neonate initially affected by the mode of delivery (Maria et al., 2010). This susceptibility of the gut microbiota to the be shaped by environmental microorganisms has also been observed in neonate piglets, whose fecal bacteria is influenced by the microbes from the slatted floor and the sows nipple (Chen et al., 2018). Although current poultry productions perform practices that minimize the exposition to maternal microbiota, environmental bacteria from the hatchery plant were identified that colonize the gastrointestinal tract of newly hatched chickens (Craven et al., 2001) and a complex structure of the resident gut bacteria was found in chicks before placement (Pedroso et al., 2005). Enteric bacteria variations have been observed between chickens from different locations, rearing regimens, and caging system (Kaakoush et al., 2014; Ludvigsen et al., 2016; Nordentoft et al., 2011). In fact, the rearing conditions is highly influential and shape the indigenous gut bacteria of the chickens at placement since studies suggest the house litter greatly contribute to the horizontal transmission of bacteria species (Cressman et al., 2010; Montrose et al., 1985; Wang et al., 2016). For instance, the ileum bacteria community of chickens placed in

new litter is dominated by *Lactobacillus* spp. while the chickens placed in reused litter develop more group of *Clostridia* (Cressman et al., 2010). The influence of new and reused litter analyzed with pyrosequencing methods at 10 and 35 days of age reported that chickens in reused litter were enriched in *Enterococcus* at day 10 and *Lactobacillus* at day 35 while an opposite pattern was observed in chickens in new litter (Wang et al., 2016). Considering chickens are coprophagic species and have steady cloacal reflexes it is very likely they uptake environmental microbes and are affected by the community of bacteria inhabiting the litter (Van der Sluis et al., 2009). These evidences demonstrate chickens are prone to be colonized by the microorganisms associated with the environment, not only from the house farm but also from the hatchery plant.

The different rearing systems have been defined as a contributor of the indigenous bacteria population of the chicken. The bacteria population of the ileum and cecum from organic farms are enriched in *Clostridium perfringens* compare to conventional chickens (Bjerrum et al., 2006). A comparative study elucidated the variations in the gut bacteria taxonomy and functionality between chickens reared in a space-limiting or conventional and chickens in roaming condition or free range (Mancabelli et al., 2016). That experiment reported that the free-range chickens developed higher bacteria diversity with more bacteria representatives from the phylum *Bacteroidetes* and *Proteobacteria*, while the conventional chickens were dominated by *Firmicutes* bacteria (Mancabelli et al., 2016; Xu et al., 2016). A metagenome investigation revealed the functional categories of the gut bacteria of free-range chickens favored the production of acetate while conventional reared chickens had more genes associated with the production of formate (Mancabelli et al., 2016). Adult chickens also appear to be influenced by the environmental microorganisms. According to Nordentoft et al. (2011), egg-laying hens placed in aviary system developed higher bacteria diversity that those in reared in cages. A room effect was observed in

microbiome experiments replicating with homogenous methodology. The study conducted by Ludvigsen et al. (2016) showed the enteric bacteria among chickens from different rooms treated with homogenous methods developed differences in the abundance of the less dominant bacteria species. Stanley et al. (2013b) consecutive replicated three times the same experiment utilizing the same chickens' provider, the same batch of diet, and the same experimental room. Interestingly, dissimilarity in the structure of the bacterial communities was reported from batch to batch (Stanley et al., 2013b), suggesting that the microorganisms from the environment may influenced and changed from one experiment to another. Based on these evidences, the environment is a confounding factor that can induce the colonization and architecture of the chicken gut microbiota. Thus, it is very likely chickens placed in different pens may develop variations in their gut microbial structure despite of equal genetic background. The same environment should be procured as much as possible when the research question is to elucidate the effect of other factors on the avian gut microbiome.

**Host genetic factors modulating the avian gut microbiota**

An analysis identified that 19% of the gut microbial variations encountered across various murine laboratories were explained by the host genetic factor (Hildebrand et al., 2013). A comparative study of the fecal microbial population between 59 mammals and humans markedly clustered the bacterial community structure based on the host phylogenetic (Ley et al., 2008). Microbiome studies with productive animals, whose genetic background is manipulated to improve feed efficiency, have demonstrated dissimilarities in the community structure of the gut bacteria based on their genetic variations. Lumpkins et al. (2010) compared the gut microbial profile of three broiler lines of chickens (two modern broilers and one historic broiler from 1957)

and showed the modern breeds of chickens clustered together in term of their bacteria architecture, whereas the historic lines of chickens clustered separated. That study concluded the similarity in growth rate of the modern broiler breeds could be responsible for the relatedness of the bacteria communities since both strains of chickens may bear comparative metabolic demands (Lumpkins et al., 2010). Notwithstanding, a gut microbial analysis with popular breeds of commercial broilers, sharing comparative growth rate (Cobb 500, Ross 308, and Hubbard), demonstrated variations in the proportion of the major phylum of bacteria (Kim et al., 2015). That study identified the ileal bacteria of Ross and Cobb broilers developed similar proportions of *Firmicutes*, *Proteobacteria,* and *Cyanobacteria,* whereas Hubbard chickens was mostly dominated by *Firmicutes* species. They also found that bacteria representatives of the phylum *Bacteroidetes* were exclusive in Cobb and *Actinobacteria* were absent in Ross chickens (Kim et al., 2015). Another study explored the associations between the host genetic background and the early microbial communities between two genetically distinct chickens with similar body weight and feed conversion ratio (Schokker et al., 2015). That study found the newly chickens of the two breeds were distinct in the abundance of the most dominant bacteria genera *Enterococcus*, *Escherichia*, and *Lactobacillus*. At 4-days of age the proportion of the bacteria *Lactobacillus* varied between breeds, and *Enterococcus* and *Escherichia* were higher in one breed of chickens at day 16. These observations demonstrate the collection of the host genes can shape the composition of the indigenous bacteria even among breeds of chickens genetically selected for similar productive purposes although with distinct genetic selection strategies.

Animals species contrasting in productive traits have been reported to differed in the membership and abundance of their enteric bacteria. For instance, dissimilarity in the fecal bacteria population was noted between lean breeds of sows compared to a local phylogenetically obese line

(Yang et al., 2014). Similar host-genetic influences were reported in mature chickens with contrasting abdominal fat deposition. Two lines of chickens, derived from a common founder population selected for high and low 56-days body weight over 54 generations, were explored regarding the composition and functionality of their enteric bacteria (Ding et al., 2016; Meng et al., 2014; Zhao et al., 2013). The variations reported in their gut microbial composition were noted even when the hens were maintained in the same house and with the same feeding regimen (Ding et al., 2016; Zhao et al., 2013). The genetic selection in commercial chickens may affect the establishment and composition of the indigenous gut bacteria, these variations might be notable between chickens with contrasting body weight and distinct productive purposes.

*Composition of the intestinal bacteria promoter of host weight gain*

The link between gut microbiota and body weight gain have reached popularity in both: human and livestock animal production. Although investigations in this topic have been driven by opposite purposes (the control in humans and the promotion in productive animals), both lines of research have coincided that the gut microbiota ecology contribute to some extent with the improvement of body weight. An experiment conducted with gnotobiotic and conventional animals encountered the gut microbiota can affect the uptake and storage of energy (Bäckhed et al., 2004). The conventionalization of germ-free mice with bacteria inoculum from an obese donor increased 60% of the body fat within the first 14 days, this was regardless of the food intake (Bäckhed et al., 2004). That experiment also observed that the conventionalized mice increased the production of hepatic triglyceride and its storage in the adipocytes, suggesting gut microbiota have a regulatory effect on the energy metabolism of the host. It appears enteric bacteria are indispensable for the host to develop a diet-induced obesity. This was noted when germ-free mice

did not become overweight when consuming a high caloric, high-fat diet (Bäckhed et al., 2007). Ley et al. (2005) also reported the intestinal microbial ecology of the leptin deficient mice (*ob/ob*); which is a genetically obese mouse, differed from the lean model by having a greater abundance of bacteria representatives of the phylum *Firmicutes* at expenses of *Bacteroidetes*. Similar *Firmicutes* : *Bacteroidetes* ratio was also identified in obese humans (Ley et al., 2006) and pigs (Yan et al., 2016a). It was identified the gut microbial population of lean subjects express more anti-inflammatory properties than an overweight counterpart (Andoh et al., 2016). The mucin-degrader bacterium *Akkermansia muciniphila*, which protect the host during inflammation by enhancing the host lipoprotein metabolism (Shen et al., 2016), is reported to be in low abundance in obese subjects (Everard et al., 2013). A review paper elucidated that a state of low-grade inflammation facilitates the incorporation of LPS to lipoproteins and increases the delivery of the LPS-rich lipoprotein in adipocytes, macrophages and other target tissue cells (Hersoug et al., 2016). The abundance of some bacteria have been correlated with the improvement of feed efficiency, weight gain, and eggs production in commercial poultry species (Angelakis and Raoult, 2010; Yan et al., 2017). Positive outcomes are observed when the proportion of *Lactobacillus* spp. is enriched in the gut profile, this is associated with a raise in the production of lactic acid. Others bacteria species associated with good performance in chickens belong to the genus *Ruminococcus* Stanley et al. (2012a), *Gallibacterium, Enterobacteriaceae, Clostridiales*, and *Lachnospiraceae* (Torok et al., 2011). Therefore, the variations encountered between sibling broilers with high and low productive performance suggest that there is a performance gut microbiota inducing growth rate (Angelakis and Raoult, 2010). Two studies authored by Singh et al. (2012); (2014a) investigated the profile of the performance-related microbiota with pyrosequencing and shotgun metagenomic sequencings. The results of those publications showed chickens with contrasting

feed conversion developed marked bacterial differences in the predominant bacteria phylum, but the identified that the phyla *Firmicutes* was always enriched in chickens with high-performance in both experiments. This suggest that high proportion of *Firmicutes* bacteria promotes the growth rate in commercial broiler chickens.

Investigators have pursued to elucidate the mechanisms by which the indigenous gut bacteria orchestrate the energy metabolism of the host. It was detected gut bacteria from overweight subjects and animals have an enhanced mechanism to extract energy from the food (Stanley et al., 2013a; Turnbaugh et al., 2006). Turnbaugh et al. (2006) used a bomb calorimeter and showed the fecal energy from obese mice contain less energy compared to the lean counterpart. The increased capacity to uptake monosaccharides of the overweight mice microbiota significantly elevated the glucose concentration and upregulated the expression of key enzymes for *de novo* fatty acid biosynthesis: acetyl-CoA carboxylase and fatty acid synthase (Bäckhed et al., 2004). The microbial enzyme bile salt hydrolase (**BSH)** have been shown to regulate the lipid metabolism of the host. High BSH activity correlated with lowering plasma cholesterol and liver triglyceride, thus, subjects with high BSH activity usually tend to reduce body weight (Joyce et al., 2014). Most bacterial phyla inhabiting the humans intestine ((Brian et al., 2008) and some strains of probiotics bacteria (Grill et al., 1995; Pereira et al., 2003) contain BSH enzymatic activity. Bile salt hydrolases from lactic acid bacteria from genetically lean chickens was higher compared to commercial chickens (Julendra et al., 2017), while another study compiled information that the suppression of BSH have a positive effect at improving growth rate in broilers fed antibiotic growth promoters (Lin, 2014). Another proposed mechanism by which enteric microorganisms can influence the energy metabolism encompasses the regulation of some host genes that participate in the lipid metabolism. According to Bäckhed et al. (2004) the indigenous gut bacteria

can regulate the fasting induced adipose factor, *Fiaf,* genes in the small intestine and when the expression of this gene is suppressed, it inhibits the activity of the lipoprotein lipase enzyme which result in the accumulation of more energy (Bäckhed et al., 2004). In fact, pharmacological applications have been proposed to target the *Fiaf* gene as an feasible intervention for the control of overweight in humans (Mandard et al., 2006). Although all previous gut microbial mechanisms have been identified using chicken independent models, they all show the potential that enteric bacteria own to regulate weight gain and fat deposition in the host. This calls for more research to determine the causes and the potential mechanisms of which gut bacteria influence contrasting productive performance poultry species.

*Molecular methods to study the avian gut microbiota*

Previous methods to characterize the bacteria communities from an environment include plating and culturing procedures where the bacterial classification was based on morphological and biochemical features (Sarangi et al., 2018). Molecular methods are popular approaches to reconstruct the bacteria ecology from an environment that skip the culturing steps and allow a direct sequence of the bacteria community. This is very advantageous since only a small fraction of the bacteria (0.1 to 10%) can survive laboratory conditions and the plating technique (Theron and Cloete, 2000). Molecular methods use the genetic material of the microorganisms to estimate the population in real time including those species already death. These methods integrate the sequencing and amplification of marker genes that are present in the vast majority of targeted microorganisms. The popular marker gene used to reconstruct the bacteria phylogeny is the 16S rDNA gene since it is ubiquitous in all prokaryotes and contains conserved and variable regions (Woese and Fox, 1977). This gene encodes for the 16S ribosomal RNA that is part of the 30-small

subunit of the ribosome and participates in the initiation of the synthesis of proteins (Wimberly et al., 2000). It comprises 1500 nucleotides grouped in nine hypervariable regions (V1-V9) that are flanked by the conserved domains. The conserved regions of the 16S rRNA have showed littler evolution among prokaryote species (Ludwig et al., 1994) retaining its functionality (Tsukuda et al., 2017). Therefore, the conserved regions are the target sites for the attachment of universal primers since they remain constant across different taxonomic branches of prokaryotes species. On the other hand, the hypervariable regions retain unique information that are used as the species signature and grouping since closely related species share similarities in these regions. Different regions of the 16S rRNA have been targeted over the past decade to reconstruct the indigenous gut bacteria of avian species. Studies had used either a single region, such as the V3 (Lei et al., 2012; Luo et al., 2013; Mourand et al., 2014), V4 (Best et al., 2016; Meng et al., 2014; Mon et al., 2015; Reed et al., 2015), and V6 (Perumbakkam et al., 2014), or multiple regions, such as V1-V5 (Singh et al., 2012), V1-V3 (Choi et al., 2014; Oakley et al., 2014), V5-V6 (Van der Hoeven-Hangoor et al., 2013), V6-V8 (Neumann and Suen, 2015) to classify the indigenous bacteria of chickens. A study conducted by Youssef et al. (2009) sequenced bacteria samples using eight candidate fragments of the16S rRNA gene and a nearly full-length fragment. They reported the fragment targeting the V4, V5-V6, and V6-V7 regions provided comparable estimates of the taxonomic signature with the nearly full-length fragment, thus, any of the aforementioned are recommended candidate fragments to construct the bacteria taxonomy from a sample.

Prior to the high throughput sequencing technology, recombinant DNA techniques were used to study the gut microbiota of commercial avian species; and bacterial fingerprinting techniques have provided insightful information in this regard. Researchers were able to determine the collection of the genes in the enteric bacteria exceed those that made up the host genotype (Liu

et al., 1997; Muyzer et al., 1993). Implementing bacterial fingerprinting it was detected the spatial bacteria variability among the different gut sections of avian species that vary in number of bacteria and in diversity (Van der Wielen et al., 2002). The two most popular bacterial fingerprinting tests used to explore the chicken gut microbiota are terminal restriction fragment length polymorphisms (**T-RFLP**) and denaturing gradient gel electrophoresis (**DGGE**) (Gong et al., 2002b; Hammons et al., 2010). Briefly, these two techniques amplify DNA fragments with PCR followed by a separation of the amplicon in an agarose gel. The amplification in T-RFLP differed from DGGE in the type of the restriction enzymes utilized to cut the DNA at specific sequence motifs (Phadke et al., 2017). While the DNA separation in DGGE is performed in a polyacrylamide gel that contains a gradient of denaturing agents, such as urea and formamide, T-RFLP uses gel electrophoresis (Phadke et al., 2017). The information obtained from these two techniques have low sensitivity to detect bacteria species in low abundance and cannot estimate diversity parameters (Muyzer et al., 1993). Therefore, a better insight into the microbial ecology from an environment has been obtained applying high throughput sequencing technology, also known as next generation sequencing (**NGS**).

Next generation sequencings are modifications of the Sanger sequencing but are less labor intense since Sanger sequencing clone the DNA fragment separated from the amplification steps (Sanger et al., 1977), while NGS combines the cloning and sequencing steps that are consecutively performed in the same reaction. There are different NGS platforms contrasting in their chemistry, run time and resolution, but all of them share similar fundaments. A comprehensive review conducted by Dr Mardis (2013) explained the general principles among NGS platforms. The first common principle is that all NGS platforms require the construction of a library. The library is the preparation of the nucleic acid by PCR fragmentations of the target DNA molecule to smaller

pieces followed by the ligation with adapters that are unique on each platform. Another shared-feature mentioned in that review was that the amplification of the library occurs in a solid surface (bead or glass). This step is required in all NGS platform as every time the library is amplified the instrument reads the nucleotides recently incorporated, therefore, the sequencing and the detection steps are simultaneously occurring in the same reaction. Each sequencing instrument capture the light generated on each reaction and then it is translated to digital reads which are later used to quantify and align against a reference sequence (Mardis, 2013). Because this technology allow multiple samples to be run in the same reaction or multiplex, millions of billions of DNA fragments are generated in the same reaction, therefore, NGS is also termed "massive parallel DNA sequencing" (Mardis, 2013).

The most popular NGS platforms used for microbial analysis are the Illumina and Roche/454 FLX. In Illumina the library is constructed using Illumina-specific adapters, and the amplification of the DNA fragments occurs in a flow cell that contain complementary oligos to the adapters used to construct the library. The next step is the creation of clusters from individual library by bridge amplification. The sequencing methods is performed by the incorporation of a reversible fluorescent and terminated nucleotide (Mardis, 2008). In this step all four nucleotides and the DNA polymerase are added in the flow cell simultaneously (Mardis, 2008), however, because the nucleotide is terminated it only allows the incorporation of one nucleotide at a time. The nucleotide incorporated is excited and produces a light that is captured by the sequencing instrumentation. After the imaging, the fluorescent molecules, the polymerase and the nucleotide that were not incorporated in the reaction are washed away and a new cycle begin with the supply of new nucleotide and polymerase. This process is repeated for a specific number of cycles which determine the length of the sequences (Mardis, 2008). On the other hand, Roche/454 FLX

sequencing uses pyrosequencing technology that releases a pyrophosphate every time a nucleotide is incorporated, with this it is initiated a series of reactions that end up with the production of light (Mardis, 2008). The instrument captures the intensity of the light which is proportional to the amount of each nucleotide that has been incorporated. The DNA is broken into fragments with restriction enzymes following the attachment of adaptors, then the library fragments are attached to an agarose beads that contain complementary oligonucleotides. The process of DNA amplification is conducted on agarose beads and generate identical DNA strands within the same bead. Individual beads are placed into a well of a sequencing plate together with the DNA polymerase, the primers, and one type of nucleotide at a time. Each well will produce a light that is specific to the nucleotide recently incorporated (Mardis, 2008).

The advent of sequencing technologies is supported by advanced computer programs, which enable the development of algorithms to filter and align the massive amount of data generated by each sequencing technology. Bioinformatics discipline combines Statistics and Mathematics to create programs for the analysis of biological data and to update databases for genome references. A popular open-source software to process raw microbiome DNA data, is QIIME (Quantitative Insights Into Microbial Ecology). This program combines existing and new algorithms (Navas-Molina et al., 2013) to perform a variety of measurement for microbial ecology (Caporaso et al., 2010). Data in QIIME is analyzed in two stages denominated an upstream stage; that includes processes for cleaning, filtering, removal of primers and barcodes sequences, checking and removal of chimera, and a downstream stage to make taxonomic summaries and measurements of ecological diversity (Navas-Molina et al., 2013). The operational taxonomic unit (**OTU**) is assigned based on a similarity threshold and QIIME uses 97% cutoff against a template gene (Navas-Molina et al., 2013). The alignment of the sequences in QIIME can be performed

using different databases, such as Greengenes (DeSantis et al., 2006), RDP (Cole et al., 2009), SILVA (Quast et al., 2013), or a custom database. There are three options for OTU picking in QIIME: 1) an open-reference approach which cluster reads against a reference sequence and the reads that did not hit the references sequence are clustered de novo 2) a close-reference approach that matches the sequences with a references sequence and exclude those read without a hit and 3) de novo approach that clusters reads against one another without an external reference sequence (Navas-Molina et al., 2013). According to Navas-Molina et al. (2013) the recommended OTU picking strategy is open-references because it provides the best trade-off between the time and the ability to discover novel diversity. The last step of the upstream-stage is the construction of the OTU table, that is a matrix with the taxonomic prediction for each OTU (Navas-Molina et al., 2013). The downstream analysis consumes less time and diversity indices and taxa summary can numerically and graphically be depicted from the OTU table.

Diversity parameters to measure microbial ecology from a sample are adapted from ecological investigations conducted in large organisms. Although the definition of a species for microorganisms is somewhat complex species are clustered based on shared genetic information which threshold of similarity is defined by the researcher. This threshold can be defined at 95% or 97% of similarities. The OTUs are assigned based on the percentage of the sites that agree in a pair-wise comparison (Nguyen et al., 2016). The most common similarity threshold utilized to construct OTUs is 97%. This level was defined in Konstantinidis and Tiedje (2005) study that found 70 closely related bacteria sharing 97% of similarity of the 16S rRNA gene. The literature uses the term OTU interchangeably with species, since both explain a specie or a group of species based on shared DNA similarity (Bohannan and Hughes, 2003).

Ecology diversity seek to investigate how many species and to describe its abundance and distribution in a sample. Alpha diversity indices estimate the total number of microorganisms or the species richness in a chosen assemblage (Magurran, 2004c). Species richness can be estimated by the extrapolation from known species accumulation-curves, which are graphs demonstrating the rate at which new species are found, in combination with non-parametric approaches (Magurran, 2004c). The use of non-parametric methods to calculate species richness is considered a powerful tool that are highly encouraged as they are not based on the abundance of the species (Magurran, 2004c). A simple parameter within this category is the Chao 1 that sum the absolute number of species from the sample with the ratio of singletons (the number of species represented in just one sample) and doubletons (the number of species represented in two sample) (Hughes et al., 2001; Magurran, 2004c). Therefore, the addition of the singleton/doubleton in the Chao1 formula will generate a greater number than the observed species richness that is generated by the presence/absence method. There are other alpha diversity parameters that consider the phylogenetic relatedness and the abundance of the OTUs; therefore, ecological diversity should be supported by the combination of different alpha estimates that considered the species richness and the abundance of the species. The Shannon diversity index provide is an alpha estimate that combines the species richness and evenness. Shannon index calculates the number of observed species in the sample weighted by their abundance, which is a measurement for heterogeneity (Magurran, 2004a). The Simpson index, is another diversity index that emphasizes in the dominant species, this means the higher the value the lower the diversity (Magurran, 2004a).

Alpha diversity indices describe the diversity of a defined sample but they do not compare the community structure across different environment. This comparison is achieved with the beta diversity that calculates the distance or dissimilarity between two or more samples (Magurran,

2004b). Different distance metrics can be used to determine the distance between communities varying in the formula to calculate the distance. The Jaccard distance, for instance, calculates the beta diversity in a binary approach and uses the presence/absence criteria of the shared taxa between samples. Another distance metric is the Bray-Curtis that calculate dissimilarity based on the relative abundance of the shared taxa (Bray and Curtis, 1957). The Euclidean distance, calculate beta diversity with straight lines distances between two points without weighing the abundance of the species, instead it uses the proportion of the species that are observed in the environment once and twice (Bohannan and Hughes, 2003). An approach developed to calculated the phylogenetic distances between microbial community is the Unique fraction metric popular knowns as UniFrac (Lozupone and Knight, 2005). The UniFrac metric computes the phylogenetic distances between communities as the fraction of the branch length of the tree that leads to descendants from either one of the two communities (Lozupone and Knight, 2005). Additional features of the UniFrac distance is that it can be weighed the relative abundance of the OTUs (Weighted UniFrac), which provides information about the dominant species, and also measure the phylogenetic distances based on the presence/absence information (Unweighted UniFrac) which information includes the low abundant or rare species (Lozupone and Knight, 2005). The open-source QIIME provides a repertoire of scripts to calculate various alpha diversity parameters and dissimilarities between communities with non-phylogenetic and phylogenetic distances metrics. This standardizes microbiome analysis among different investigation branches and, in some extent, facilitates the process to those investigators with littler bioinformatics background.

To summarize, the gut microbiota is an important area defining the health and productivity of commercial avian species that can be explored with high throughput sequencing methods. Various factors have been identified to modulate the intestinal bacteria of chickens including diet,

the chickens genetic and environment. The influence of the host genetic and the environment have been less explored compared to the diet factor in commercial chickens. Our understanding of the host genetic gut microbiota interaction is of relevance since the poultry industry is constantly improving the productivity through the genetic selection. Some genetic programs result in the need to make special accommodation to extend the chicken's productivity, such as feed restriction in broiler breeder hens which may have implications on animal welfare. However, ad libitum feeding impairs the metabolism and shorten the lifespan of broiler breeder hens. The intestinal microbiota has not been explored in the overfed hen model. It is known environmental factors can promote bacteria variations between pens and even among sibling chickens in homogenous conditions and be the source of pathogenic bacteria, such as *Clostridium perfringens* that produce necrotic enteritis. Gut microbial information in avian species exploring the host genetic and environment factors is lagged behind compared to investigations in mammals, therefore, more studies addressing these factors are needed in commercial chickens. Therefore, three experiments were designed to study the effects of host-biology and environmental conditions on the gut bacterial composition:

1. Two genetically divergent chicken lines (meat-type and egg-type) sharing the same rearing area and diet
2. Individually caged broiler breeder hens maintained with feed-restricted or fed-satiated regimen
3. Early gut microbial changes in chickens with necrotic enteritis in a naturally induced model

# CHAPTER II

# COMPARATIVE STUDY OF THE FECAL BACTERIA OF GENETICALLY DIVERGENT MEAT- AND EGG-TYPE CHICKENS WHEN CO-HOUSED

## *Introduction*

The avian intestinal microbiota has driven more investigations to comprehend its interaction with current farming practices, avian health, and productive parameters. Many factors have been detected to affect the gut microbiome of commercial chickens (Choi et al., 2018; Hammons et al., 2010; Hou et al., 2016; Kers et al., 2018). Both biology and the environment are key factors recognized as important drivers of the gut bacterial composition of commercial chickens (Cressman et al., 2010; Ding et al., 2017; Ding et al., 2016). The genetic background of chickens and the composition of the bacteria in the rearing pen can define the colonization patterns and the succession of the enteric microorganisms in a flock (Schokker et al., 2015). Bacteria species colonize the gut of newly hatched chickens in the hatchery, and by the time of placement, chickens own a complex bacterial community (Pedroso et al., 2005). The enteric microorganisms are further shaped by the conditions encountered in the rearing area (Ludvigsen et al., 2016). For instance, a different gut microbial population is promoted when chickens are reared with fresh and reused litter (Cressman et al., 2010; Wang et al., 2016), and between conventional and free range regimens (Mancabelli et al., 2016; Xu et al., 2016). A comparative microbial analysis of the ceca bacteria between free-range and cage-range regimens was conducted with two local breeds of meat-type chickens from China (Sun et al., 2018). That study found the phylum *Firmicutes* dominating the bacterial profile in both systems but the proportion of *Lactobacillus* spp. was higher in the caged-system regardless of the genetic background of the experimental chickens. Another

distinctions between the two rearing systems were reported at the phylum *Bacteroidetes* that was enriched in the free-range chickens whereas *Proteobacteria* species were more abundant in the cage-raised chickens (Sun et al., 2018). The modulatory effect of the environment on enteric microorganisms was also noted in rearing cage systems of adult hens (Nordentoft et al., 2011). In that study, the ileum and the bacterial community of the ceca clustered together depending on the cage system hens were placed; either conventional, furnished cage or aviary. All previous observations highlight the influential role of the environmental conditions in shaping the indigenous bacteria of chickens.

The contribution of the chicken genotype in shaping the indigenous bacteria remains elusive. A handful of scientific investigations have explored the genetic-gut bacteria interaction in experimental models that uses various breeds of meat-type chickens (Kim et al., 2015; Lumpkins et al., 2010; Schokker et al., 2015) and chickens selected for high and low 56-day body weight (Ding et al., 2016; Meng et al., 2014; Zhao et al., 2013). A study that used popular breeds of chickens for meat production (Cobb 500, Ross 308, and Hubbard) demonstrated that the major gut bacteria phyla were different among the chicken breeds despite of same diet and homogeneous methodology (Kim et al., 2015). That study identified the ileal bacteria of Ross and Cobb broilers shared similar proportions of *Firmicutes*, *Proteobacteria,* and *Cyanobacteria,* while Hubbard chickens were mostly dominated by *Firmicutes* species. Additional findings were observed in the representative bacteria of the phylum *Bacteroidetes,* that exclusively inhabited the ileum of Cobb chickens, and *Actinobacteria* that was absent in Ross chickens (Kim et al., 2015). A different report found that newly hatched broiler chickens of two type of strains were distinct in the abundance of the dominant bacteria genera *Enterococcus, Escherichia*, and *Lactobacillus*. At 4 days of age, the proportion of *Lactobacillus* varied between breed of chickens and at day 16 *Enterococcus* and

*Escherichia* were enriched in one of the breed than the other (Schokker et al., 2015). Since both breeds of chickens proceeded from different parent flocks, the initial bacterial difference found at hatch might be influenced by maternal components that drove the variations at the later age. However, it has been reported that chickens with similar growth rate develop a similar gut bacterial community. According to Lumpkins et al. (2010), the bacterial community of two modern lines of chickens (multipurpose and high-yield) shared more similarities between each other than with a slow growth rate historic line of chicken (Athens Canadian Random Bred). A series of experiments that investigated whether the enteric bacteria of the chicken can be associated with its fat deposition were conducted utilizing two lines of chickens derived from a common founder population selected for more than 50 generations for high and low body weight (Ding et al., 2016; Meng et al., 2014; Zhao et al., 2013). Next generation sequencing detected the contrasting selection criteria affected 29 bacteria species at 35 weeks of age (Zhao et al., 2013). Meng et al. (2014) reported that the chickens selected for low body weight had bacteria species with moderate heritability, like *Bacillaceae*, *Flavobacteriaceae*, *Helicobacteraceae*, *Comamonadaceae*, *Enterococcaceae*, and *Streptococcaceae*, while the chickens selected for higher body weight had zero bacteria heritability. Furthermore, the whole microbial genome sequencing analysis conducted by Ding et al. (2016) annotated some variations in the microbial functionality between the high and low body weight hens. Altogether, this information shows that the quantitative genetic background of the chickens can drive differences in the community structure of the gut bacteria between long-term genetic selected chickens for contrasting purposes. Differences may be noticeable between breeds with variations in body weight and metabolism and selected for meat and egg production.

Mainly, commercial chickens are selected for two purposes, to yield meat or lay eggs. The prolonged genetics selection has made these two lines of chickens phenotypically and metabolically distinct. Meat type or broiler chickens are characterized by their rapid growth rate, feed efficiency to convert lean protein, and hyperphagia compared to the slow growth and lower feed intake of egg-type chickens. Differences between meat- and egg-type chickens have been noted in their mechanisms governing appetite and feed consumption (Cline et al., 2009; Cline et al., 2008), and in their immune responses. Broilers and egg-laying chickens respond differently against infections such as *Campylobacter jejuni* (Han et al., 2016), infectious bursal disease virus (Aricibasi et al., 2010) and *Salmonella enterica* serovar Enteritidis (Abasht et al., 2009). In fact, significant differences in their intestinal immunity have been recorded while in their health state; layer chickens have higher counts of CD4+ and CD8+ T lymphocytes in the lamina propria and greater expression of proinflammatory IL-8 and IL-6 in the ceca than broiler chickens regardless of the age (Han et al., 2016). Such inherent immunological differences between broiler and layer chickens make these two breeds of chickens a good animal model to study the relationship between innate immune function and the intestinal microbiota. Microbiome analyses have been mostly investigated in broiler species. A recent literature review revealed only two publications elicited gut microbial information that include both meat-type, or broiler chickens, and egg-type, or layer chickens, within the same experiment (Kers et al., 2018). One of the publication was authored by Videnska et al. (2014a) and constructed a core microbiota of chickens combining the fecal bacteria information from a healthy 62-weeks old egg-laying flock with 3.5-weeks average age broiler chickens. This difference in the age of chickens is a disadvantage to make gut microbial comparisons, not only because the bacteria profile is dominated by different bacteria species but also the complexity of the microbial population increases with the age of the chickens (Awad et

al., 2016; Ballou et al., 2016; Ranjitkar et al., 2016). For instance, the ceca of broiler chickens from 2 to 4 weeks of age is nearly dominated by the phylum *Firmicutes,* egg-laying hens older than 7 months have an equal distribution of the bacteria from the phylum *Firmicutes* and *Bacteroidetes* (Videnska et al., 2014b). The second publication was authored by Han et al. (2016) and utilized same age broiler-type (Ross-308) and layer-type (Lohmann Selected Leghorn) chickens to compare the ceca bacterial community when fed either a commercial broiler or layer diet. The aforementioned study found that the intestinal bacterial communities were affected by both: the diet and the genetic background of the chickens. Notably, experimental trials including broiler and layer chickens remain easily identifiable beginning at 1-week of age showing contrasting body weights, 150g vs 63g. This enable both strains of chicken to be co-housed to minimize environmental effects product of different rearing pens and annotate the possible contributions of the host genetics in shaping gut microbial parameters. Therefore, the effect of the host genetics on gut microbial populations can be study combining meat-type and egg-type chickens in the same rearing pen and feeding a common diet.

Studies comparing enteric bacteria among different breeds of chickens are scarce. Importantly, available publications have used an experimental design that may inadvertently influence outcomes because while each breed is kept in the same house, they reside in physically separate pens. This design is not sufficiently rigorous to exclude variables such as differences in hatchery and environment microbiota that could alter initial microbiota exposure at chick placement. In this study, it was proposed to compare the fecal microbial population between broiler and layer chickens exposed to the same environment from hatchery to finishing. It was hypothesized that the differences metabolism defining growth rate and feed consumption on those chicken breeds can drive variations in the composition and architecture of the indigenous gut

bacteria. Two independent experiments were conducted, where chickens were co-incubated and co-housed and the nutrition was standardized, with both lines of chickens consuming a phased broiler diet. Since in-feed antibiotic growth promoters alter the community structure of the gut microbiota of chickens (Araujo et al., 2014; Wise and Siragusa, 2007), one of the experiment was supplemented with this antimicrobial product to model potential enteric bacteria responses under commercial conditions. Since this is a longitudinal experiment, the community structure and composition of the fecal microbiota was obtained by re-sampling the same bird at different time-points. The bacterial composition was constructed by sequencing the 16S ribosomal RNA (rRNA) gene with Illumina platform and bioinformatic processing in QIIME.

*Materials and Methods*

**Animal husbandry**

Animal husbandry was done at the Poultry Science Research, Teaching, and Extension Center, Texas A&M University (TAMU) and approved by the Institutional Animal Care and Use Committees in accordance with the Animal Welfare Act (IACUC 2015-0160), the Public Health Service Policy and the Humane Care and use of Laboratory Animals.

Two independent experiments were conducted using the same breeds of chickens, incubation and rearing procedures. Embryonated Hubbard-Hubbard broiler eggs were acquired from Ideal Poultry Breeding Farms (Cameron, TX) and Hy-Line fertile eggs were taken from a stock maintained at the Poultry Science Research facility, Texas A&M University. Foam incubators (HovaBator) were loaded with half broiler eggs (~ 20 eggs) and half layer eggs. Fertile eggs were removed from the egg turn-tray and were placed into the wire incubator floor at day 18. The eggs were kept physically separated by breed by placing a middle wire-wall in the incubators

machine. At hatch, chickens from both strains were individually identified using wing banding and placed in the same rearing area, which consisted of a single 12 x 10-foot floor pen with new litter shavings.

**Diets**

A phased feeding program formulated for broiler chickens was provided in both experiments. Chickens had free access to feed and water.

***Experiment 1***. 10 broiler and 10 layer chickens were co-housed for a period of 4-weeks. Chicks were provided with a commercial starter diet containing 227g/Ton amprolium and 50 g/Ton bacitracin methylene disalicylate from hatch to week 2 (Table 1) and with a locally manufactured corn-soy broiler grower diet from week 3 to the end of the experiment (Table 2).

***Experiment 2***. 5 broiler and 5 layer chickens were co-housed for a period of 7-weeks. Birds were raised with a three phases diet, locally manufactured without antimicrobial growth promoters. The starter diet was provided from hatch to week 2, the grower I from week 3 to 5, and grower II diet from week 6 to 7 (Table 3).

**Table 1. Calculated nutrient composition of commercial broiler starter diet in Experiment 1 (Show Broiler starter pellet)**

| Nutrients[1] | wt% |
|---|---|
| Crude Protein | 26 |
| Lysine | 1.50 |
| Methionine | 0.55 |
| Crude fat | 6.00 |
| Crude fiber | 3.00 |
| Salt | 0.45 |
| Calcium | 1.20 |
| Analyzed nutrient | |
| Metabolizable energy (kcal/kg) | 3156 |

[1]Active drug ingredient Amprolium 227g/ton and Bacitracin Methylene Disalicylate 50g/ton

**Table 2. Ingredients composition and calculated nutrient values of local broiler grower diet in Experiment 1 (15-29d)**

| Ingredients | wt% |
|---|---|
| Yellow corn | 63 |
| Dehulled soybean meal | 30 |
| Fat blended (animal/vegetable) | 3.35 |
| Salt | 0.31 |
| DL-methionine | 0.24 |
| Lysine HCL | 0.14 |
| Limestone | 1.59 |
| Monocalcium phosphate | 1.40 |
| Trace mineral [1] | 0.05 |
| Vitamins [2] | 0.25 |
| Calculated nutrient composition | |
| Metabolizable energy (kcal/kg) | 3131 |
| Protein | 20 |
| Lysine | 1.15 |
| Methionine | 0.55 |
| TSAA (methionine + cystine) | 0.88 |
| Crude fat | 6.03 |
| Crude Fiber | 2.54 |
| Calcium | 0.91 |
| Available phosphorus | 0.41 |

[1] Vitamin premix/kg diet 11023 IU vitamin A, 3858 IU vitamin $D_3$, 46 IU vitamin E, 0.0165 mg $B_{12}$, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 261.2 mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin.

[2] Trace mineral premix/kg diet 149 mg manganese, 125 mg zinc, 17 mg iron, 7 mg copper, 1.0 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium.

[3] Data is presented in mass percentages unless otherwise indicated

**Table 3. Ingredients composition and calculated nutrient values of diets formulated for broiler chickens in Experiment 2**

| Ingredients (wt%) | Starter | Grower I | Grower II |
|---|---|---|---|
| Yellow corn | 60.34 | 64.73 | 64.55 |
| Dehulled soybean meal | 32.74 | 29.33 | 27.25 |
| Fat blended (animal/vegetable) | 2.43 | 2.0 | 4.25 |
| Salt | 0.46 | 0.48 | 0.38 |
| DL-methionine | 0.28 | 0.07 | 0.25 |
| Lysine HCL | 0.30 | 0.02 | 0.16 |
| Limestone | 1.57 | 1.46 | 1.57 |
| Monocalcium phosphate | 1.57 | 1.58 | 1.28 |
| Trace minera[1] | 0.50 | 0.05 | 0.05 |
| Vitamins[2] | 0.25 | 0.25 | - |
| Calculated nutrient composition | | | |
| Metabolizable energy (Kcal/Kg) | 3047 | 3055 | 3199 |
| Protein | 21.45 | 19.80 | 19 |
| Lysine | 1.35 | 1.05 | 1.10 |
| Methionine | 0.60 | 0.38 | 0.54 |
| TSAA (methionine + cystine) | 0.95 | 0.72 | 0.86 |
| Crude fat | 5.04 | 4.74 | 6.95 |
| Crude Fiber | 2.60 | 2.57 | 2.48 |
| Calcium | 0.95 | 0.90 | 0.89 |
| Available phosphorus | 0.45 | 0.45 | 0.38 |

[1] Vitamin premix/kg diet 11023 IU vitamin A, 3858 IU vitamin $D_3$, 46 IU vitamin E, 0.0165 mg $B_{12}$, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 261.2 mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin.
[2] Trace mineral premix/kg diet 149 mg manganese, 125 mg zinc, 17 mg iron, 7 mg copper, 1.0 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium.
[3] Data is presented in mass percentages unless otherwise indicated

## Chicken sampling

Fecal samples were collected by placing individual birds in a five-gallon plastic bucket, previously cleaned and disinfected with 70% ethanol and freshly voided feces were sampled with a sterile disposable spatula (Corning, NYS 14831). The samples were placed in a 5mL sterilized screw-cap tube (Argos Tech, IL 60061) frozen and stored at -80$^{o}$C without any additives or pretreatment prior to DNA extraction. Fecal samples were obtained from the same bird throughout the experimental period in both experiments. In Experiment 1, the fecal samples were collected at

1, 2, 3, and 4 weeks of age; in Experiment 2, collection occurred at 1, 3, 5, and 7 weeks of age. Fecal samples were sent to MrDNA laboratory (www.mrdnalab.com, Shallowater, TX, USA) for the extraction of the 16S rRNA bacterial gene and bacteria sequencing.

**Bacterial DNA isolation and sequencing**

The fecal bacterial DNA was extracted using PowerSoil DNA isolation kit (QIAgen, USA). Polymerase chain reaction was used to amplify the V4 hypervariable region of the 16S rRNA bacterial gene with the forward primer 515F: GTGCCAGCMGCCGCGGTAA and the reverse primer 806R: GGACTACHVGGGTWTCTAAT using the HotStar Taq Plus Master Mix Kit (QIAgen, USA) following the manufacturer's instructions. The thermal cycling was an initial denaturation at 98°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s, and elongation at 72°C for 1 min, and 72°C for 5 min. The PCR products were checked in a 2% agarose gel and then pooled in equal proportions based on the molecular concentration. The samples were pooled and calibrated with Ampure XP beads. The pooled samples and purified PCR products were used to prepare the Illumina library. The massive parallel sequencing was conducted in a Miseq Illumina following the manufacturer's guidelines. The bacterial DNA extraction, amplification and sequencing was completed by MrDNA laboratory (www.mrdnalab.com, Shallowater, TX, USA).

**Bioinformatics analysis**

The raw sequences were processed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline v 1.9 (Caporaso et al., 2010). First, the sequences were de-multiplexed and barcodes removed following the default QIIME setting for quality filtering. Chimera sequences were identified and filtered with the USEARCH 6.1 pipeline (Edgar, 2010). Operational taxonomic

units, **OTUs**, were constructed with open-reference approach and the taxonomic assignment was performed against the Greengenes 13.8 database with a 97% cutoff (DeSantis et al., 2006). In this process of picking OTUs with open references the reads are aligned against a reference sequence and the new reads are grouped based on De novo strategy. In De novo OTUs picking the reads are grouped together without a reference genome. According to Navas-Molina et al. (2013) the recommended OTU picking approach is open-references, because it provides the best trade-off between the time and the ability to discover novel diversity. Sequences assigned to chloroplast, mitochondria, and OTUs with < 0.01% abundance were removed. Rarefaction curves were constructed to reduce variability among the length of the reads. Rarefaction in Experiment 1 was performed at a depth of 13,260 reads per sample and in Experiment 2, at 70,750 reads per sample. Those rarefaction levels complied with the minimum depth recommended to dataset sequenced in Illumina platform (1000 sequences/sample) (Navas-Molina et al., 2013). Estimation of the bacteria richness and diversity within samples were conducted with the alpha estimates Chao1, Observed species, and the diversity index Shannon with the QIIME default scripts. The bacterial community structures between samples, or beta diversity, was calculated with the Weighted and Unweighted UniFrac distances and the distance matrices were collapsed into a 3D principal coordinate analysis (**PCoA**) generated with QIIME default (Lozupone and Knight, 2005). Because there is a strong correlation between phylogeny and biomolecular functions, **PICRUSt** (Phylogenetic investigation of Communities by Reconstruction of Unobserved states) tool was used to infer the gene family abundance from each organism utilizing the KEGG pathway database (Langille et al., 2013). The prediction was generated with the online Langille Lab PICRUSt server (http://galaxy.morganlangille.com/) (Langille et al., 2013). Additionally, the accuracy of the

prediction was estimated with the nearest sequence taxon index (**NSTI**) that quantify the availability of the nearest genome representative for each sample (Langille et al., 2013).

**Statistical Analysis**

Alpha diversity estimates and taxa abundance were evaluated for normality with Shapiro-Wilk Test, and, due to a failed normality assumption they were assessed using Kruskal-Wallis (JMP Pro 13, SAS software Inc.). Linear discriminant analysis with effect size (**LEfSe**) was performed to elucidate differences in the bacterial taxa abundance between broilers and layers (Segata et al., 2011) using the Galaxy workflow (http://huttenhower.sph.harvard.edu/galaxy/) with $\alpha = 0.05$ and LDA score = 3.0. This is a statistical tool to identify the differential features between groups with high-dimensionality data, in this case the differentially abundant OTUs between broiler and layer chickens. LEFSe initially detects the differences in taxonomic abundance with Kruskal-Wallis sum-rank test following by a pairwise test Wilcoxon rank-sum test and the features with significant differences are used to perform a linear discriminant analysis model to estimate the effect size of each differentially abundant feature (Segata et al., 2011). The similarity in microbial communities was numerically computed with ANOSIM (Analysis of Similarity) test in PRIMER 6 software (PRIMER-E ltd., Luton, UK) between chicken breeds, samplings points, and phases of diet.

Non-parametric univariate test, Kruskal-Wallis test (JMP Pro 13, SAS software Inc.) was performed to compared relative abundance between breed of chickens at each taxonomic level. It was used a Dunn method for joint ranking that uses Bonferroni adjustment for multiple comparisons to compared the temporal variations and the effect of the diet on bacteria diversity

indices and to obtain the connecting letters (JMP Pro 13, SAS software Inc.). Statistical significances were considered at $P$ value < 0.05.

### *Results*

The objective of these experiments was to assess and compare the fecal bacteria community between the genetically divergent broiler and layer chickens when co-incubated, co-housed and provided with identical feed. Two independent experiments were conducted at different places and time utilizing the same breeds of chickens. The bacterial composition of fecal samples was investigated by sequencing the 16S ribosomal RNA gene and several alpha estimates and beta diversity were generated with QIIME pipeline.

*Experiment 1, Comparison of the fecal bacterial communities between broiler and layer chickens with antimicrobial supplementation in starter diet*

The total number of fecal samples was 80 (n=10 broiler and 10 layers) collected at 1, 2, 3, and 4 weeks of ages. The sequencing analysis yielded 3,536,497 quality sequences (mean ± SD = 44,206 ± 27,324). The range of the reads of more than 90% of the samples were between 13,260 and 141,754. In order to capture most of the relevant diversity present in that 90% of the population the rarefaction depth was performed at 13,260 (Figure 2, panel A). Seven samples were excluded since they contained less than 13,260 reads and they were 2 samples of layers from week 1, 2 layers and 2 broilers from week 3, and 1 layer sample from week 4. The bacteria richness estimated with Chao1 and Observed species and the diversity index Shannon, there were no statistical differences in these parameters between broiler and layers chickens. See Table 4 for the numeric values. A principal coordinate analysis (PCoA) was computed with the Unweighted UniFrac distance to visualized variations in the community structure between broiler and layer chickens.

As it is observed in Figure 2, panel B, both breeds of chickens clustered together. However, according to the analysis of similarity ANOSIM test, the community structure of the fecal bacteria of broilers differed from layers at 4-weeks of age with both Unweighted (R = 0.37 and P value = 0.001) and Weighted UniFrac distances (R = 0.25 and P value = 0.005).

The abundance of several bacterial taxa differed between broilers and layers, especially at week 3 and 4, although the univariate test detected the phylum and class *Actinobacteria* enriched in layer-type chickens (Kruskal-Wallis test $P = 0.04$ and $< 0.001$ respectively) at week 1 and 2. Based on the linear discriminant analysis effect size, LEfSe, there were 12 bacterial taxa differently abundant between breeds at week 3. The bacterial families: *Sphingomonadaceae*, *Pasteurellaceae*, *Enterobacteriaceae* and *Lactobacillaceae* were overabundant in the meat-type chickens than the egg-type chicken (Figure 3, panel A). LEfSe revealed the microbiota in layer-type chickens were enriched in 8 bacterial taxa, and broiler-type in 4 taxa at 4-weeks of age. Some of the bacterial families more abundantly present in layer-type chickens were *Staphylococcaceae* and *Corynebacteriaceae*, while broiler-type chickens were enriched in the family *Pasteurellaceae* (Figure 3, panel B). The functionality of the bacteria was predicted with the online tool PICRUSt. To calculate the accuracy of the PICRUSt prediction the NSTI were considering before performing any statistical comparisons. A large NSTI (> 0.20) indicate a low accuracy prediction, since the NSTI in experiment 1 was 0.05 ± 0.005 it suggests that about 95% of the identified species in the samples could be predicted from the reference genome. Thereby, PICRUSt information was used to make inferences of the microbial functionality in this experiment. Statistical differences in functional pathways were reported at week 3 and 4 between broiler and layer-type chickens Table 5.

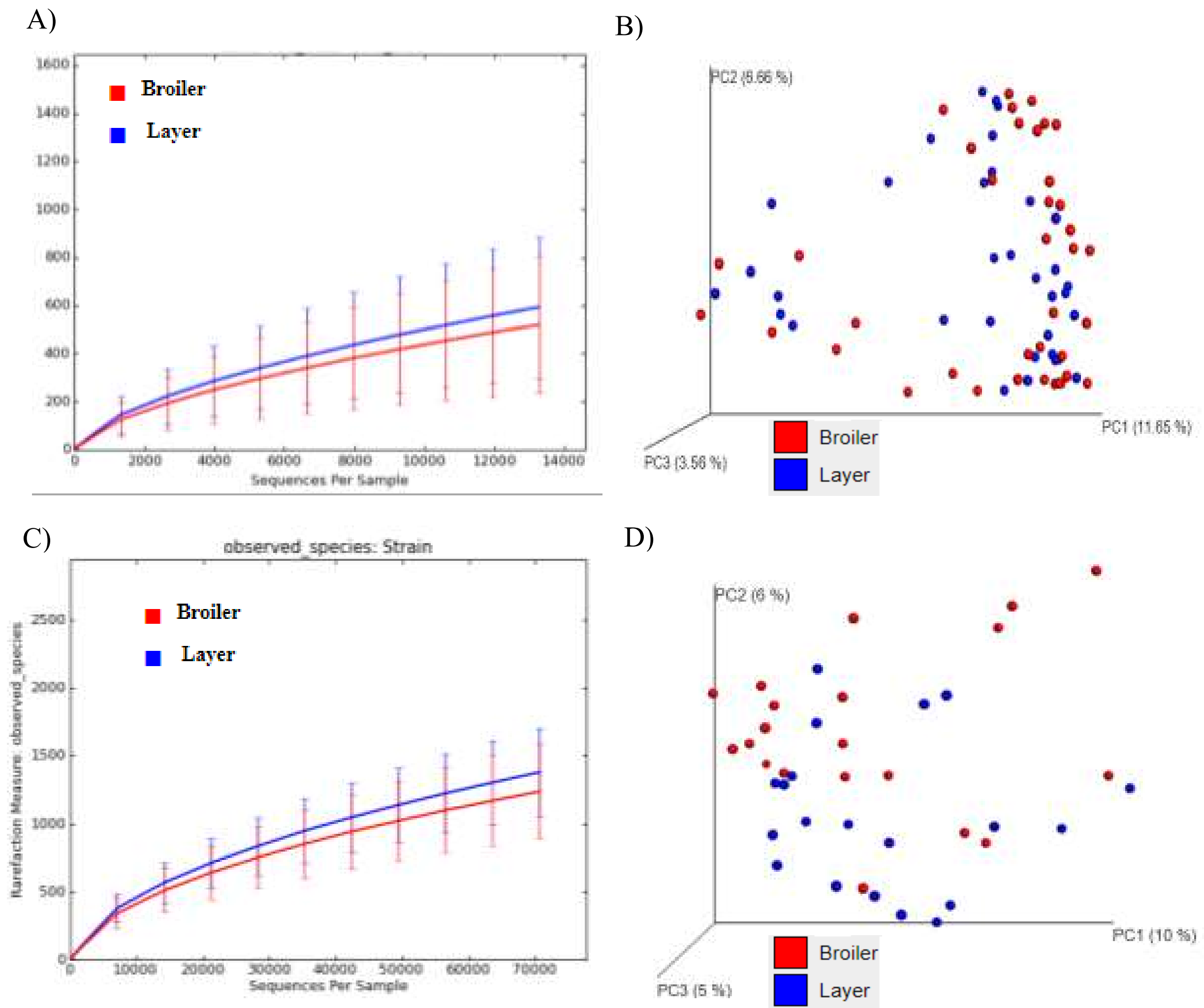


**Figure 2. Fecal bacteria diversity of broiler- and layer-type chickens. Alpha diversity rarefaction curves measured by the number of observed species of the 16S rRNA gene in Experiment 1 on a random subset at 13,260 reads per samples (A), and Experiment 2 in a random subset at 70,750 sequences per samples (C). Each line represents the mean of each chickens' strain and the error bars the standard deviation. Beta diversity visualized with Principal coordinates analysis (PCoA) plots measured with Unweighted UniFrac distances of the 16S rRNA genes, each point represents the fecal community of individual birds. Panel B, the community structure of bacteria in Experiment 1, and Panel D, the community structure of bacteria in Experiment 2. PCoA plots reveals similarity in the fecal microbial community between broilers and layers chickens.**

**Table 4. Summary of alpha diversity estimates of broiler- and layer- type chickens at different ages**

| Index | Strain | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | wk1 | wk2 | wk3 | wk4 | wk1 | wk3 | wk5* | wk7 |
| Chao 1 | Broiler | 1192 | 1212 | 1432 | 1236 | 2738 | 2036 | 2202 | 2928 |
| | Layer | 1094 | 1350 | 1780 | 1518 | 2641 | 2179 | 3130 | 3149 |
| Observed Species | Broiler | 500 | 486 | 584 | 521 | 1454 | 958 | 1075 | 1464 |
| | Layer | 468 | 539 | 770 | 606 | 1295 | 1055 | 1528 | 1644 |
| Shannon | Broiler | 3.36 | 3.31 | 4.40 | 3.66 | 4.90 | 3.07 | 3.90 | 4.00 |
| | Layer | 3.40 | 3.70 | 5.40 | 4.11 | 4.20 | 3.20 | 4.80 | 5.00 |

* Statistical differences in all alpha parameters between broilers and layers at week 5 ($P < 0.05$, Kruskal-Wallis test)

*Experiment 2, Comparison of the fecal bacterial communities between broiler and layer type chickens without antimicrobial supplementation*

The total number of fecal samples was 40 (n=5 broilers and 5 layers) collected at 1, 3, 5, and 7 weeks of age. The sequencing analysis yielded 3,846,800 of quality sequences (mean ± SD = 96,170 ± 14,813). The number of reads per sample ranged from 73,166 to 127,163, thus, the rarefaction depth performed in this experiment was based on the sample with the minimal reads (70,750 reads/samples). Consequently, no sample was excluded for the diversity analysis (Figure 2, panel C). Significant differences in the bacteria richness and diversity evaluated with Chao1, Observed species, and Shannon index were observed between breeds at week 5 only (Table 4). The non-parametric Kruskal-Wallis test found the layer-type chickens with greater number of total bacteria species and higher diversity than the broiler-type chickens at 5-weeks of age (*P* value Chao1 = 0.016, Observed species = 0.016, and Shannon = 0.028). The community structure of the fecal bacteria evaluated with Unweighted UniFrac distance and depicted into the 3D PCoA showed these two breeds of chickens developing similarity (Figure 2, panel D). Based on the ANOSIM test with the Unweighted UniFrac distance broiler- and layer-type chickens developed dissimilarity only at week 5 ($R = 0.28$ and $P$ value = 0.048). This result supported the differences in the alpha diversity estimates found at week 5, since the Unweighted UniFrac distance uses the

presence/absence approach and consider the rare species with low abundance. No differences in the community structure of the fecal bacteria was found between breeds and across all sample ages with the Weighted ANOSIM.

Based on LEfSe analysis, the order *Clostridiales* and the class *Clostridia* were more abundant in broiler-type chickens at 1 week of age compared to layer-type chickens. More variations in the bacteria abundances between chicken strains were reported at 5 and 7 weeks of age, frequently identified to be more abundantly present in layer-type chickens (Figure 4). At 5-weeks of age, the layer-type chickens were enriched in 9 bacteria taxa compared to broiler. Based on LEfSe and Kruskal-Wallis test bacteria representatives from the phylum *Bacteroidetes* ($P$ = 0.04) and *Actinobacteria* ($P$ = 0.009), and the families *Enterobacteriaceae*, *Brevibacteriaceae* and *Staphylococcaceae* (Figure 4**,** Panel A) were overabundant in layer-type chickens. At 7-weeks of age LEfSe identified 15 bacteria taxa differentially abundant between chicken breeds. The bacterial class *Bacilli* and the order *Lactobacillales* were enriched in broiler-type chickens, while layer-type birds were more abundant in bacteria from the class *Clostridia* and families *Clostridiaceae*, *Turicibacteraceae,* and *Helicobacteraceae* (Figure 4, Panel B). The predicted bacteria functionality, according to PICRUSt, showed various pathways differing between chicken breeds at 7 weeks. Interestingly, most of the significant pathways were enriched in the layer-type chickens (Table 6). Statistical analysis with the predicted bacteria metagenome was conducted since the NSTI in this experiment was 0.05 ± 0.005 which indicate that 95% of the identified species in the samples can be predicted from the reference genome database.

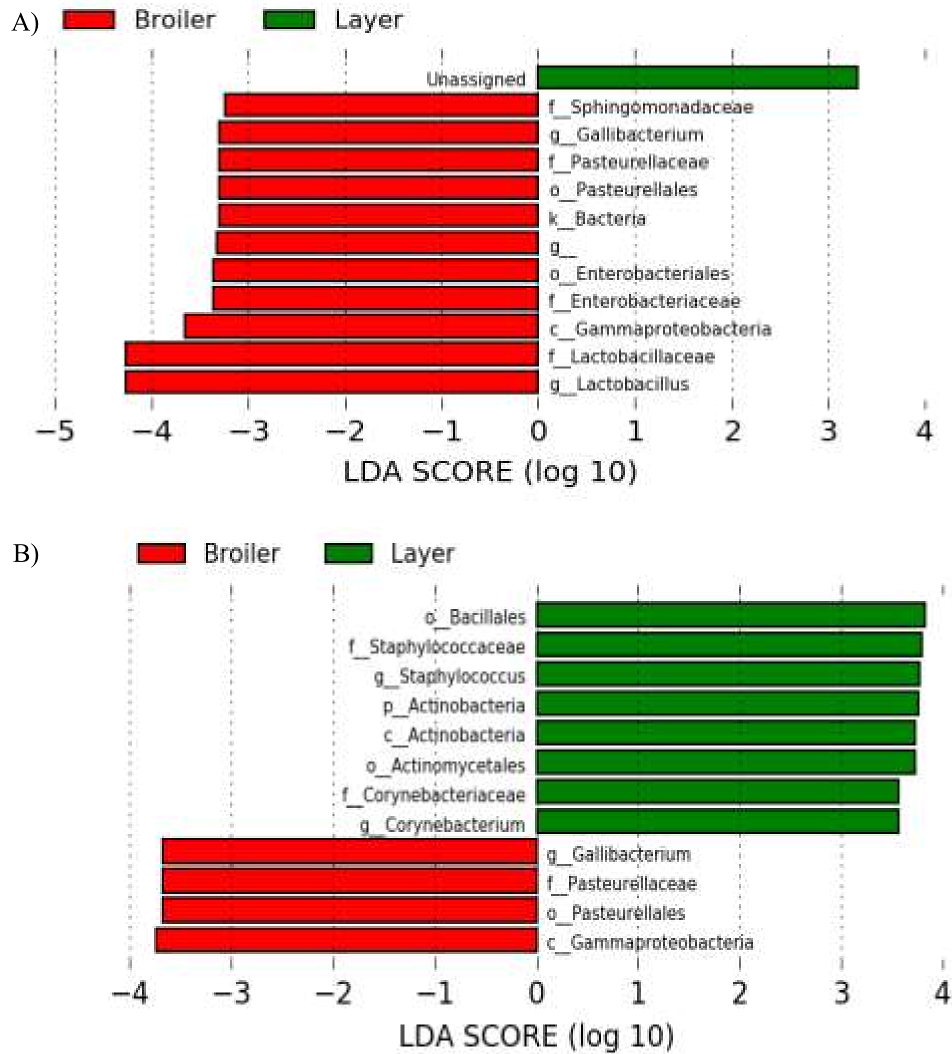


**Figure 3. Linear Discriminant Analysis Effect Size (LEfSe) in Experiment 1 of differentially abundant taxa between broiler- and layer-type chickens. Panel A, abundance of bacterial taxa most likely to explain statistically differences between broiler and layers at 3 weeks of age. Panel B, abundance of bacterial taxa significantly differing between broiler and layers at 4 weeks of age. LEFSe calculated with alpha = 0.05 and LDA score > 3.0.**

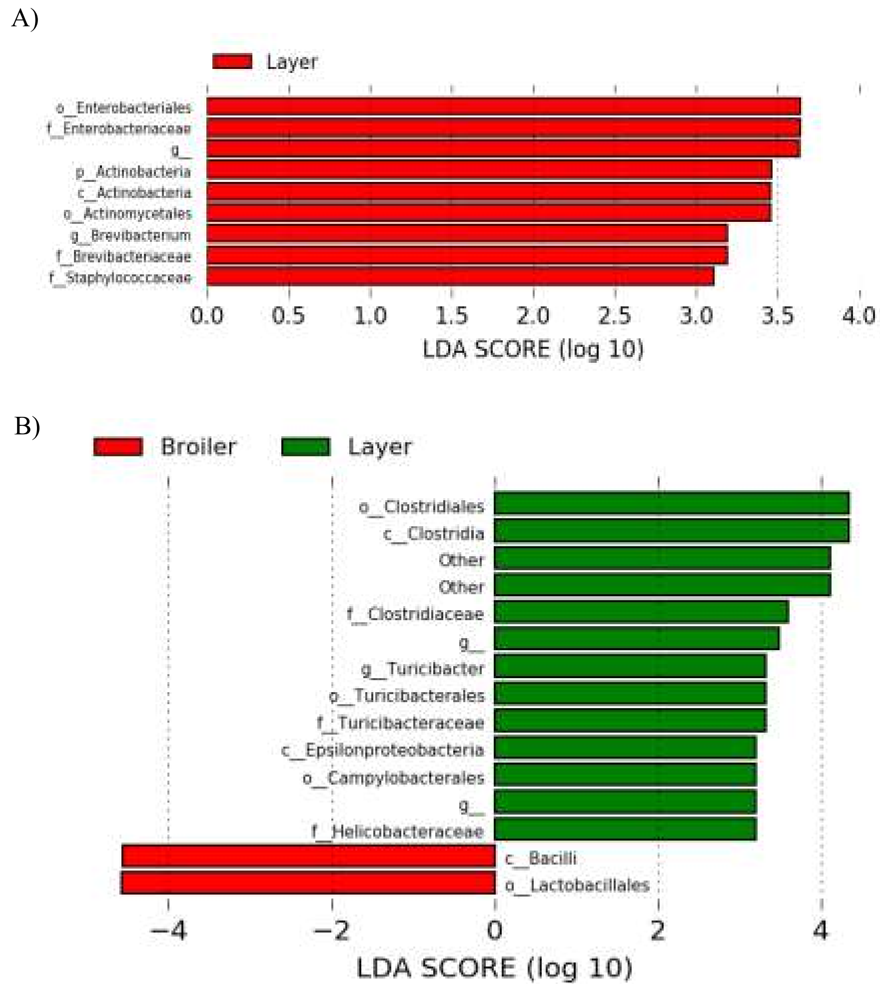


**Figure 4. Linear Discriminant Analysis Effect Size (LEfSe) in Experiment 2 of differentially abundant taxa between broiler- and layer- type chickens. LEFSe was constructed with alpha = 0.05 and LDA score > 3.0. Panel A, differentially abundant taxa enriched in layer-type chickens at 5-weeks of age and no abundance of bacteria taxa was found abundant in broilers. Panel B, abundance of bacterial taxa statistically different between broiler-type and layer-type at 7-weeks of age.**

**Table 5. Metagenome prediction of the fecal bacteria of broiler and layer chickens with PICRUSt tool Experiment 1 (third level of the KO hierarchy)**

| KEGG pathway | WEEK 1 | | | WEEK 2 | | | WEEK 3 | | | WEEK 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Amino Acid Metabolism | 423,564 | 470,443 | NS | 398,982 | 427,707 | NS | 519,009 | 661,390 | NS | 418,218 | 424,028 | NS |
| Biosynthesis of Other Secondary Metabolites | 39,060 | 38,656 | NS | 36,036 | 40,163 | NS | 38,341 | 52,095 | NS | 37,925 | 35,033 | NS |
| Cancers | 6,210 | 6,351 | NS | 6,035 | 6,044 | NS | 5,607 | 6,398 | NS | 5,222 | 5,346 | NS |
| Carbohydrate Metabolism | 633,977 | 674,720 | NS | 61,062 | 628,663 | NS | 662,056 | 762,305 | NS | 608,172 | 616,157 | NS |
| Cardiovascular Diseases | 8 | 16 | NS | 15 | 29 | * | 31 | 19 | NS | 11 | 37 | NS |
| Cell Communication | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS |
| Cell Growth and Death | 33,543 | 36,480 | NS | 33,562 | 34,602 | NS | 31,142 | 34,978 | NS | 29,738 | 31,581 | NS |
| Cell Motility | 70,852 | 84,731 | NS | 57,057 | 60,475 | NS | 105,333 | 95,273 | NS | 66,274 | 54,058 | NS |
| Cellular Processes and Signaling | 203,420 | 222,136 | NS | 181,670 | 187,493 | NS | 209,579 | 264,981 | * | 175,690 | 173,674 | NS |
| Circulatory System | 25 | 36 | NS | 1240 | 88 | NS | 1,094 | 109 | NS | 52 | 116 | NS |
| Digestive System | 556 | 473 | NS | 8582 | 1,041 | NS | 1,536 | 1,911 | NS | 1,652 | 1,363 | NS |
| Endocrine System | 8,656 | 9,208 | NS | 8,460 | 9,548 | NS | 11,524 | 18,187 | NS | 9,128 | 9,199 | NS |
| Energy Metabolism | 29,330 | 318,092 | NS | 283,045 | 293,725 | NS | 317,493 | 373,632 | NS | 262,813 | 272,968 | NS |
| Environmental Adaptation | 7,651 | 8,969 | NS | 7,404 | 7,813 | NS | 8,336 | 9,229 | NS | 6,562 | 6,830 | NS |
| Enzyme Families | 131,342 | 141,325 | NS | 12,688 | 129,666 | NS | 116,992 | 144,705 | NS | 108,404 | 11,750 | NS |
| Excretory System | 1,423 | 1,679 | NS | 883 | 1,160 | NS | 866 | 2,192 | 0.006* | 739 | 9250 | NS |
| Folding, Sorting and Degradation | 140,040 | 149,775 | NS | 132,555 | 137,921 | NS | 139,745 | 164,579 | NS | 118,770 | 126,557 | NS |
| Genetic Information Processing | 160,653 | 176,559 | NS | 161,842 | 162,185 | NS | 1,646 | 173,420 | NS | 154,118 | 150,152 | NS |

**Table 5 Continued**

| KEGG pathway | WEEK 1 | | | WEEK 2 | | | WEEK 3 | | | WEEK 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Glycan Biosynthesis and Metabolism | 118,158 | 120,952 | NS | 114,370 | 116,408 | NS | 109,579 | 119,119 | NS | 102,509 | 96,140 | NS |
| Immune System | 1,785 | 1,772± | NS | 1,686 | 1,966 | NS | 3,037 | 3,525 | NS | 2,782 | 1,567 | * |
| Immune System Diseases | 4,592 | 4,947 | NS | 4,417 | 4,359 | NS | 3,162 | 3,977 | NS | 2,755 | 3,981 | ** |
| Infectious Diseases | 26,306 | 28,408 | NS | 24,822 | 25,464 | NS | 26,585 | 28,272 | NS | 22,022 | 24,639 | * |
| Lipid Metabolism | 168,233 | 184,365 | NS | 16,524 | 168,287 | NS | 185,994 | 207,124 | NS | 169,896 | 169,303 | NS |
| Membrane Transport | 757,309 | 824,223 | NS | 704,713 | 724,622 | NS | 742,914 | 875,056 | NS | 629,434 | 714,774 | NS |
| Metabolic Diseases | 6,823 | 7,186 | NS | 6,443 | 6,768 | NS | 5,658 | 7,131 | 0.035* | 5,366 | 5,897 | NS |
| Metabolism | 136,653 | 146,135 | NS | 132,650 | 134,277 | NS | 146,994 | 166,389 | NS | 137,727 | 130,401 | NS |
| Metabolism of Cofactors and Vitamins | 193,055 | 222,216 | NS | 176,594 | 187,114 | NS | 219,978 | 293,478 | 0.035* | 74,079 | 178,945 | NS |
| Metabolism of Other Amino Acids | 85,139 | 94,080 | NS | 82,073 | 84,031 | NS | 92,331 | 108,160 | NS | 80,054 | 84,313 | NS |
| Metabolism of Terpenoids and Polyketides | 93,348 | 100,938 | NS | 93,628 | 96,065 | NS | 106,997 | 122,501 | NS | 94,111 | 96,962 | NS |
| Nervous System | 5,096 | 5,671 | NS | 4,343 | 4,838 | NS | 4,547 | 7,267 | 0.008* | 3,848 | 4,376 | NS |
| Neurodegenerative Diseases | 4688 | 5,377 | NS | 4,513 | 4,620 | NS | 10,492 | 8,568 | NS | 4,662 | 5,240 | NS |
| Nucleotide Metabolism | 281,488 | 306,173 | NS | 277,994 | 282,537 | NS | 256,871 | 289,982 | NS | 239,297 | 261,283 | NS |
| Poorly Characterized | 290,566 | 312,735 | NS | 272,912 | 280,181 | NS | 285,846 | 339,183 | NS | 245,719 | 265,707 | NS |
| Replication and Repair | 599,065 | 648,455 | NS | 593,142 | 605,634 | NS | 532,952 | 614,913 | NS | 501,171 | 546,750 | NS |
| Sensory System | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS |

**Table 5 Continued**

| KEGG pathway | WEEK 1 | | | WEEK 2 | | | WEEK 3 | | | WEEK 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Signal Transduction | 77,197 | 85,429 | NS | 67 941 | 67,768 | NS | 89,623 | 102,740 | NS | 63,590 | 66,028 | NS |
| Signaling Molecules and Interaction | 12,631 | 13,338 | NS | 13,138 | 13,216 | NS | 102,65 | 12,457 | * | 11,198 | 12,320 | NS |
| Transcription | 161,533 | 176,014 | NS | 146,606 | 151,735 | NS | 153,558 | 193,768 | NS | 140,446 | 142,940 | NS |
| Translation | 376,886 | 409,402 | NS | 373,494 | 384,057 | NS | 352,276 | 388,373 | NS | 32,324 | 350,388 | NS |
| Transport and Catabolism | 7,641 | 7,161 | NS | 6,688 | 7,657 | NS | 9,748 | 17,092 | * | 7,715 | 7,075 | NS |
| Xenobiotics Biodegradation and Metabolism | 112,763 | 126,753 | NS | 124,725 | 121,070 | NS | 153,909 | 154,387 | NS | 14,819 | 141,879 | NS |

NS=No significant differences
*Statistical differences < 0.05 with Kruskal-Wallis test
**Statistical differences < 0.01 with Kruskal-Wallis test

**Table 6. Metagenome prediction of the fecal bacteria of broiler and layer chickens with PICRUSt tool Experiment 2 (third level of the KO hierarchy)**

| KEGG pathway | WEEK 1 | | | WEEK 3 | | | WEEK 5 | | | WEEK 7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Amino Acid Metabolism | 2,750,755 | 2,193,077 | NS | 2,237,709 | 2,006,809 | NS | 1,998,780 | 2,452,250 | NS | 2,397,415 | 3,149,399 | * |
| Biosynthesis of Other Secondary Metabolites | 246,219 | 196,859 | NS | 175,737 | 165,283 | NS | 163,025 | 191,747 | NS | 205,895 | 247,158 | NS |
| Cancers | 31,163 | 27,450 | NS | 30,451 | 19,555 | NS | 29,163 | 33,809 | NS | 29,242 | 38,749 | * |
| Carbohydrate Metabolism | 3,840,995 | 3,440,150 | NS | 3,018,113 | 2,868,934 | NS | 3,003,835 | 3,193,691 | NS | 3,384,656 | 3,549,785 | NS |
| Cardiovascular Diseases | 25 | 27 | NS | 20 | 26 | NS | 20 | 84 | NS | 23 | 54 | ** |
| Cell Communication | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS |
| Cell Growth and Death | 195,655 | 171,523 | NS | 157,516 | 168,212 | NS | 161,287 | 163,384 | NS | 160,204 | 174,405 | NS |
| Cell Motility | 435,433 | 318,703 | NS | 818,761 | 720,026 | NS | 459,529 | 484,031 | NS | 445,998 | 843,799 | * |
| Cellular Processes and Signaling | 1,223,982 | 964,624 | NS | 1,208,567 | 1,013,071 | NS | 903,502 | 1,103,589 | NS | 1,060,507 | 1,446,648 | * |
| Circulatory System | 156 | 147 | NS | 1,826 | 881 | NS | 3,597 | 1,715 | NS | 444 | 2,153 | * |
| Digestive System | 8,791 | 5,284 | NS | 3,610 | 3,360 | NS | 3,281 | 4,860 | NS | 8,265 | 5,614 | * |
| Endocrine System | 69,161 | 44,799 | NS | 41,251 | 35,829 | NS | 34,562 | 61,024 | NS | 52,365 | 78,735 | * |
| Energy Metabolism | 1,764,187 | 1,498,673 | NS | 1,496,967 | 1,326,422 | NS | 1,420,496 | 1,544,795 | NS | 1,488,876 | 1,877,486 | * |
| Environmental Adaptation | 51,855 | 40,095 | NS | 51,746 | 59,529 | NS | 41,468 | 41,201 | NS | 39,858 | 55,785 | * |
| Enzyme Families | 741,268 | 671,207 | NS | 627,006 | 587,442 | NS | 6,220,031 | 669,725 | NS | 609,738 | 734,189 | NS |
| Excretory System | 5,875 | 4,892 | NS | 4,192 | 1,646 | NS | 4,171 | 8,980 | NS | 3,933 | 8,291 | * |
| Folding, Sorting and Degradation | 799,190 | 695,695 | NS | 730,063 | 662,689 | NS | 665,798 | 734,456 | NS | 689,304 | 882,828 | * |

**Table 6 Continued**

| KEGG pathway | WEEK 1 | | | WEEK 3 | | | WEEK 5 | | | WEEK 7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Genetic Information Processing | 906,487 | 803,889 | NS | 894006 | 794,367 | NS | 795,034 | 832,422 | NS | 855,651 | 993,918 | * |
| Glycan Biosynthesis and Metabolism | 715,754 | 588,643 | NS | 676604 | 533387 | NS | 555,794 | 572,448 | NS | 590,949 | 700,311 | NS |
| Immune System | 15,976 | 7,225 | NS | 11,548 | 5,980 | NS | 8,961 | 10,617 | NS | 15,863 | 23613 | NS |
| Immune System Diseases | 25,806 | 25,387 | NS | 20,438 | 20,538 | NS | 22,039 | 22,890 | NS | 15,986 | 16,375 | NS |
| Infectious Diseases | 140,684 | 133,256 | NS | 163,548 | 131,492 | * | 129,602 | 137,372 | NS | 127,726 | 148,155 | NS |
| Lipid Metabolism | 990,663 | 866,643 | NS | 892,569 | 836,415 | NS | 783,816 | 924,281 | NS | 922,208 | 989,348 | NS |
| Membrane Transport | 4,289,570 | 4,175,960 | NS | 3,936,434 | 3,724,594 | NS | 3,701,793 | 4,029,450 | NS | 3,759,752 | 4,573,702 | * |
| Metabolic Diseases | 37,534 | 33,447 | NS | 33,314 | 30,709 | NS | 29,516 | 31,740 | NS | 28,633 | 34,280 | * |
| Metabolism | 799,298 | 739,035 | NS | 706,674 | 601,296 | NS | 656,025 | 72,167 | NS | 766,405 | 865,821 | * |
| Metabolism of Cofactors and Vitamins | 1,209,521 | 934,050 | NS | 1,006,479 | 805,353 | NS | 854,702 | 1,049,502 | NS | 1,015,824 | 1,427,279 | * |
| Metabolism of Other Amino Acids | 495,580 | 443,030 | NS | 464,444 | 422,632 | NS | 407,047 | 477,311 | NS | 444,844 | 514,982 | * |
| Metabolism of Terpenoids and Polyketides | 561,439 | 500,261 | NS | 483,522 | 465,349 | NS | 464,616 | 540,428 | NS | 511,967 | 56,1705 | * |
| Nervous System | 30,838 | 23,317 | NS | 18,949 | 17,562 | NS | 20,834 | 26,023 | NS | 21,007 | 30,998 | * |
| Neurodegenerative Diseases | 26,100 | 23,738 | NS | 44,361 | 35,038 | NS | 34,372 | 38770 | NS | 32,306 | 53,360 | * |
| Nucleotide Metabolism | 1,514,9869 | 1,418,134 | NS | 1,306,057 | 1,271,321 | NS | 1,372,416 | 1394017 | NS | 1,322,140 | 1,491,702 | NS |
| Poorly Characterized | 1,538,590 | 1,484,636 | NS | 1,521,576 | 1,339,118 | NS | 1,360,531 | 1,551,382 | NS | 1,432,714 | 1,779,224 | NS |
| Replication and Repair | 3,335,860 | 3,083,180 | NS | 2,881,927 | 2,921,751 | NS | 2,953,809 | 3,035,265 | NS | 2,796,165 | 3,300,803 | NS |

**Table 6 Continued**

| KEGG pathway | WEEK 1 | | | WEEK 3 | | | WEEK 5 | | | WEEK 7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* | Broiler | Layer | *P* |
| Sensory System | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS | 0 | 0 | NS |
| Signal Transduction | 445,459 | 567,717 | NS | 405,720 | 438,806 | NS | 470,360 | 388,503 | NS | 455,912 | 441,954 | NS |
| Signaling Molecules and Interaction | 58,333 | 55,611 | NS | 66,034 | 64,779 | NS | 73,612 | 68,839 | NS | 63,853 | 63,609 | NS |
| Transcription | 854,704 | 850,240 | NS | 815,589 | 903,234 | NS | 930,128 | 743,471 | NS | 810,376 | 840,591 | NS |
| Translation | 1,830,690 | 1,952,759 | NS | 1,900,321 | 2,040,266 | NS | 2,076,309 | 1,949,576 | NS | 1,866,245 | 1,908,532 | NS |
| Transport and Catabolism | 46,127 | 48,573 | NS | 42,273 | 43,642 | NS | 67,162 | 27,601 | NS | 49,744 | 46,531 | NS |
| Xenobiotics Biodegradation and Metabolism | 686,138 | 581,739 | NS | 612,460 | 762,463 | * | 656,546 | 552,282 | NS | 778,329 | 591,416 | NS |

NS=No significant differences
*Statistical differences < 0.05 with Kruskal-Wallis test
**Statistical differences < 0.01 with Kruskal-Wallis test

## Temporal bacterial variation

A comparative analysis of the species richness and diversity parameters was performed from one time-point to another to visualize the temporal bacteria variations taking place during a normal broiler growing period considering the bacteria information from both breeds of chickens. As observed in the rarefaction curve by week in both experiments (Figure 5, panel A and C) there was a tendency to increase the total number of species as the chickens grew older. The microbial information in Experiment 1 was taken weekly for four weeks and based on the alpha estimates evaluated with Chao1, Observed species, and Shannon index, higher bacteria richness and diversity was reported at 3-weeks of age (Table 7). The community structures of the fecal bacteria or beta diversity visualized in the 3D PCoA (Figure 5, panel B) showed the bacteria community structure clustering based on the time-point samples were collected, this information was additionally numerically computed with both Weighted and Unweighted ANOSIM global P = 0.001. This suggest that the bacterial membership and its abundance differed from one week to another in Experiment 1. Samples from Experiment 2 were taken at week 1 and then by-weekly for seven weeks. Based on Chao 1, Observed species, and Shannon index, chickens were statistically the same in the total number of species and in diversity at every sampled week except for a significant reduction observed at week 3 (Table 8). The community structure of the fecal bacteria or beta diversity visualized in the 3D PCoA (Figure 5, panel D) showed dissimilarity from one time-point to another with both Unweighted (R = 0.378 and P = 0.001) and Weighted (R = 0.268 and P = 0.001) ANOSIM.

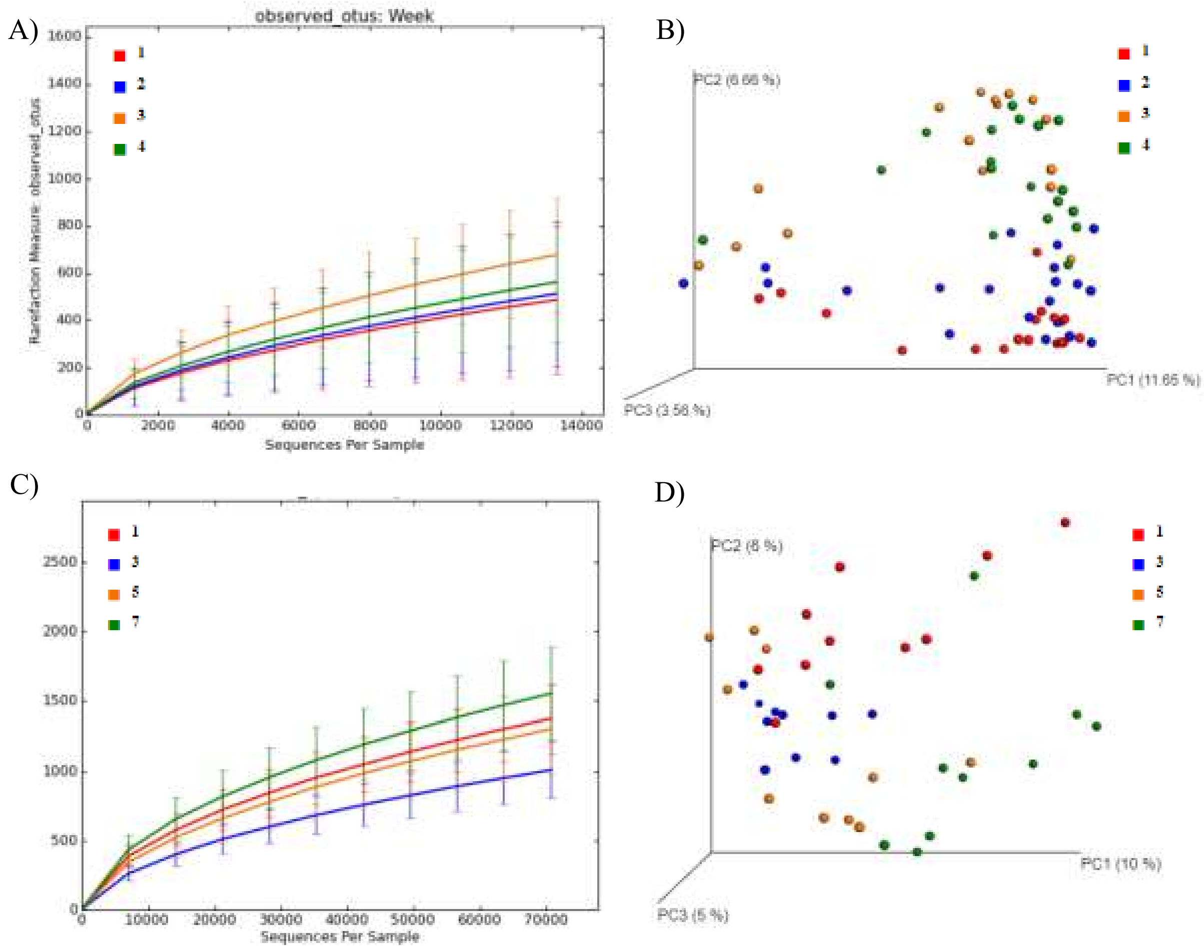


**Figure 5. Bacterial richness and microbial community evaluated by timepoint from experiment 1 and 2. Panel A, alpha diversity rarefaction curves measured by the number of observed species of the 16S rRNA gene in Experiment 1 on a random subset at 13,260 reads per samples. Panel C, total number of species in a random subset at 70,750 sequences per samples in Experiment 2. Each line represents the mean of each chickens' strain and the error bars the standard deviation. Panel B, beta diversity of Unweighted UniFrac in Experiment 1, global P value of ANOSIM test with both Unweighted and Weighted UniFrac = 0.001. Panel D, beta diversity of Unweighted UniFrac distance of Experiment 2, global ANOSIM P value = 0.001 of both Weighted and Unweighted distance. The variations of the communities are visualized in the 3D Principal coordinate analysis (PCoA) each point represents the bacteria community of one chicken at one timepoint.**

**Table 7. Species richness and diversity estimates by timepoint of the fecal samples of Experiment 1**

| Experiment 1 | Chao 1 | Observed OTUs | Shannon Index |
|---|---|---|---|
| Week 1 | 1148.93 ± 164 [y] | 486.50 ± 76 [y] | 3.38 ± 0.31 [y] |
| Week 2 | 1281.88 ± 157 [xy] | 512.40 ± 69 [y] | 3.53 ± 0.34 [y] |
| Week 3 | 1606.47 ± 130 [x] | 677.43 ± 62 [x] | 4.90 ± 0.31 [x] |
| Week 4 | 1369.88 ± 144 [xy] | 561.68 ± 60 [xy] | 3.87 ± 0.32 [xy] |
| P value | ** | ** | ** |

[1]Data are given in mean ± SEM
[2]Different letter within a column represents statistical differences ($P < 0.05$) utilizing nonparametric comparison for all pairs with Dunn method for joint ranking that uses Bonferroni adjustment
**Statistical differences < 0.01 with Kruskal Wallis test

**Table 8. Species richness and diversity estimates by timepoint of the fecal samples of Experiment 2**

| Experiment 2 | Chao 1 | Observed OTUs | Shannon Index |
|---|---|---|---|
| Week 1 | 2689.74 ± 107 [xy] | 1374.50 ± 81 [x] | 4.54 ± 0.22 [x] |
| Week 3 | 2107.59 ± 147 [y] | 1006.70 ± 66 [y] | 3.15 ± 0.28 [y] |
| Week 5 | 2666.54 ± 196 [xy] | 1301.60 ± 107 [xy] | 4.36 ± 0.24 [x] |
| Week 7 | 3038.64 ± 223 [x] | 1554.40 ± 110 [x] | 4.55 ± 0.39 [x] |
| P value | ** | ** | ** |

[1]Data are given in mean ± SEM
[2]Different letter within a column represents statistical differences ($P < 0.05$) utilizing nonparametric comparison for all pairs with Dunn method for joint ranking that uses Bonferroni adjustment
**Statistical differences < 0.01 with Kruskal Wallis test

### The effect of diet in fecal microbiota

The fecal bacteria variations based on the diet phase (starter, grower I and II) were explored in experiment 1 and 2. The rarefaction plots and the beta diversity by the diet in phases are depicted in Figure 6, Panel A and C. In experiment 1, the alpha diversity parameters significantly increased when feeding the grower diet ($P < 0.05$) (Table 9) and dissimilarity of the community structure between diets was qualitative and quantitative reported with the ANOSIM test, P value = 0.001 in both Unweighted ($R = 0.302$) and Weighted ($R = 0.179$) distances (Figure 6, panel B). The species richness and diversity in Experiment 2 showed the lowest values of alpha parameters when chickens were eating grower I Table 9. The bacterial community structure of the fecal developed by each type of diet was dissimilar and in the PCoA they clustered separated (Figure 6, panel D).

These differences were also numerically computed with ANOSIM test with both Unweighted (R = 0.304 and P = 0.001) and Weighted (R = 0.16 and P = 0.02) UniFrac. However, similarities were recorded between the starter and grower I with only Weighted ANOSIM, suggesting differences were based on the total number of the species rather than the abundance.

Stacked area charts were constructed to visualize the bacterial shifts at the family level with those bacteria with >1% of relative abundance (Figure 7 and Figure 8). The changes in the bacteria abundance were markedly observed when birds transited from one diet to another. The phyla *Firmicutes* was generally reduced in both experiments the week following the transition of one phase of diet to another. This was evident at week 3 where both experiments and both type of chickens reduced the bacteria members of the phyla *Firmicutes* with the transition from a starter to a grower, and at 7-weeks in experiment 2 with the transition of a grower I to a grower II diet.

**Table 9. Diversity estimates of the fecal bacteria community evaluated by the diet in phases.**

| | Experiment 1 | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|
| | Starter | Grower | *P* | Starter | Grower I | Grower II | *P* |
| Chao 1 | 1218 ± 112 | 1478 ± 99 | ** | 2689 ± 107 $^{ab}$ | 2387 ± 135 $^{b}$ | 3038 ± 223 $^{a}$ | * |
| Ob. OTUs | 500 ± 50 | 614 ± 43 | ** | 1374 ± 81 $^{ab}$ | 1154 ± 70 $^{b}$ | 1554 ± 110 $^{a}$ | ** |
| Shannon | 3.46 ± 0.23 | 4.34 ± 0.24 | ** | 4.54 ± 0.22 $^{a}$ | 3.76 ± 0.22 $^{a}$ | 4.55 ± 0.39 $^{a}$ | * |

[1]Data are given by mean ± SEM

[2]Different letters within a row in Experiment 2 represents statistical differences (P < 0.05) utilizing nonparametric comparison for all pairs with Dunn method for joint ranking that uses Bonferroni adjustment

[*]Statistical differences < 0.05 with Kruskal Wallis test

[**]Statistical differences < 0.01 with Kruskal Wallis test

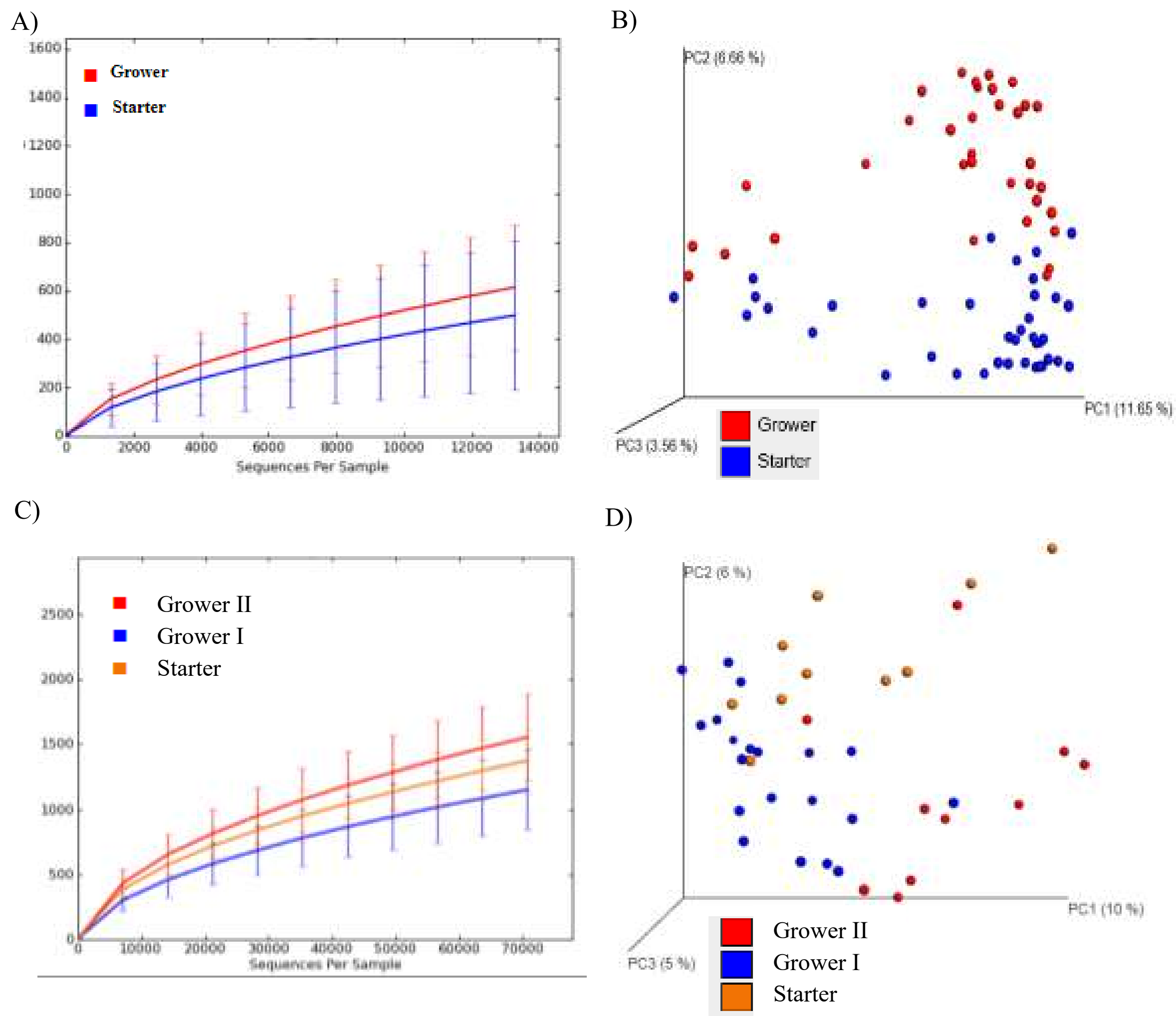


**Figure 6. Bacterial diversity evaluated by the diet in phases. Alpha rarefaction diversity of the 16S rRNA gene evaluated with numbers of observed species in Experiment 1 (A) and Experiment 2 (C). Each line represents the mean of diversity based on the different type of diet and error bars the standard deviation. PCoA plots of Unweighted UniFrac distance by type of diet in Experiment 1 (B) and Experiment 2 (D). PCoA plot revealed clustering of bacteria community based on type of diet.**

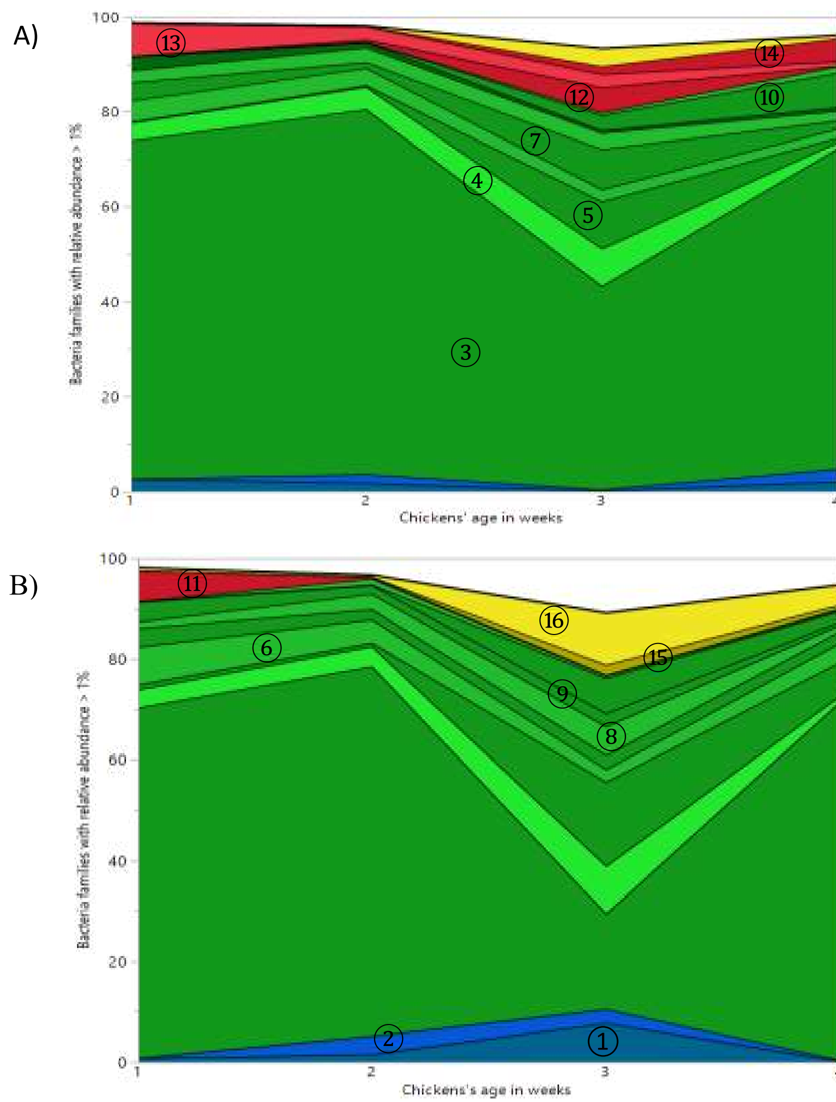


**Figure 7. Temporal variation of the fecal microbiota of chickens in Experiment 1. Panel A, fecal bacteria profile in broiler chickens. Panel B, fecal bacteria profile in layer chickens. From bottom to top, both panels have the same bacteria order. Different colors represent different bacteria phylum. Blue color, bacteria families within Bacteroidetes, green color, families within Firmicutes, red color, bacteria families within Proteobacteria, yellow color, bacteria families within Actinobacteria. 1 – *Bacteroidaceae*, 2 – *Rikenellaceae*, 3 – *Lactobacillaceae*, 4 – *Ruminococcaceae*, 5 – *Staphylococcaceae*, 6 – *Clostridiaceae*, 7 – *Enterococcaceae*, 8 – order *Clostridiales*, 9 – *Lachnospiraceae*, 10 – *Peptostreptococcaceae*, 11 – *Veillonellaceae*, 12 – *Helicobacteraceae*, 13 – *Enterobacteriaceae*, 14 – *Pasteurellaceae*, 15 – *Dermabacteroceae*, 16 – *Corynebacteriaceae*.**

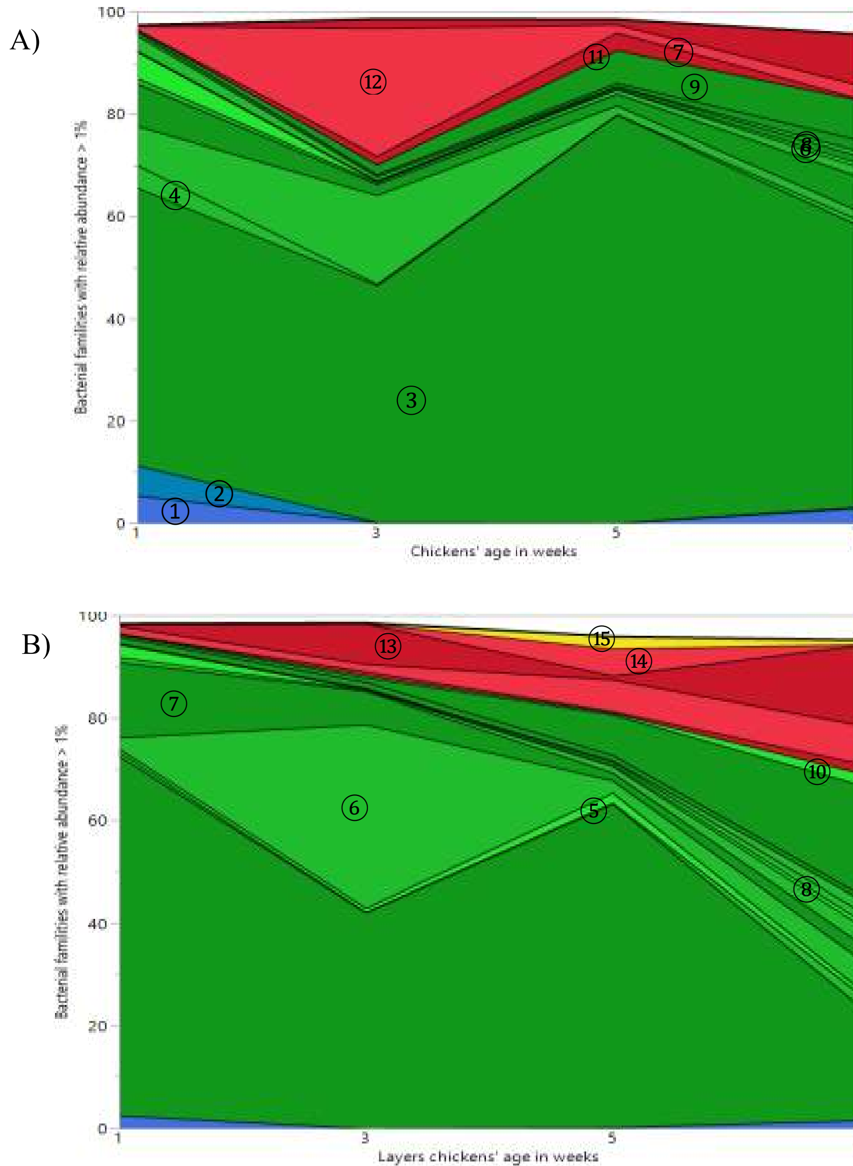


**Figure 8. Temporal variation of the fecal microbiota of chickens in Experiment 2. Panel A, fecal bacteria profile in broiler chickens. Panel B, fecal bacteria profile in layer chickens. From bottom to top, both panels have the same bacteria order. Different colors represent different bacteria phylum. Blue color, bacteria families within Bacteroidetes, green color, families within Firmicutes, red color, bacteria families within Proteobacteria, yellow color, bacteria families within Actinobacteria. 1 – *Bacteroidaceae*, 2 – *Porphryromonadaceae*, 3 – *Lactobacillaceae*, 4 – *Ruminococcaceae*, 5 – *Staphylococcaceae*, 6 – *Clostridiaceae*, 7 – *Enterococcaceae*, 8 – family group composed by *Clostridiales*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Veillonellaceae* and *Planococaceae*, 9 – *Clostridiales*; Other, 10 – *Turicibacteraceae*, 11 – *Helicobacteraceae*, 12 – *Enterobacteriaceae*, 13 – *Pasteurellaceae*, 14 – *Moraxellaceae*, 15 – *Brevibacteriaceae*.**

*Discussion*

The gut bacteria population in chickens has been studied mainly in broiler species to associate the composition of the intestinal bacteria with the health and productive parameters during normal growing period of broiler. Few microbiome experiments have been conducted with young layers chickens, and even less have sought to compare the gut microbial communities between genetic divergent chickens such as meat-type and egg-type breeds. The objective of this study was to compare the composition of the fecal microbiota between broiler-type and layer-type chickens when co-housed and fed similarly to identified possible host genetic influences on gut bacteria populations. Additionally, the temporal bacteria shift and the effect of a diet program in phases were evaluated. This is the first experiment combining broiler and layer chickens in the same rearing pen and diet during a broiler growing period (4-7 weeks). Two independent experiments were conducted adopting standard diets in phases for broiler chickens, and the starter diet on Experiment 1 was supplemented with antimicrobial growth promoter. Fecal samples were obtained weekly in Experiment 1 and by-weekly in Experiment 2. The taxonomic assignment based on the Greengenes 16S rRNA gene database reported these genetically divergent meat- and egg-type chickens initially shared fecal bacteria composition and abundance, but the differences were noted as the chickens grew older.

The gut microbiota of chickens is known to increase in species richness and diversity as the bird ages (Chang et al., 2016; Juricova et al., 2013; Oakley and Kogut, 2016). The most dramatic microbial changes reported take place during the first week post-hatch (Crhanova et al., 2011) but continuous microbial changes occurs over the whole life of the chicken (Videnska et al., 2014b). We compared the community structure of the fecal bacteria of the broiler and layer chickens and found they were qualitatively and quantitatively similar at all sampled-point, except

for wk 4 in Experiment 1 and at wk 5 in Experiment 2. The similarity in alpha and beta diversity parameters observed in both experiments suggest a possible homogenization of the bacteria ecology, perhaps as a consequence of the high plasticity of the gut microbiota in the young host (Kerr et al., 2015). Notably, the inoculum of bacteria that chickens first encounter in the rearing area can precede a healthy or detrimental gut bacteria population (Yin et al., 2010). This is influenced by the fact chickens are coprophagic species and have steady cloacal reflexes promoting the uptake of environmental microbes (Van der Sluis et al., 2009), causing the community of bacteria inhabiting the litter to shape the indigenous gut bacteria (Locatelli et al., 2017). The similarity in the species richness and community structure found in these experiments may partly be influenced by the co-incubation and co-habitations occurring during a critical period for gut microbial development which make environmental factor highly influential on intestinal bacteria.

In keeping with a previous report (Perumbakkam et al., 2014), the community of the fecal bacteria of chickens in this study was dominated by the phylum *Firmicutes,* followed by *Proteobacteria*, see Figure 7 and Figure 8. Studies have found *Firmicutes* species dominating all gut section of the growing broiler chickens (Ranjitkar et al., 2016) and in egg-type chickens from 2 to 8 weeks of age (Videnska et al., 2014b). The bacterial group *Bacteroidetes,* occasionally is found as second in dominance of the fecal microbial profile (Meng et al., 2014; Schokker et al., 2015), and in our two experiments and in combination with the phyla *Actinobacteria* they were the ones that least dominated the microbial profile of the fecal samples. We believe the subtherapeutic dose of antimicrobial added in the starter diet of Experiment 1 had some influence at shaping the gut microbial population in both chicken strains since the early in-feed antibiotic perturbs numerous genes of the intestinal mucosa (Schokker et al., 2017) and can suppress T cell mediate response (Gao et al., 2017) which could have promoted the similarity in the intestinal

environment in broilers and layers. Furthermore, there are evidence showing antibiotic growth promoters altered the composition of the indigenous gut bacteria of chickens (Geier et al., 2011; Torok et al., 2011) and some of recurrent bacterial changes comprises a reduction in the species richness and diversity (Pourabedin et al., 2014; Wise and Siragusa, 2007). The use of in-feed antibiotic can also diminish species of bacteria with beneficial influences such as *Lactobacillus* spp. (Lin et al., 2013; Murai et al., 2016) while pathogenic bacteria can take advantage of this situation and overgrow (Simon et al., 2016). A reduction in *Lactobacillus* spp. could imply a decline on the bacteria bile salt hydrolases activity (Wang et al., 2012), which elevated activity has been associated with leanness in mammals and reported to be higher in genetically lean chickens (Julendra et al., 2017). In fact, a proposed mode of action of the antibiotic growth promoters associated with the improvement of feed efficiency is a reduction of bacteria specie with bile slat hydrolases activity (Lin, 2014). Our results also showed the bacteria genus *Gallibacterium* overgrowing following the removal of the in-feed antibiotic. Members of this genus have been identified as commensal bacteria of chickens (Bojesen et al., 2003) and its high abundance was associated with high-fat deposition in mature hens (Ding et al., 2016). Notwithstanding, this genus also includes infectious bacteria species such as *Gallibacterium anatis* which has been associated with reproductive infection in egg-laying hen, gastrointestinal and respiratory diseases in broiler chickens (Bojesen et al., 2004; Persson and Bojesen, 2015; Singh et al., 2016). The suppression of antibiotic growth promoter also appeared to spur the growth of other potential pathogenic bacteria such as the family *Enterobacteriaceae* previously reported to increase abundance in the ceca of 14-days old broilers following antibiotic removal (Wise and Siragusa, 2007). Interestingly, broiler chickens were more abundant in *Gallibacterium* and *Enterobacteriaceae* compared to layer chickens after removing the in-feed antibiotic, this suggest these breeds of chickens own distinct

intestinal immune mechanisms to cope with the intestinal alteration caused by the use of antibiotic growth promoter.

Broiler- and layer-type chickens have different immune reactions in health and illness situations (Han et al., 2016) and own a distinct intestinal functionality (Shires et al., 1987; Sklan et al., 1975). Perhaps these variations exposed the intestinal bacteria to different microenvironment and mediated a distinct gut bacteria composition. For instance, the length of the small intestine relative to the body weight is longer in broiler than layers (Shires et al., 1987) to support the higher feed ingestion taking place in the broiler type chickens (Noy and Sklan, 1999; Sklan, 2001). Conversely, layer-type chickens have greater grinding and fermentative capacity in the crop and ceca that has been associated with a greater retention time of the digesta (Shires et al., 1987). Then why our results showed the bacteria differences only after chickens were older than two weeks? we can only attempt to answer with probable reasons. We have already mentioned that a shared environment when the gut microbiota is plastic and prone to environmental conditions can induce homogenization of the gut bacteria community. Another possibility, which is concomitant to the gut microbial maturation, is the development of the intestinal morphology that in broiler-type chickens take two weeks after hatch (Ranjitkar et al., 2016; Van der Wielen et al., 2002). According to Lumpkins et al. (2010) the gut microbial population among three breeds of broiler chickens were similar during the first two weeks of age and that the differences in the bacteria community appeared after this period. In fact, the maturation of enteric bacterial population is another distinction between broiler and layer chickens since the literature reported the indigenous gut bacteria in meat-type chickens reach stability earlier than egg-type chickens (Den Hartog et al., 2016; Mead, 1989; Ranjitkar et al., 2016). Of note, maternal antibodies are transferred in the egg yolk and protect the hatchling against pathogenic microorganisms for two or three weeks post-

hatch while chickens acquire their own humoral immunity (Grindstaff et al., 2003; Hamal et al., 2006). This immune protection could have shaped the gut bacteria community differently since it depends on the humoral immunity of the hens and some breeds of hens transfer more concentration of antibodies than other (Hamal et al., 2006). All these observations could lead to different intestinal immune regulations and drive differentiation once chickens reach intestinal maturation.

In this study, the functional pathways of the fecal bacteria were predicted based on the 16S rRNA genes profile utilizing the online PICRUSt. Based on the associated KEGG orthologous markers, most of the significant functional pathways differing between chicken breeds were enriched in layer-type chickens. Among the predicted functions more abundantly expressed by layers chickens at 7 weeks of age were amino acid metabolism, energy metabolism, lipid metabolism and cell motility. Interestingly, these same functional gene categories were previously reported abundant in lean hens compared to a heavy counterpart applying the same predictive tool (Ding et al., 2016). The enrichment in amino acid metabolism pathway may be either because the diet we used had more protein content or because layer-type chickens have accelerated whole-body protein synthesis and degradation compared to meat type chickens (Muramatsu et al., 1987). It is evident that broiler chickens have a rapid lean tissue deposition and bone formation than same age egg-laying hen, in this process the insulin like growth factor (**IGF**)-1 was identified to modulate protein synthesis and fat deposition in chickens (Tomas et al., 1998). In fact, intramuscular delivery of IGF-1 on the breast muscle of a 7-days old Single Comb White Leghorn (layer-type) accelerated the muscle synthesis rate in this type of chickens (Conlon and Kita, 2002), evidencing the role of IGF-1 in the formation of skeletal muscle in commercial chickens. Studies with murine mice have recognized the gut microbiota can influence the synthesis and the serum concentration of circulating IGF-1 (Van Wyk and Smith, 1999; Yan and Charles, 2018; Yan et al.,

2016b). In fact, positive correlations were reported in chickens supplemented with *Lactobacillus plantarum* and the expression of IGF-1 in the liver (Kareem et al., 2016) and gut bacteria short-chain fatty acids and the production of IGF-1 (Yan et al., 2016b). Among all the taxonomic differences reported in both experiments, the order *Lactobacillales* was always identified enriched in broiler-type chickens which may be an indicative this group of bacteria supports the rapid lean deposition.

The temporal bacterial variations were studied in both experiments by comparing the bacterial richness and diversity, and the community structure between sampling-time. No temporal variation based on bacteria richness and diversity was observed in our experiments. Interestingly, the bacteria diversity at 3-week of age increased in Experiment 1 but was reduced in Experiment 2. Since week 3 may represent the bacteria population of a transition from starter to grower diets, we believed this reflects a diet associated alteration. According to Torok et al. (2011) the age of the chickens is an influential factor when experimental chickens are provided with one type of diet but is age is not significant when fed a diet in phases. A typical broiler diet in phases transit from a nutrient and protein-rich, as it is starter, to one with fewer nutrients and filled with more calories, typical of finisher diets. A study reported that the bacteria community of chicken when fed starter diet cluster apart from four other experimental grower diets (Ranjitkar et al., 2016). The reduction in protein content and the increment of dietary energy may have caused the bacteria alterations observed at week 3 in both experiments and those observed at week 7 in Experiment 2. Changes in the gut microbial population due to a program in phases were previously reported in broiler chickens fed a commercial diet and diet supplemented with antimicrobial (Kumar et al., 2018). It was observed that the microbial population of the ceca is highly affected by a feeding program in phases (Kumar et al., 2018) and such alteration can be reflected in the fecal microbiota (Stanley et

al., 2015). The protein and energy requirements are lower in layer-type chickens than in broiler chickens The provision of a diet with greater protein content may alter the bacteria metabolites involved in amino acid degradation and reduce butyrate concentration as those observed in humans (Beaumont et al., 2017). This could have affected layer-type chickens since potential bacterial family (bacteria family *Enterobacteriaceae*, *Clostridiaceae*, and *Campylobacter*) were more abundant in layer-type chickens in Experiment 2. However, the bacteria succession of layer-type chickens in this second experiment followed the bacterial shifts reported in Videnska et al. (2014b) where the *Firmicutes* division, from 8th to 38th weeks, is replaced by *Proteobacteria* species (Panel B of Figure 8). The temporal bacteria variations were more pronounced due to dietary factors than the age of the chickens however, the differences reported between broiler and layer chickens may be outside the jurisdiction of the diet and be a host-related consequence.

Murine studies have accounted 19% of the bacterial variations of various laboratories facilities was explained by the host genetic and that 31.7% could be explained by the caging system (Hildebrand et al., 2013). Our findings support that a shared environment, and a common diet in phases may have shaped the indigenous gut bacteria of these genetically divergent chickens, however, as the chickens grew older, the host genetics may influence the fecal bacteria profile. This might be driven by the differential intestinal immunity, feed consumption and digestion patterns reported between broiler and layer chickens. Nevertheless, some limitation of this experiment is that it was performed in a limited sample size and utilizing one protocol for the sequence of the fecal bacteria. Furthermore, there is a limited number of experiments comparing intestinal bacteria between genetically divergent chickens, providing very few options for comparison. Though the rarefaction depth in experiment 1 excluded 7 samples, a parallel analysis was performed using all samples rarified to 2500 reads, which still complied with the minimum

recommended depth for dataset sequenced in Illumina platform (1000 sequences/samples) (Navas-Molina et al., 2013). Similar tendency on bacteria richness and diversity were also observed at that lower rarefaction depth as with the sequencing depth used to in this study.

In conclusion, this experiment showed divergent meat- and egg-type chickens exposed to a common area and same diet developing similar fecal microbial population, but showed taxonomic variation as the chickens grew older, suggesting a possible host related influence at a later stage of a broiler growing period. Further work is needed to detect the contribution of the host genetic and the confounding environment factor in the development of the gut microbiota of commercial chickens. Follow-up steps should associate the intestinal bacteria of this experimental model with productive traits such as lean deposition, feed conversion and bone development.

# CHAPTER III

# MICROBIOTA CHANGE IN THE CLOACA OF FEED-SATIATED BROILER BREEDER HENS

## *Introduction*

Obesity is produced by a surplus of positive energy balance over time that consequently is stored in the visceral and subcutaneous adipose tissues. With the advent of high throughput sequencing technology, the composition and the functionality of the indigenous gut bacteria population has been associated with the pathogenesis of obesity (Romieu et al., 2017). The association between enteric bacteria and the development of obesity has been investigated with germ-free mice deliberately colonized with bacteria from obese donors, which they became obese regardless of the dietary energy intake (Bäckhed et al., 2004). A subsequent study reported that a high-caloric and high-fat diet did not produce the obesity symptoms in germ-free mice than the same diet fed to conventional mice (Bäckhed et al., 2007). Various mechanisms have been proposed by which gut microbes contribute to the development of obesity. In the experiment of Bäckhed et al. (2004), germfree mice conventionalized with microbiota from overweight donor improved the uptake of monosaccharides. This change in carbohydrate absorption led to elevated glucose concentration and increased expression of key enzymes for de novo fatty acid biosynthesis: acetyl-CoA carboxylase and fatty acid synthase. Other studies demonstrated the obese-microbiota is more efficient at extracting energy from the ingesta (Stanley et al., 2013a; Turnbaugh et al., 2006). Turnbaugh et al. (2006) used a bomb calorimeter and reported that the feces from obese mice contain significantly less energy than those from lean littermates. One possible way the gut bacteria might enhance the energy extraction from the ingesta is by improving

its fermentative capacities and generate more short-chain fatty acids (SCFA). Short-chain fatty acids are absorbed by the host and can be metabolized in the liver and converted in an intermediate of the citric cycle (Cheeke and Dierenfeld, 2014; Peng, 2007). It was documented that SCFA mediated by the colonic fermentation of pectin can increase hepatic lipogenesis (Rolandelli et al., 1989) and that germ-free animals oral supplemented with SCFA increased body weight (Furuse et al., 1991). It has been found the concentration of SCFA in obese individuals is elevated compared to their lean counterparts (Schwiertz et al., 2010). Another mechanism by which gut bacteria could induce body weight gain is by the regulation of host genes that participate in the lipid metabolism. For instance, in the experiment of Bäckhed et al. (2004) the obese associated microbiota was found to suppress the expression of the fasting induced adipose factor (*Fiaf*) gene in the small intestine. The *Fiaf* gene inhibits the activity of the lipoprotein lipase and consequently prolong the energy storage in the adipocytes. Although it is still unknown whether the symbiotic gut bacteria are a cause or a result of the development of obesity, evidence suggest that those bacteria may contribute to an enhanced body weight gain.

**Gut bacterial changes associated with body weight gain in chickens**

Alterations in the balance of the indigenous bacteria have been identified in subjects with metabolic disorders such as obesity (Andoh et al., 2016; Bakker et al., 2015) and diabetes (Baothman et al., 2016). The bacterial profile in subjects with metabolic disorders is generally reported to contain lower species richness and diversity compared to their lean counterparts, and keystone bacteria become disproportionate and possibly replaced by pathobionts (Vangay et al., 2015), in a situation termed “dysbiosis.” Early studies conducted in mammals identified a positive correlation between an increment in body weight and an increase of *Firmicutes : Bacteroidetes*

(Ley et al., 2005; Ley et al., 2006). Ley et al. (2005) reported the intestinal microbial ecology of the leptin deficient mice (*ob/ob*); which is a genetically obese mouse, differed from the lean model by having a greater abundance of bacterial representatives of the phylum *Firmicutes* at the expense of *Bacteroidetes*. Similar *Firmicutes : Bacteroidetes* was also identified in obese humans (Ley et al., 2006), fat pigs (Yan et al., 2016a), and chickens treated with the antibiotic penicillin (Singh et al., 2013). However, based on the meta-analysis performed by Angelakis et al. (2012) this bacterial ratio is not universally true in overweight subjects; the literature shows other bacteria phyla can also be associated with higher body weight such as *Actinobacteria* and *Proteobacteria*. Adult hens with high abdominal fat had reduced bacteria from the phylum *Fusobacteria* but increased *Proteobacteria* compared to hens with low body fatness (Ding et al., 2016). The microbial functionality predicted with PICRUSt in Ding et al. (2016) revealed that the microbiota of the fat line hens were enriched in signal transduction mechanisms and fatty acid biosynthesis when compared to the lean line of hens. The lipid metabolism of chickens may be influenced by the activity of the bacterial associated enzyme bile salt hydrolases (BSH), which activity was noted to be high in genetically lean chickens (Julendra et al., 2017) and in mammals this high activity represent a reduction of plasma cholesterol and liver triglyceride (Joyce et al., 2014). Avian studies have reported that BSH in lean chickens can be provided from lactic acid bacteria (Julendra et al., 2017) and its suppression was proposed as a mode of action of antibiotic growth promoter to improve body weight gain (Lin, 2014).

There are key gut bacteria species that has been associated with the promotion of body weight gain in commercial chickens. Some bacteria species identified with a positive influence on broiler chickens belong to the genus *Lactobacillus*, *Ruminococcus* (Stanley et al., 2012a), *Gallibacterium, Enterobacteriaceae, Clostridiales*, and *Lachnospiraceae* (Torok et al., 2011). The

improvement observed by the feed supplementation with direct fed microbial (DFM) products or probiotic is accompanied by an alteration in the composition of enteric microorganisms. The productive benefits accounted by the in-feed probiotic is attributed to set up the condition for the overgrowth of the lactic acid species (Samli et al., 2007) and butyrate producer bacteria (De Maesschalck et al., 2015; Tiihonen et al., 2010; Wang et al., 2017). The literature additionally shows a notable difference in the gut microbial profile of chickens that contrast in productive performance (Singh et al., 2012; 2014a) and some enteric bacteria population have been correlated with enhanced fat deposition in adult hens (Ding et al., 2016; Meng et al., 2014; Zhao et al., 2013). All these evidences suggest that the indigenous bacteria of chickens can influence weight gain at various developmental stages of the bird.

**Broiler breeder hens and feed restriction**

Modern broiler chickens have long been selected for rapid lean tissue growth as juveniles and as a result grow from 46g at hatch over 2000g, market weight, in 4 to 6 weeks (Cobb-Vantress, 2018). This rapid growth rate is a desired trait in the progeny, unfortunately, it is also expressed by the broilers parents and in adulthood is associated with a number of metabolic and physical compromises that limit fecundity and livability (Liu et al., 2014; Walzem and Chen, 2014). Genetic selection for growth rate has negatively impacted reproductive outcomes in broiler type chickens. Compared to similar egg-type laying hens, broiler breeder hens may only reach 50% of the egg production since they have reduced ovulation rate and greater fat deposition (Griffin and Goddard, 1994). Broiler breeder chickens are prone to hyperphagia and to become obese (Barbato, 1994; Richards, 2003) due to their growth potential and the excess of appetite that is desired in the progeny. The central and peripheral mechanisms that regulate food intake seems to be altered when

genetic selecting chickens targeting for growth rate improvement (Barbato, 1994; Richards, 2003), furthermore the hypothalamic mechanism that regulate satiation also appears to become impaired (Burkhart, 1983). Thereby, the hyperphagia of the broiler breeders chickens are counteracted by varying forms of feed restriction regimens to ensure reproductive fitness in male and female breeders (Hocking et al., 1989). Feed restriction is a practice that caloric restrict broiler breeder chickens where chickens allowing the consumption of 1/3 of their voluntary feed intake which could predisposed hens to chronic hunger and stress (Dixon et al., 2014). Though, feed restriction ration ensures the provision of the nutrients necessary for growing, maintenance, and reproduction during rearing and breeding periods. Since the onset of the first laid egg is triggered by weight-dependent factors rather than the animal age (Bornstein et al., 1984), hens allowed free access to feed at rearing reach sexual maturation earlier (Heck et al., 2004; Robbins et al., 1988). The unregulated feed allowance at rearing can permanently impair eggs productivity and increase the incidence of erratic oviposition, defective eggs, and multiple ovulation (Yu et al., 1992). Experimental animals feed restricted for more than 48 hours developed morphological alterations of the digestive system such as a reduction of the protein mass of the intestine and liver (Ju and Nasset, 1959), reduction of energy expenditure in the jejunum (Park et al., 1998), and the induction of mucosal sloughing (Bayer et al., 1981). The side effects of intense feed restriction could predispose birds to intestinal inflammation and increase endotoxin permeability (Pearce et al., 2013) which can altered the gut immunity and the architecture of the enteric population.

Previous studies from our laboratory documented broiler hens allowed to feed to satiety for a period of 10-days significantly increased body weight, abdominal fat, developed fatty liver, lipotoxicity, and ovarian dysfunction (Chen, 2004). The satiated hens in that experimental model developed hyperglycemia (high blood sugar), hyperinsulinemia (high level of circulating insulin)

and insulin resistance (Chen et al., 2006). The 10-days feed satiated hens expressed 2-fold more hepatic triacylglycerol, elevated concentration of plasma glucose, non-esterified fatty acid, and higher concentrations of cholesterol, plasma insulin, and leptin (Chen, 2004). This model of overfed hens identified two types of hens within the feed-satiated group as follows: 1) half of the hens that continued laying eggs and 2) half of then stopped production after a 10-days trial. The feed-satiated hens that discontinued laying eggs developed ovarian abnormalities such as expanded follicular hierarchies, atretic or type 3 follicles had heavier abdominal fat pads and liver weights, greater concentrations of plasma leptin and triacylglycerol compared to the feed-satiated hens with normal ovaries that continued laying eggs (Chen et al., 2006). It is evident that an increase in feed consumption can impair the normal metabolism and reproduction of broiler breeder hens in just 10 days. Dietary changes have a dominating role in altering the gut microbial composition of avian species (Zhang et al., 2010). Chicken independent studies have also reported that a calories-restricted regimen enriched the phylum *Bacteroidetes* (Walker et al., 2011) and reduced butyrate producer bacteria (Santacruz et al., 2009). The overfed broiler hen model remains uncharacterized regarding its gut microbial population.

The purpose of this study was to evaluate possible gut microbial changes in mature broiler breeder hens when increasing feed intake without changing the macronutrients distribution. The gut microbial ecology of cloacal swabs was evaluated by measuring the 16S rRNA bacterial gene. Considering that half of overfed hens in Chen et al. (2006) experiment discontinued laying eggs because they developed ovarian abnormality, we used a sample size of n=30 mature broiler breeder hens to visualize the associations between the indigenous gut bacteria and productive outcomes of three groups of hens: 10 feed restricted, 10 overfed hens with normal ovaries and 10 overfed hens with abnormal ovaries. This information is relevant to associate shift in gut bacteria species due to

alterations in metabolic functions in broiler breeder hens. Since caloric restriction altered the composition and architecture of the gut microbiota (Wang et al., 2018b), it was hypothesized that overfeeding causes changes in the gut microbiota consistent with increased inflammation, altered ovarian function and egg production in broiler breeder hens previously feed-restricted.

## *Materials and Methods*

### Animal husbandry

Animal husbandry was done at the Poultry Science Research, Teaching, and Extension Center, TAMU University. This animal experiment was approved by the Institutional Animal Care and Use Committees (IACUC 2015-0140) in accordance to the Animal Welfare Act, the Public Health Service Policy and the Humane Care and use of Laboratory Animals. A total of 30 commercially reared broiler breeder hens (Ross 708 strain) at 29.3 weeks of age initiating peak of production (80% eggs/hens/day) were purchased from a local commercial farm and raised in single cages. The hens were individually housed in a wire cage (24" x 19" x 24") and fed a soy- and corn-based mash breeder layer diet containing 2750 Kcal of ME/kg (Table 10). The Aviagen (2016) recommends feeding hens age 29 to 33 weeks 444 kcal/bird/day, therefore, with a diet containing 2750 kcal the restricted ration was 167g/day/hens. The hens were fed restricted prior to acquisition and continued feed restriction regimen for 15 days of which the first 5 days were considered as an adaptation period to the individual cages, followed by 10 days used to measure baseline eggs production. After this period, hens, with no statistical differences in body weight ($P = 0.509$) and laying rate ($P = 0.713$), were randomly allocated into 2 groups. Ten hens continued with the recommended feed restriction (**FR**) and 20 hens were provided with double the portion of feed restriction 334g/day/hen (**UR**) for 10 days. See Figure 9. The uneven numerical distribution of

hens was based on the experiments conducted by Chen et al. (2006) that showed that in this acute re-feeding model half of the fed-satiated hens developed ovarian dysfunction and stopped laying eggs and the other half maintained a normal ovarian morphology and continued laying eggs despite consumption of the excess of energy. Information on microbiota composition of the cloaca was sought in three groups of hens varying in energy intake, egg production and ovarian morphology. Feed was placed between 8:00 - 9:00 am with a photo-schedule of 15 light hours (on/off times). Unrestricted hens wasted feed when the whole feed ration was given at the morning, therefore, from day 3 onward the ration was provided in two portions with the second feeding placed between 2:00 and 3:00 pm. Water was provided ad libitum.

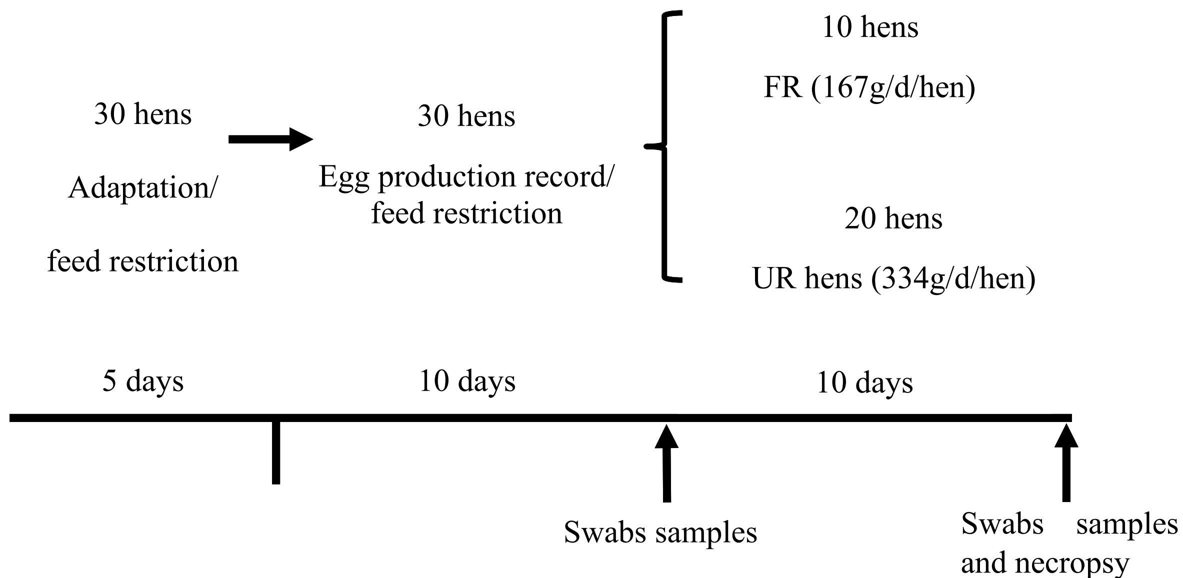


**Figure 9. Timeline of events showing the adaptation, egg production, and treatment periods.**

**Table 10. Ingredient composition of local broiler breeder hen diet**

| Ingredients | % |
|---|---|
| Yellow corn | 75.81 |
| Dehulled soybean meal | 18.37 |
| Sodium bicarbonate | 0.25 |
| Salt | 0.28 |
| DL-methionine | 0.21 |
| Lysine HCL | 0.05 |
| Limestone | 4.26 |
| Monocalcium phosphate | 0.25 |
| Trace minera[1] | 0.05 |
| Vitamins[2] | 0.25 |
| Calculated nutrient composition | |
| Metabolizable energy (Kcal/Kg) | 2750 |
| Protein | 15.52 |
| Lysine | 0.77 |
| Methionine | 0.46 |
| TSAA (methionine + cystine) | 0.73 |
| Crude fat | 3.06 |
| Crude Fiber | 2.38 |
| Calcium | 3.28 |
| Available phosphorus | 2.38 |

[1] Vitamin premix/Kg diet 11023 IU vitamin A, 3858 IU vitamin D3, 46 IU vitamin E, 0.0165 mg B12, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 261.2mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin.
[2] Trace mineral premix/Kg diet 149 mg manganese, 125 mg zinc, 17 mg iron, 7 mg copper, 1.0 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium.
[3] Data is presented in mass percentages unless otherwise indicated

**Egg production and necropsy**

All 30 hens were necropsied at the end of the trial to record the weight of the following organs: liver, ovary, hierarchical follicles, and abdominal fat pad. Liver hemorrhages were judged using a 5-points scale (Wolford and Polin, 1972). A score of 1 represents a liver without hemorrhage, a score of 2 represents a liver containing from 1 to 5 hemorrhages, and a score of 3, 4, and 5 represent a liver with > 6 hemorrhages (which is an indicative of severe liver damage). The liver fat was also graded based on the color scoring scheme of Polin and Wolford (1977), where a score of 1 indicates a firm liver with mahogany coloration, a score of 2 represents a slighter

yellow liver, a score of 3 is assigned to a less firm liver with more yellow areas and a little mahogany, and a score of 4 is a liver that rupture easily and is 100% yellow. A fat liver score of 3 and 4 is considered a fatty liver.

**Bacterial DNA isolation and sequencing**

Cloaca swabs from all 30 hens were taken twice in this experiment by rubbing with circular movements within the hen's cloaca a sterile cotton swab (Puritan, USA) for 10 seconds. Swab samples were taken at the end of the adaptation period, before hens were allowed to access to double the feed ration, and 10 days after this point. The microbial DNA from swabs was extracted using PowerSoil DNA isolation kit (QIAgen, USA). The V4 hypervariable region of the 16S rRNA gene was PCR amplified from the microbial genomic with the forward primer 515F: GTGCCAGCMGCCGCGGTAA and reverse primer 806R: GGACTACHVGGGTWTCTAAT using the HotStar Taq Plus Master Mix Kit (QIAgen, USA) and following the manufacturer's instructions. The thermal cycling consisted on an initial denaturation at 98°C for 3 minutes followed by 28 cycles of denaturation at 94°C for 30 s, the annealing was at 53°C for 40 s, and the elongation phase at 72°C for 1 minutes followed by 72°C for 5 minutes. The PCR products were checked in 2% agarose gel and then pooled in equal proportions based on the molecular concentration. The pooled samples were purified with calibrated Ampure XP beads and Illumina library were prepared using the pooled sample and purified PCR products. Illumina sequencing was performed on a Miseq following the manufacturer's guidelines. Bacterial DNA extraction, amplification and sequencing of 16S rRNA V4 variable region was completed by MrDNA laboratory (www.mrdnalab.com, Shallowater, TX, USA).

**Bioinformatics analysis**

The raw data was processed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline v 1.9 (Caporaso et al., 2010). The sequences were de-multiplexed, and the barcodes removed following quality filtering using the default QIIME setting. Chimera sequences were identified and filtered with the USEARCH 6.1 pipeline (Edgar, 2010). Operational taxonomic units, OTUs, were constructed with an open-reference approach and the taxonomic assignment was performed against the Greengenes 13.8 database with a 97% cutoff (DeSantis et al., 2006). In this process of picking OTUs with open references the reads are aligned against a reference sequence and the new reads that did not hit any sequence are grouped based of De novo strategy. In De novo OTUs picking the reads are grouped together without a reference genome. According to Navas-Molina et al. (2013) the recommended OTU picking approach is open-references because it provides the best trade-off between the time and the ability to discover novel diversity. Sequences assigned to chloroplast, mitochondria, and low abundance OTUs with < 0.01% abundance were removed. Rarefaction curves were conducted to reduce variability among the length of the samples and balanced at 31,000 reads per samples. This rarefaction level complied with the minimum depth recommended to dataset sequenced in Illumina platform (1000 sequences/sample) (Navas-Molina et al., 2013). Diversity estimates for alpha and beta diversity were generated using the QIIME default scripts. Estimation of the bacteria richness and diversity within samples were conducted with the alpha estimates Chao1, Observed species, and the diversity indices Shannon and Simpson. The community structure of the bacteria between samples or beta diversity was calculated with the Weighted and Unweighted UniFrac distances and the distance matrix was collapse into a 3D principal coordinate analysis (PCoA) generated with QIIME default (Lozupone and Knight, 2005).

**Statistical analysis**

Data for body weight, organs weight, egg production, weight of egg produced, and liver scores were compared between restricted (**R**) and unrestricted (**UR**) hens with Least Square Means in JMP Pro 13 (SAS software Inc). Three groups of hens were created to analyze microbial data as follows: Control 1 are the 30 hens fed restricted at week 31, Control 2 are the 10 fed-restricted hens at 33 weeks, and the group Released included the 20 hens fed twice the recommended ration. Alpha diversity estimates and taxa abundance were evaluated for normality with Shapiro-Wilk Test in JMP Pro 13 (SAS software Inc), and because they failed the normality assumption, they were assessed using Kruskal-Wallis test followed by Dunn method for joint ranking that uses Bonferroni adjustment for multiple comparisons. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to assess the bacterial taxa differing in its abundance among the Control 1, Control 2, and Released groups, applying the Galaxy workflow (http://huttenhower.sph.harvard.edy/galaxy/) with $\alpha = 0.05$ and LDA score = 2. This is a statistical tool to identify the differential features between groups with high-dimensionality data. LEFSe initially detects the differences in taxonomic abundance with Kruskal-Wallis sum-rank and consistency is investigated with a pairwise test Wilcoxon rank-sum test and then apply a linear discriminant analysis to estimate the effect size of each differentially abundant features (Segata et al., 2011). An analysis of similarity, ANOSIM, was used to compare the community structure between samples at different ages with PRIMER 6 software (PRIMER-E ltd., Luton, UK). Statistical differences were considered at $p$ value $< 0.05$.

**Productive parameters**

At day one, more than 50% of feed-satiated hens finished the 334g of feed. A similar event occurred on day two and three. From day four onward, the number of hens finishing the 334g declined (See Figure 10). Hens that were provided twice the recommended feed ration unexpectedly selected and wasted feed, thus, the 334g had to be split and provided twice a day in the morning and the afternoon from day three onward.

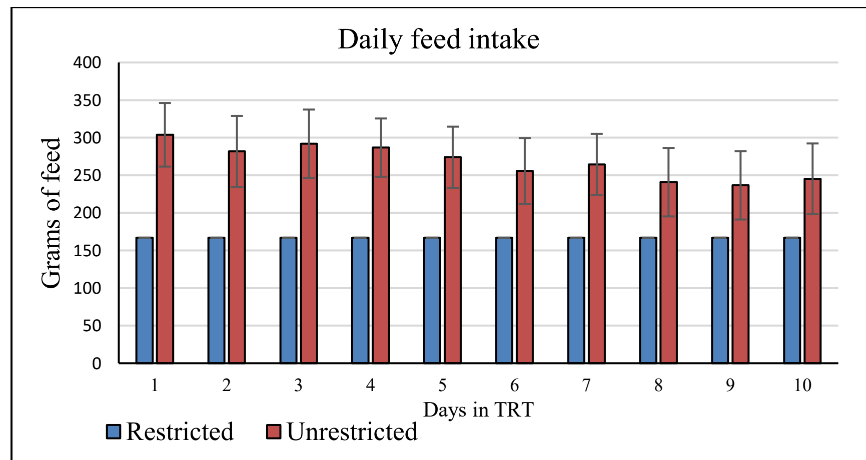


**Figure 10. Average daily feed consumption in group of hens fed Restricted (167g/day/hen) and twice the recommended ration (334 g/day/hen).**

Interestingly, doubling the feed portion for 10 days did not affect the body weight of the hens, P = 0.390. The average egg production during the 5-days adaptation period was 76.0%, during the basal periods was 79.0% and during the 10-days trial 76.3% (Table 12). Egg production was not affected by the increase in caloric intake, no significant difference was found in the egg production among periods, P = 0.628. However, a tendency to reduce egg production in unrestricted (**UR**) hens compared to restricted (**R**) hens (P = 0.05, 73% vs 83%) was observed during the treatment period. The excess of calories did not affect egg weight, P = 0.846. Egg weight

appeared to be affected by the age of the hens as the average weight increased from 56.24g in the basal period to 59.36g during treatment. See Table 12.

At the end of the trial, necropsy investigations showed UR hens developed higher absolute and fractional liver weight compared to restricted, P < 0.001. (See Table 11). The average score for liver hemorrhages was 1.11 in R hens and 1.50 in UR hens, however, this liver hemorrhages score in the feed-restricted hens was between 1 (88%) and 2 (11%) points, whereas, the scores in UR hens were distributed among 1 (80%), 3 (15%), and 5 (5%) points. The average score for fatty liver was not significant between groups of hens, P > 0.10. Most of the R hens had a liver score for fat between 1 and 2 points, whereas more than 50% of UR hens scored between 3 and 4. No differences in the absolute and fractional weight of the abdominal adipose tissue were found between feeding regimen. Feed-satiated hens had an expanded follicular hierarchy of large follicles and the absolute and fractional ovarian weight was significantly greater in UR hens compared to R hens, P < 0.001. (See Table 11) Furthermore, UR hens significantly developed 2.45 more hierarchical follicles than feed restricted hens, P value < 0.001. There were 3 overfed hens with an expanded hierarchical follicle reaching the F12 rank, moreover, all the hierarchical follicles in UR hens were significantly heavier than R hens.

**Swabs microbiota results**

A total of 60 cloacal swabs were taken, 30 at 31 weeks, and 30 at 33 weeks of age. The sequencing analysis yielded 4,980,610 of quality sequences (mean ± SD = 83,010 ± 30,988), the range of the number of read/samples were between 14,026 and 160,422. The rarefaction depth was performed at 31,000 reads per sample; of which 2 samples from the control group at 31 weeks, were excluded for the diversity analysis (< 15,600 reads). After rarefaction, richness estimates

computed with Chao 1 and Observed OTUs and the evenness and diversity indices Shannon and Simpson revealed significant differences between hens based on the age. The connecting letter in Table 13 demonstrate all evaluated alpha parameters similar between Control 2 and Released hens. A significant increase in bacteria richness and diversity indices was observed based on the time hens were in the experimental environment regardless of the feeding regimen. The 2D principal coordinate analysis, PCoA, with Unweighted UniFrac distance (See Figure 11, panel B and C) depicts a slight variation in the bacterial communities of hens based on the time hens were in the experimental environment rather than the feeding regimen. The PC1 and PC2 accounted for 15.43% and 7.5% of the variation, thus, a second PC depicting the PC1 and PC3 was additionally included to explain 5.20% more of the variation.

**Table 11. Body weight, organs weight, and liver scores of mature broiler breeder hens, ROSS 708**

| | Control hens | Released hens | P-Value‡ |
|---|---|---|---|
| Initial BW, Kg | 3.75 ± 0.06 | 3.69 ± 0.04 | 0.509 |
| Final BW, Kg | 3.89 ± 0.08 | 3.98 ± 0.05 | 0.390 |
| Weight gain, g | 145.00 ± 67 | 285.10 ± 47 | 0.099 |
| Liver weight, g | 73.15 ± 8.85 | 120.50 ± 6.25 | *** |
| Liver/BW, % | 1.84 ± 0.22 | 3.03 ± 0.15 | *** |
| Hemorrhage Score | 1.11 ± 0.31 | 1.50 ± 1.50 | 0.312 |
| Fat Color Score | 2.00 ± 0.29 | 2.60 ± 0.21 | 0.110 |
| Abdominal Adipose Tissue (AAT) weight, g | 94.42 ± 10.5 | 86.83 ± 7.43 | 0.560 |
| AAT weight/ BW, % | 2.39 ± 0.28 | 2.20 ± 0.20 | 0.575 |
| Ovary weight, g | 59.90 ± 4.70 | 88.28 ± 3.37 | *** |
| Ovary/BW, % | 1.14 ± 0.10 | 2.21 ± 0.07 | *** |

‡Least Square Means P Value
***Parameters with statistical significances, P value ≤ 0.001
Data is presented as mean ± SEM

**Table 12. Egg production and ovarian morphology of mature broiler breeder hens, ROSS 708**

| | Control, n=10 | Released n=20 | P-Value‡ |
|---|---|---|---|
| Basal egg production, % | 80.00 ± 3.30 | 78.50 ± 2.33 | 0.713 |
| Egg weight, g | 55.61 ± 0.99 | 56.56 ± 0.70 | 0.441 |
| Egg production TRT, % | 83.00 ± 4.06 | 73.00 ± 2.87 | 0.054 |
| Egg weight, g | 59.18 ± 1.09 | 59.44 ± 0.77 | 0.846 |
| Yolk weight, g | 16.96 ± 0.30 | 17.50 ± 0.21 | 0.160 |
| Hierarchical follicles, | 6.10 ± 0.39 | 8.55 ± 0.28 | *** |
| | Hierarchical follicles weight, g | | |
| F1 | 16.21 ± 0.44 | 17.87 ± 0.31 | ** |
| F2 | 13.50 ± 0.65 | 16.05 ± 0.46 | ** |
| F3 | 9.38 ± 0.77 | 12.96 ± 0.54 | *** |
| F4 | 5.92 ± 0.81 | 10.54 ± 0.57 | *** |
| F5 | 3.02 ± 0.82 | 7.96 ± 0.58 | *** |
| F6 | 0.98 ± 0.71 | 5.39 ± 0.47 | *** |
| F7 | 0.95 ± 1.46 | 3.02 ± 0.47 | * |
| F8 | - | 2.21 ± 0.38 | |
| F9 | - | 1.45 ± 0.30 | |
| F10 | - | 1.15 ± 0.38 | |
| F11 | - | 0.65 ± 0.05 | |

‡Least Square Means P Value
*Parameters with statistical significances, P value ≤ 0.05
**Parameters with statistical significances, P value ≤ 0.01
***Parameters with statistical significances, P value ≤ 0.001
Data is presented as mean ± SEM

The analysis of similarity, ANOSIM, numerically confirmed with the Unweighted UniFrac, dissimilarity between the community of bacteria based on the time hens were in the experimental environment (global P = 0.003 and R = 0.18) and the Unweighted UniFrac ANOSIM reported the bacterial community in Control 1 significantly different from both Control 2 (R= 0.27, P = 0.001) and Released hens (R= 0.22, P 0.01). This suggests that the differences from week 31 to 33 were attributed to variations in the phylogenetic composition rather than relative abundance since similarity among the groups was found with Weighted UniFrac. No differences were reported with Weighted and Unweighted UniFrac ANOSIM between the Control 2 and Released hens (Weighted P= 0.71 Unweighted P = 0.91).

**Table 13. Summary of alpha diversity estimates of the bacteria community of the cloaca of mature broiler hens**

| | 31 Weeks | 33 weeks | | *P* value |
|---|---|---|---|---|
| | Control 1(n=28)‡ | Control 2(n=10) ‡‡ | Released (n=20) | |
| Chao 1 | 1941.58 ± 69[b] | 2705.47 ± 234[a] | 2690.84 ± 156[a] | *** |
| Observed OTUs | 843.39 ± 24[b] | 1288.40 ± 109[a] | 1271.45 ± 84.81[a] | *** |
| Shannon Index | 4.15 ± 0.15[b] | 5.70 ± 0.46[a] | 5.59 ± 0.33[a] | *** |
| Simpson Index | 0.80 ± 0.02[b] | 0.88 ± 0.04[a] | 0.89 ± 0.03[a] | ** |

[1]Numbers are given by Mean ± SEM
[2]Means with different superscript letters within a row are statistically different
‡Control 1 restricted hen at week 31
‡‡Control 2 restricted hen at week 33
**P Value: Kruskal-Wallis test with alpha < 0.01
***P Value: Kruskal-Wallis test with alpha < 0.001

## Bacterial taxonomy

The dominant bacterial phyla across all the samples were: *Firmicutes* 52%, *Proteobacteria* 18%, *Actinobacteria* 18%, and *Bacteroidetes* 10%. No significant differences were noted in dominant bacteria phyla among groups of hens. Linear discriminant analysis effect size, LEfSe, identified 12 bacterial taxa differentially abundant among the three groups of hens (See Figure 12). Based on LEfSe, the low abundance phylum *Verrucomicrobia* and *Cerasicoccaceae* were enriched in the Control 2. Kruskal-Wallis test also found *Verrucomicrobia* in addition to *Cyanobacteria* significant enriched in the Control 2 and Released hens (See Table 14).

Differences in taxonomic bacteria with relative abundance greater than 1% were identified at the family and genus level. Kruskal-Wallis test detected the genus *Lactobacillus*, from the family *Lactobacillaceae*, underabundant in the Released hens. Two bacterial genera that were reduced in the Released hens were *Bacteroides* and an unclassified bacterium from the family *Ruminococcaceae*. One interesting genera LEfSe identified differing among groups of hens was *Akkermansia*, which was found to be more abundantly present in the Control 2 (See Figure 12, panel B).

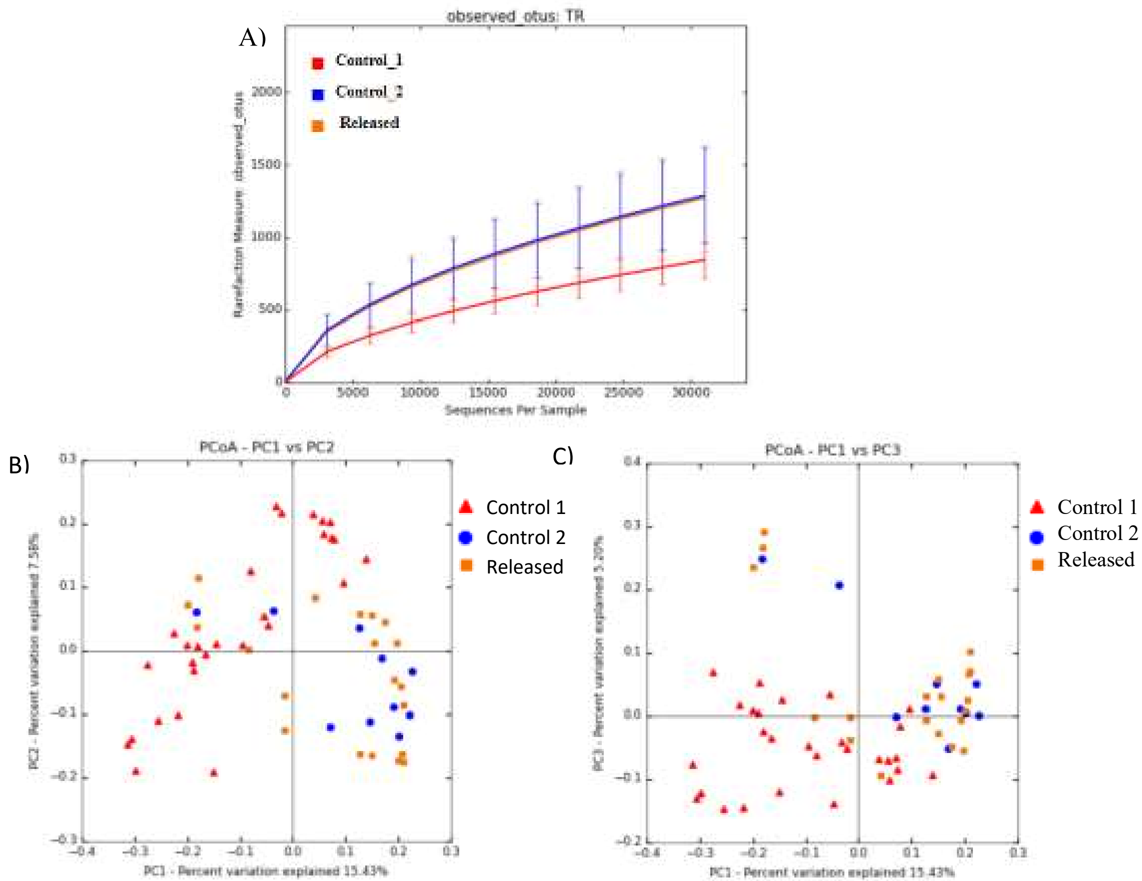


**Figure 11. Alpha and beta diversity of the 16S rRNA bacterial gene of the cloaca community of broiler breeder hens. Diversity analysis was conducted in a rarefied dataset at 31,000 read/samples, A). Principal coordinate analysis, PCoA, based on Unweighted UniFrac graphed in two PCs: PC1 and PC2 explained 23.01% of the variation, B), and PC1 and PC3 explained 20.63% of the variation, C). PCs figures show a slighted spatial heterogeneity between age of the hens.**

**Table 14. Relative abundance of the most dominant bacteria phyla, family, and genus.**

| | Range (min% - max%) | | | Median (%) * | | | |
|---|---|---|---|---|---|---|---|
| **Taxonomy** | **Control 1** | **Control 2** | **Released** | **Control 1** | **Control 2** | **Released** | **P** |
| ***Phylum*** | | | | | | | |
| Firmicutes | 2.84 - 98.81 | 6.23 - 85.79 | 1.76 - 97.86 | 54.03 | 44.54 | 44.32 | 0.524 |
| Proteobacteria | 0.54 - 9 5.28 | 0.74 - 91.77 | 0.48 - 96.08 | 5.20 | 10.61 | 9.24 | 0.252 |
| Actinobacteria | 0.30 - 55.59 | 1.34 - 28.61 | 0.49 - 56.37 | 13.64 | 16.39 | 13.28 | 0.974 |
| Bacteroidetes | 0.10 - 51.53 | 0.60 - 28.89 | 0.12 - 28.41 | 3.21 | 11.02 | 8.06 | 0.236 |
| Fusobacteria | 0 - 2.86 | 0.02 - 3.97 | 0 - 9.12 | 0.09 | 0.13 | 0.31 | 0.209 |
| Verrucomicrobia | 0 - 0.09 | 0 - 5.40 | 0 - 2.78 | 0 [b] | 0.08 [a] | 0.02 [a] | *** |
| Cyanobacteria | 0 - 0.46 | 0 - 0.86 | 0 - 1.48 | 0.01 [b] | 0.05 [a] | 0.06 [a] | *** |
| ***Family*** | | | | | | | |
| Planococcaceae | 0.13 - 86.96 | 0.18 - 71.87 | 0.14 - 82.71 | 0.51 | 0.53 | 1.00 | 0.098 |
| Lactobacillaceae | 0.16 - 90.48 | 0.18 - 88.47 | 0.27 - 81.42 | 7.60 [a] | 2.78 [ab] | 1.42 [b] | * |
| [Tissierellaceae]‡ | 0.15 - 29.62 | 0.09 - 35.18 | 0.05 - 30.47 | 9.22 | 5.88 | 1.30 | 0.237 |
| Actinomycetaceae | 0.08 - 27.20 | 0.36 - 35.25 | 0.07 - 33.96 | 5.98 | 9.20 | 1.52 | 0.255 |
| Porphyromonadaceae | 0.06 - 17.52 | 0.13 - 25.91 | 0.04 - 51.15 | 4.58 | 4.54 | 0.64 | 0.227 |
| Corynebacteriaceae | 0.17 - 27.43 | 0.23 - 16.69 | 0.06 - 35.52 | 4.76 | 3.51 | 1.25 | 0.270 |
| Pasteurellaceae | 0.10 - 59.34 | 0.07 - 31.08 | 0.10 - 10.45 | 0.77 | 0.34 | 0.29 | 0.108 |
| Moraxellaceae | 0.06 - 51.92 | 0.04 - 3.70 | 0.04 - 78.38 | 0.65 | 0.18 | 0.16 | 0.054 |
| Pseudomonadaceae | 0.04 – 85.77 | 0.05 - 93.07 | 0.07 - 42.21 | 0.28 | 0.10 | 0.14 | 0.147 |
| Enterobacteriaceae | 0.06 - 24.7 | 0.06 - 1.29 | 0.07 - 88.8 | 0.62 | 0.24 | 0.22 | 0.061 |
| Staphylococcaceae | 0.13 - 44.67 | 0.11 - 39.38 | 0.17 - 14.57 | 0.48 | 0.46 | 1.61 | 0.511 |
| Aerococcaceae | 0.14 – 38.0 | 0.03 - 5.46 | 0.16 - 16.90 | 1.30 | 1.17 | 1.92 | 0.243 |
| Enterococcaceae | 0.05 - 5.24 | 0.22 - 11.70 | 0.1 - 31.10 | 0.90 | 0.91 | 1.80 | 0.595 |
| Ruminococcaceae | 0.06 - 25.30 | 0.05 - 7.40 | 0.05 - 0.67 | 1.18 [a] | 0.35 [ab] | 0.16 [b] | *** |
| Streptococcaceae | 0.02 - 81.88 | 0.04 - 5.00 | 0.03 - 0.52 | 0.19 | 0.08 | 0.10 | 0.096 |
| Bacteroidaceae | 0.01 - 17.33 | 0.02 - 7.28[ab] | 0.01 - 0.30 | 0.53 [a] | 0.22 [ab] | 0.05 [b] | *** |
| Lachnospiraceae | 0.03 - 9.13 | 0.06 - 3.59 | 0.02 - 0.91 | 1.21 [a] | 0.35 [ab] | 0.08 [b] | *** |
| Comamonadaceae | 0.01 - 43.65 | 0 - 1.41 | 0 - 1.13 | 0.30 | 0.17 | 0.08 | * |
| ***Genera*** | | | | | | | |
| Lactobacillus | 0.16 - 90.48 | 0.48 - 88.46 | 0.27 - 81.40 | 7.60 [a] | 2.78 [ab] | 1.42 [b] | * |
| Sporosarcina | 0.10 - 69.26 | 0.08 - 45.98 | 0.11 - 78.86 | 0.35 | 0.38 | 0.69 | 0.065 |
| Actinomyces | 0.07 - 23.91 | 0.27 - 33.23 | 0.07 - 32.72 | 5.32 | 8.66 | 1.32 | 0.268 |
| Porphyromonadaceae ‡‡ | 0.02 - 17.12 | 0.13 - 25.9 | 0.02 - 51.12 | 4.52 | 4.12 | 0.64 | 0.289 |
| Corynebacterium | 0.17 - 27.43 | 0.23 - 16.69 | 0.06 - 35.52 | 4.76 | 3.51 | 1.25 | 0.270 |
| Gallibacterium | 0.10 - 59.30 | 0.07 - 31.04 | 0.10 - 10.43 | 0.73 | 0.33 | 0.29 | 0.140 |
| Acinetobacter | 0.05 - 51.88 | 0.04 - 3.61 | 0.04 - 78.33 | 0.64 | 0.15 | 0.15 | * |
| Enterobacteriaceae ‡‡ | 0.05 - 24.4 | 0.05 - 127 | 0.07 - 87.96 | 0.61 | 0.24 | 0.22 | 0.062 |
| Staphylococcus | 0.10 - 43.84 | 0.1 - 38.82 | 0.14 - 13.72 | 0.37 | 0.37 | 1.25 | 0.471 |
| Helcococcus | 0.02 - 7.77 | 0.02 - 10.03 | 0 - 13.3 | 2.07 | 2.16 | 0.38 | 0.296 |
| Pseudomonas | 0.03 - 54.60 | 0.02 - 55.50 | 0.04 - 24.11 | 0.18 | 0.07 | 0.09 | 0.133 |
| Enterococcus | 0.04 - 5.13 | 0.20 - 11.59 | 0.09 - 30.98 | 0.88 | 0.88 | 1.76 | 0.644 |
| Peptoniphilus | 0 - 9.19 | 0.01 - 9.15 | 0 - 7.35 | 1.87 | 0.70 | 0.29 | 0.131 |
| [Tissierellaceae]; ph2 | 0 - 6.55 | 0.03 - 7.12 | 0.01 - 4.53 | 1.74 | 0.97 | 0.26 | 0.286 |
| Lactococcus | 0.02 - 81.75 | 0.02 - 4.43 | 0.01 - 0.51 | 0.16 | 0.08 | 0.10 | 0.113 |
| Pseudomonadaceae ‡‡ | 0 - 31.10 | 0.01 - 37.36 | 0.02 - 18.03 | 0.06 | 0.03 | 0.04 | 0.203 |
| Aerococcus | 0.03 - 35.97 | 0.02 - 4.90 | 0.05 - 16.64 | 0.21 | 0.20 | 0.30 | 0.498 |
| [Tissierellaceae] ‡‡ | 0.04 - 5.17 | 0 - 4.72 | 0 - 5.81 | 1.13 | 0.50 | 0.20 | 0.203 |
| Bacteroides | 0.01 - 17.33 | 0.02 - 7.28 | 0.01 - 0.30 | 0.53 [a] | 0.23 [ab] | 0.05 [b] | *** |
| Planococcaceae ‡‡ | 0.01 - 1.25 | 0.01 - 7.91 | 0.01 - 32.61 | 0.06 | 0.05 | 0.14 | 0.164 |
| Ruminococcaceae ‡‡ | 0.04 - 18.08 | 0.03 - 4.69 | 0.02 - 0.48 | 0.83 [a] | 0.21 [ab] | 0.10 [b] | *** |
| Lysinibacillus | 0 - 16.44 | 0 - 17.6 | 0 - 19.45 | 0.05 | 0.06 | 0.14 | 0.102 |
| Comamonas | 0.01 - 43.23 | 0 - 1.4 | 0 - 1.07 | 0.26 | 0.16 | 0.30 | * |

[1] Median with different superscript within a row differ (P<0.05, Dunn's multiple comparisons)
‡ Squared brackets = Taxonomic group QIIME v.1.9.0 proposed based on Greengenes v.13.8 database
‡‡ Unclassified bacteria unidentified genera with 97% similarity to a reference sequence
**P Value: Kruskal-Wallis test with alpha < 0.01
***P Value: Kruskal-Wallis test with alpha < 0.001

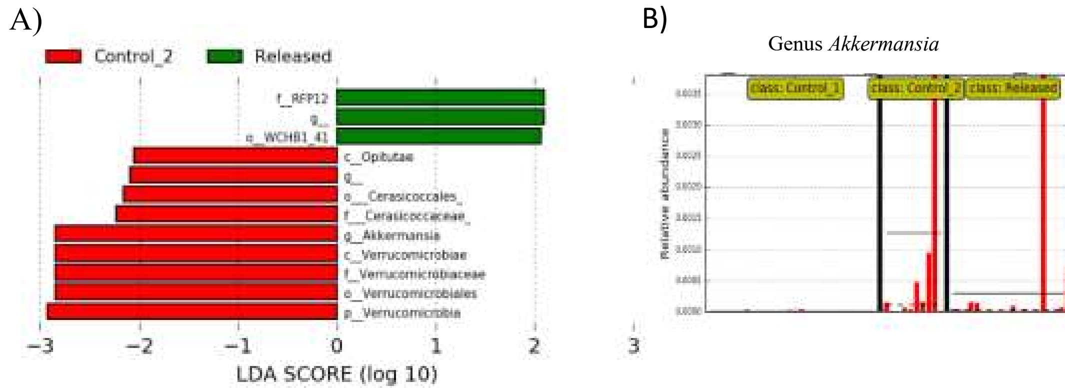


**Figure 12. Differentially abundant bacteria taxa of the cloaca community between overfed and feed-restricted mature hens ROSS 708. Abundance of bacteria taxa statistically different among Control 1, Control 2, and Released hens (A). There were no bacteria taxa significant abundant in Control 1 hens. Abundance of the genus *Akkermansia* that LEfSe identified to be overabundant in the Control 2 (B).**

## *Discussion*

In this study, an experimental design that allows broiler breeder hens to consume twice the recommended feed restriction ration was replicated for a 10-days period to annotate the changes in the gut bacteria diversity and taxonomy. Reproductive parameters were compared between a control group of hens that continued with the feed restriction regimen and the feed-satiated hens. Previously, this overfed hen model was reported to increase body weight, abdominal tissue, have impair egg production and ovarian function (Chen, 2004). Conversely, this experiment found the body weight, abdominal tissue and eggs production unaffected by the provision of more caloric intake (Table 11 and Table 12). Notably the strain of broiler hen, being Ross 708, differed from the Cobb 500 used in prior studies. Nevertheless, voluntary feed intake affected the absolute and fractional weights of liver and ovary. Additionally, more than 50% (12 out of 20) of the feed-satiated hens developed liver steatosis and presented an expanded follicular hierarchy of large follicles that were heavier at each comparable classification (F1, F2 etc.). The 16S ribosomal-

RNA-encoding gene recorded similarity in the community of bacteria of the cloaca between restricted and unrestricted groups of hens. The major alterations in bacteria diversity were found in low abundant bacteria but alterations in the gut microbial composition were more pronounced based on the time hens were in the experimental environment than on the consumption of excess of calories.

The negative impact that voluntary feeding have on egg production has been described in various breeds of hens for meat purposes (Chen et al., 2017; Chen et al., 2006; Pan et al., 2014). Our study was based on in which Cobb 500 hens were allowed free access to feed for a 10-day period and reported overfeeding induced diabetic conditions, lipotoxicity, and impairment in egg production. While the overfed hens in Chen (2004) reduced egg production within three days following excess caloric intake, egg production in this experiment was stable throughout the10-day period. Research in energy-satiated hen model have generally conducted used longer periods of ad libitum access to feed (Heck et al., 2004; Robinson et al., 1991; Robinson and Wilson, 1996), which limits our ability to make relevant comparisons. An investigation to identify novel genes for follicular selection in broiler hens provided a comparative reproductive results since researchers used the same breed of hens at similar age (29 weeks old Ross 308) for 14 days and provide information of feed satiated and feed restricted hens (McDerment et al., 2012). In keeping with the aforementioned study, egg production was unaffected in the overfed hens and they developed expanded follicular hierarchy and heavier follicular weight. Therefore, we attribute our results were influenced by the genetics background of the hens, because several genetic lines of broiler hens have showed variations in reproductive activity when allowing excess of dietary calories (Heck et al., 2004; Robinson and Wilson, 1996; Yu et al., 1992). For instance, the dwarf broiler hens are known to tolerate *ad libitum* feeding and maintain egg productivity as opposed to

standard lines of heavy hens (Bruggeman et al., 2005; Heck et al., 2004). According to Griffin and Goddard (1994), some breeding companies may have introduced a dwarfing gene into the female lines as an approach to counteract the conflict of egg and meat production. Our finding may support the notion that Ross and Cobb broiler hens respond differently to the excess caloric intake in that Cobb appear to be highly susceptible and Ross hens more tolerant. This suggest that Ross broiler hens may persist in egg production when flock uniformity is not optimal. Nevertheless, as broiler chickens are constantly selected to improve feed conversion, and recently for animal welfare, it may not be practical to compare new results obtained with commercial breeds of 10 years ago. Thereby, more investigations utilizing both lines of hens are needed to distinguish among the responses to overconsumption of energy.

Calorie-restricted diets without malnutrition extend the lifespan and confer numerous benefits to the health of the broiler breeder parents (Zhang et al., 2013). It has been observed that concomitant to feed restriction there is an alteration in the community structure of the gut microbiota (Wang et al., 2018b) which has been proposed to be a positive change correlated with the health benefits of caloric restriction. The anti-obesity effect of mice fed 70% of a normal chow ad libitum slighted increased the proportion of the bacterial phyla *Bacteroidetes* and *Actinobacteria*, while *Firmicutes* and *Verrucomicrobia* were decreased (Wang et al., 2018b). Caloric restriction is a nutritional intervention that reduces body weight in subjects already overweight. Conversely, the purpose of this intervention in broiler breeder hens is to secure the gradual increment in body weight throughout the hens' productive lifespan. Thus, caloric intervention between feed-restricted mammals and feed-restricted broiler hens may predispose the enteric bacteria to different environmental stress and behave differently. According to chicken-independent models life-long caloric restriction strongly shapes the gut microbial composition

(Kiilerich et al., 2016). It was recently shown that the population of gut bacteria from life-long caloric-restricted mice transplanted to gnotobiotic recipients endured an obesogenic diet (Wang et al., 2018b), suggesting that the evolution of functional bacteria genes extend the benefits of caloric restriction even in the presence of an insult. Feed restricted hens may develop a microbial community influenced by the life-long caloric restriction, that could have enabled the development of anti-obesity bacterial functionality that resist to the side effect of excess of dietary energy for a short period. We speculate this could have influenced the few taxonomic variations found between feed restricted and overfed hens in our experiment. Another reason why we did not find significant microbiota changes as those reported in chicken-independent experiments when ingesting more calories might be because in those experiment the dietary fat was altered (Jumpertz et al., 2011; Wang et al., 2019) while we maintained the same portion of macronutrients. Multiple times it has been determined that alteration of the dietary fat density modified the gut microbial population of the host (Bäckhed et al., 2007; Huang et al., 2013; Jumpertz et al., 2011). This suggest that the alteration of the gut microbiota reported in those studies when feeding an obesogenic diet (high-fat diet) is caused by the alteration of the diet rather than the animal developing obesity. A recent experiment reported that the modification in the indigenous gut bacteria is mostly influenced by the dietary fat density than by an obese genotype (Xiao et al., 2017a). In this experiment, the dietary calories were increased by doubling the feeding ration without altering the macronutrient distribution, which may induce the few microbial alterations reported between over-fed and caloric restricted hens.

Major taxonomic differences between the overfed and feed restricted hens were encountered in bacterial species with relative abundance < 1%. Feed satiated hens significantly reduced the bacterial families *Rumminococcaceae* and *Lachnospiraceae,* and the genus

*Bacteroides*. Theses alterations in low abundant microbes could suggest a change in the microbiota functionality. A metagenome investigation of 39 subjects identified that the low-abundance *Escherichia* contributed over 90% with the assembly of important proteins that enable other microbes to colonize the epithelium and bind to the mucus (Arumugam et al., 2011). Furthermore, the reduction of the bacteria family *Ruminococcaceae* may affect the production of butyrate short-chain fatty acid (Medvecky et al., 2018; Yang et al., 2013), which may predispose overfed hen to a less protective gut microbiota (Antonissen et al., 2016). Interestingly, the bacteria phylum *Verrucomicrobial* that contain the genera *Akkermansia* increased with the amount of time hens were in the experimental environment, but its abundance was significantly lower in the overfed hens than in the feed restricted hens or Control 2. A similar tendency in this bacterium was reported between obese and lean subjects (Everard et al., 2013). Specifically, the mucin-degrader bacterium *Akkermansia muciniphila*, proposed to protect the host during inflammation by enhancing the host lipoprotein metabolism, has been reported to be low or absent in overweight individuals (Shen et al., 2016). Based on the findings of this study, the gut microbial profile of feed restricted hens appears to provide more protection by having more butyrate producer and bacteria species with anti-inflammatory protection.

Variations in bacteria abundance from species with > 1% median relative abundance was only observed with the genus *Lactobacillus* that decreased in the overfed hens. Since fatty liver was the predominant metabolic disorder developed in half of the feed-satiated hens, the reduction of *Lactobacillus* may be correlated with the development of fatty liver disease that was recently reported in a chicken independent experiment (Wang et al., 2018a). Microbiome analysis has implicated the participation of the gut microbiota with fat accumulation in the liver (Le Roy et al., 2013). The development of liver steatosis mediated by the gut microbiota was investigated by

Wang et al. (2018a) with the conventionalization of mice with gut microbial transplant from a genetically obese donor (Prader-Willi syndrome). The recipient mice developed liver steatosis mediated by the alterations of hepatic transcriptional profile that affected functions related to lipid anabolism such as activation of lipid absorption, uptake of cholesterol, and synthesis of steroid (Wang et al., 2018a). Those metabolic changes were concomitant with the modification of the gut microbial structure where the recipient mice that developed fatty liver were low in the abundance of 6 OTUs, including OTUs from *Bifidobacterium* and *Lactobacillus*, and had a lower ratio of acetate and higher ratio of propionate short-chain fatty acids (Wang et al., 2018a). We conducted an additional comparison of the relative abundance of the bacteria at different taxonomic levels within overfed hens to identify bacteria members possibly contributing to the onset of liver steatosis. The phylum *Bacteroidetes* ($P = 0.044$) was the only phylum that was significantly decreased in hens that developed fatty liver, which could have contributed to the formation of two groups of overfed hens regarding liver characteristic.

In summary, this study showed that the overfed model of hens may differ in reproductive outcomes depending on the breed of the hens. The breed of hen used in this experiment maintained the eggs production and appeared to direct the excess of energy to develop more and heavier hierarchical follicles. Our microbiota results showed that the provision of twice the amount of feed provided under feed restriction guidelines mediated few taxonomic alterations in the bacterial community of the cloaca. Those alterations were mostly observed in the low abundant bacteria, such as the family *Rumminococcaceae* and *Lachnospiraceae* and the genus *Bacteroides,* and the high abundant *Lactobacillus* group. The bacteria modifications found in this experiment may have altered the gut microbial functionality and assisted to the development of fatty liver in feed-satiated hens. This is the first microbiome experiment conducted in feed-satiated broiler hens, therefore,

this experiment is relevant to understand gut microbial composition with susceptibility to metabolic disorders associated with excess of energy. More investigations are needed in this area to detect enteric microbial modifications associated with variations in feed consumption in commercial broiler breeder hens.

# CHAPTER IV

# EARLY GUT MICROBIAL ALTERATIONS IN A NATURAL OCCURRING NECROTIC ENTERITIS CHICKEN MODEL

## *Introduction*

Necrotic enteritis (**NE**) is a prevalent enteric disease in commercial poultry species and the cause of dramatic economic losses. This disease has long been known in the industry, but it was under control by the use of in-feed antimicrobial agents such as antibiotic growth promoters and ionophores coccidiostats. Since the use of subtherapeutic antimicrobial has been removed from farm practices, the current economic losses have extended to an augmentation in mortality, growth retardation, and a raise in the costs associated with medical treatments (Cooper and Songer, 2009). Consequently, researchers are motivated to improve knowledge about the different putative factors participating in the pathogenesis of this disease to pursue alternative methods for its control and reduction in commercial poultry productions.

The etiological agent of NE is the bacterium *Clostridium perfringens* (***Cp***). Various isolates of *Cp* have been identified depending on the type of major toxins they produce (A, B, C, D, E). The four major toxins are: Alpha, Beta, Epsilon and Lota (Songer and Meer, 1996), and the *Cp* isolates causing NE are type A, which produces Alpha toxin, and type C, which produces both Alpha and Beta toxins (Songer and Meer, 1996). The Alpha toxin is considered the most lethal that is able to hydrolase membrane phospholipids, resulting in the lysis of red and white blood cells (Elder and Miles, 1957) while the Beta toxin has a necrotizing effect in the intestinal mucosa (Songer and Meer, 1996). *Clostridium perfringens* is a spore-forming bacterium that has been isolated from the environment and feces of animals and humans (Yadav et al., 2017). The ability

to form spores enables *Cp* to tolerate temperature fluctuations (Parish, 1961). Because *Cp* is also associated with the soil, water, and feed (Van Immerseel et al., 2004), it is very likely for chickens to get exposed to *Cp* at an early age. In fact, *Cp* were isolated from eggshell fragments, chicks fluff, and paper pads (Craven et al., 2001). Thus, it is very likely for *Cp* to be a member of the indigenous bacteria of chickens (Barnes et al., 1972).

The injurious toxins are produced only when *Cp* encounter ideal conditions, which usually includes the appearance of intestinal lesions and excess of nutrients availability. The associated NE-symptoms are generally visible in broiler chickens between 2 to 5 weeks of age (Timbermont et al., 2011; Van Immerseel et al., 2004), and the clinical signs associated with this infection include depression, dehydration, diarrhea, ruffled feathers and lower feed intake (Gazdzinski and Julian, 1992). Many predisposing factors have been identified to assist in the pathogenesis of necrotic enteritis disease. Some of them consist of, but are not limited to, co-infection with coccidia, dietary influences, immune status, stress, and even gut microbial shift (Antonissen et al., 2016). It was found that birds experimentally challenged only with a *Cp* did not replicate the same alterations in species richness and membership in the gut bacteria as the *Cp*-challenged birds additionally exposed to the predisposing factors coccidia and a diet containing fishmeal (Stanley et al., 2014). High dietary fishmeal have been reported to affect broilers only when pathogenic *Cp* is a member of the intestinal microbiota (Huang et al., 2018).

The predisposing factor coccidia is caused by the protozoa of the genus *Eimeria* spp. whose life cycle includes an exogenous and an endogenous phase. During the exogenous phase, the infected and recovered birds shed the oocyst in the dropping; the unsporulated oocyst is stable and can survive environmental conditions for several months because it is protected by a thick-wall structure (Lal et al., 2009), therefore, the eradication of this parasite from the farm is almost

impossible. After the ingestion of the oocyst, the endogenous phase is facilitated by the mechanical grinding of the gizzard that releases the sporozoite to the intestinal environment. The endogenous phase initiates when the sporozoite have penetrated the epithelial cells and initiate a series of merogony or asexual reproduction cycles to generate new sporozoites that will carry on with the merogony (Yun et al., 2000). The extrication of the new sporozoite from the intestinal cells ruptures the membrane causing lesions, inflammation and protein leakage that set up the conditions for the overgrowth of *Cp* (Yun et al., 2000). After several merogonies, a sexual reproduction or gametogony takes place and form the oocysts that are subsequently released in the dropping and are viable to infect a new host (Yun et al., 2000). In regard to nutritional predisposing factors two main dietary ingredients have been identified: a high percentage of animal protein, such as fishmeal, and high non-starch polysaccharide (**NSP**) cereals, wheat, rye, barley, and oat groats (Riddell and Kong, 1992), albeit, there are other anti-nutrients identified to promote the overgrowth of *Cp;* such as lectins, tannins, and mycotoxins (Antonissen et al., 2015; McDevitt et al., 2007). It is believed that the predisposing dietary factors alter the intestinal environment and provide optimal conditions for the proliferation of anaerobic bacteria (Hubener et al., 2002) by increasing the viscosity of the digesta, reducing the passage rate and the digestibility of the nutrients (Kermanshahi et al., 2018). This was observed when elevated concentration of crude protein and amino acids increased the cfu/g of *Cp* in the ileum and cecum of broiler chickens (Drew et al., 2004).

Alterations in the structure of the gut bacteria have been observed in chickens when NE disease is experimentally induced. Microbiota analysis with high throughput sequencing technology revealed the ceca microbial community was altered when chickens were infected with both *C. perfringens* and coccidia, or infected with coccidia and supplemented with high fishmeal

diet (Lin et al., 2017). Gut microbial changes have also been reported in NE-induced models with a highly pathogenic *Cp* strain (WER-NE36) (Lacey et al., 2018) coccidia infection significantly altered the abundance of a number of bacteria taxa in the ceca (Macdonald et al., 2017).A study reported that the abundance of the bacteria families *Lactobacillaceae* increased and *Clostridiaceae* reduced in the bacteria community of the ceca and fecal of chickens with NE disease (Lacey et al., 2018). Another *Cp*-inoculated model, that fed chickens with a diet containing 50% of fishmeal, observed the NE-induced broilers (Ross 308) reduced the population of butyrate producer bacteria (Stanley et al., 2012b). In fact, a recent review paper described that the major gut bacteria shifts in the *Cp*-induced NE chicken models consist of an reduction in the abundance of the segmented filamentous bacteria, a shift in the proportion of the genera *Lactobacillus*, and a reduction of butyrate producer bacteria (Antonissen et al., 2016). The overgrowth of *Cp* and the gut bacteria shift at different taxonomic level is a clear representation of a dysbiosis state that accompany the onset of NE disease in commercial broiler chickens. Whether this bacteria shift is a consequence or a cause of NE still remains unknown; gut microbial population is an area that should be investigated in all the stages of the pathology of the NE challenged chicken models.

The experimental induction of necrotic enteritis in chickens is on demand to evaluate putative factors aiding in the virulence of this disease and to assess new intervention methods. The precipitation of NE has been conducted combining the infection of *Eimeria* spp. and diets with high fishmeal (Stanley et al., 2014). Albeit, some investigators have successfully replicated NE only with the coccidia-based model (Prescott et al., 2016). The reproduction of this disease under research conditions has been conducted by the inoculation of *Cp* isolates from a field case with NE that produces toxin A and carries the netB gene (Kaldhusdal et al., 1999; Riddell and Kong, 1992) and the co-infection with *Eimeria* species (Immerseel et al., 2004). Occasionally,

immunosuppressed birds can substitute *Eimeria* infections (Timbermont et al., 2011), such as broiler chickens infected with infectious bursal diseases virus vaccine which are prone to secondary infections (McReynolds et al., 2004). Researchers have also induced NE-associated symptoms by only exposing birds to environmental areas (litter, feeders and drinkers) with a previous history of NE outbreak. This approach is considered very convenient since it resembles those conditions in a natural occurring NE outbreak in a commercial poultry facility. This model effectively replicates the intestinal lesions and the NE-associated symptoms, and it was successfully implemented to determine the contribution of dietary calcium sources and levels on the pathogenesis of a NE episode (Paiva et al., 2013, 2014). The experimental design in the natural occurring NE model from the aforementioned experiments were without dietary intervention or oral inoculation of *Cp*. It was reported that the recommended dietary calcium for growing broilers (0.9%) (NRC, 1994) aggravated the progression of NE disease under a natural occurring NE model (Paiva et al., 2013, 2014). They conclude that lower calcium (0.6%) should be implemented in facilities with a known history of necrotic enteritis outbreak.

The current study used the natural occurring necrotic enteritis chicken model and assessed the gut microbial changes of the ileum and cecum occurring at first sign of morbidity in the population. Since dietary calcium contribute with the pathogenesis of NE, diets with two levels of calcium, high (0.90%) and low (0.70%), and supplemented with two feed additives to ameliorate the disease (product A or B) was used as dietary treatment in this experiment. High throughput bacteria sequencing was conducted to amplify the V4 region of the 16S rRNA bacterial gene in a MiSeq Illumina platform to compare the bacterial richness and diversity between apparent healthy chickens and chickens with NE-associated signs. Usually, control birds are maintained in a distinct setting to avoid cross contamination, however, in this experiment the control birds were healthy

pen-mates selected from the same pen as sick chickens. It was hypothesized chickens with necrotic enteritis symptoms have an altered microbial community and that this may be affected by the dietary treatment.

## *Materials and Methods*

### Animal trial

Animal husbandry was done at the Poultry Science Research, Teaching, and Extension Center, Texas A&M University (TAMU) and approved by the Institutional Animal Care and Use Committees (IACUC 2015-0249) in accordance with the Animal Welfare Act, the Public Health Service Policy and the Humane Care and use of Laboratory Animals. Day-old male chickens (Cobb 500) were acquired from a local hatchery (Tenaha, TX) and were placed in a house with a concrete floor and tunnel ventilation. Chicks received the standard vaccination at the hatchery and upon arrival received 1X spray vaccination with a commercial coccidia vaccine (Advent, Huvepharma) following the manufacturer's application guidelines. Chickens remained in the hatchery tray for 30 minutes to allow the ingestion of the vaccine. The total chickens were n=2,688 allocated in 96 pens (3ft x 6ft = 18ft$^2$, 28 chickens/pen). Pens were separated with 2ft high side walls with 1" x 1" welded wire. The pens had litter from previous flock with clinical sign of NE top-dressed with fresh pine shaving.

### Experimental diets and productive parameters

The birds were fed a corn-soybean meal basal diets in phases for broiler (starter 1-14d, grower 15-35d and finisher 35-45d) formulated to meet or exceed the NRC (1994) nutrients recommendations except for calcium. One basal diet were formulated with NRC (1994) calcium

recommendations 0.95% (**Stand**), and the second basal diet formulated with 0.75% calcium (**LoCa**) Table 15. Additionally, two dietary supplements were added for the control and prevention of NE-associated symptoms, supplemental products A, which is a probiotic and prebiotic mixture, and product B that is a non-human medication. Six (6) dietary treatments were used in this experiment: a Stand, LoCa**,** two diets with 0.95% dietary calcium and supplemented with product A and B (**StandA** and **StandB**) and two diets containing 0.75% dietary calcium supplemented with product A and B (**LoCaA** and **LoCaB**). Each treatment consisted of 16 replicate pens with 28 chickens per pen. Birds had ad libitum access to feed and water.

Body weight and feed weight were performed on day 14, 35, and 45, to calculate average body weight (**BW**) gain, feed intake (**FI**), and feed conversion (**FC**). Mortality was recorded daily.

**Tissue collection**

Birds were monitored twice a day for clinical signs of NE. First NE-associated symptoms were found at day 14, and at day 18 all pens had at least one bird with NE incidence. Chickens were considered having NE-associated symptoms when had depression, ruffler feather and diarrhea. In total, 19 sick chickens together with 19 healthy pen-mate were collected at day 18 to sample the ileum and cecum for microbial analysis. Chickens were humanly slaughtered with CO2 asphyxia and the ileum and cecum were aseptically removed. Two sections of the ileum were dissected from the midpoint between Meckel's diverticulum and the ileal-ceca junction for *Cp* enumeration and for bacteria sequencing, and the content of one cecum was extruded into a clean tube by finger pressure for bacteria sequencing.

**Table 15. Ingredients composition and calculated nutrient values of experimental diets, in mass percentage unless otherwise indicated**

| | Starter | | Grower | | Finisher | |
|---|---|---|---|---|---|---|
| Item | Stand | LoCa | Stand | LoCa | Stand | LoCa |
| Corn | 57.31 | 59.16 | 65.65 | 67.48 | 69.84 | 71.21 |
| Dehulled soybean meal | 36.04 | 35.72 | 27.36 | 27.05 | 24.15 | 24.03 |
| Fat blended (animal/vegetable) | 2.85 | 2.21 | 2.67 | 2.04 | 2.84 | 2.34 |
| Pork MBM[1] | 0.00 | 0.00 | 0.85 | 0.85 | 0.00 | 0.00 |
| Salt | 0.46 | 0.46 | 0.26 | 0.25 | 0.23 | 0.23 |
| DL-methionine | 0.23 | 0.22 | 0.32 | 0.32 | 0.23 | 0.23 |
| Lysine HCL | 0.14 | 0.14 | 0.26 | 0.27 | 0.19 | 0.19 |
| L-threonine | 0.06 | 0.06 | 0.19 | 0.19 | 0.06 | 0.06 |
| Calcium Mineral | 1.77 | 1.36 | 1.41 | 1.00 | 1.42 | 1.08 |
| Monocalcium phosphate | 0.79 | 0.32 | 0.48 | 0.00 | 0.40 | 0.00 |
| Sodium Bicarbonate | 0.00 | 0.00 | 0.19 | 0.20 | 0.29 | 0.29 |
| Trace mineral[2] | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Vitamins[3] | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Phytase | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Calculated nutrient composition | | | | | | |
| Energy (ME, kcal/kg) | 3,058 | 3,058 | 3,146 | 3,146 | 3,190 | 3,190 |
| Protein | 22.67 | 22.68 | 19.88 | 19.89 | 18.00 | 18.00 |
| Lysine | 1.32 | 1.32 | 0.91 | 1.21 | 1.05 | 1.05 |
| Methionine | 0.56 | 0.56 | 0.62 | 0.62 | 0.51 | 0.51 |
| TSAA (methionine + cystine) | 0.94 | 0.94 | 0.94 | 0.94 | 0.82 | 0.82 |
| Crude fat | 5.36 | 4.79 | 5.51 | 4.95 | 5.71 | 5.26 |
| Crude Fiber | 2.67 | 2.69 | 2.54 | 2.56 | 2.48 | 2.50 |
| Calcium | 0.95 | 0.75 | 0.85 | 0.65 | 0.75 | 0.58 |
| Available phosphorus | 0.47 | 0.37 | 0.42 | 0.32 | 0.37 | 0.29 |

[1]Meal and Bone Meal
[2]Vitamin premix/kg diet 11023 IU vitamin A, 3858 IU vitamin $D_3$, 46 IU vitamin E, 0.0165 mg $B_{12}$, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 261.2 mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin.
[3]Trace mineral premix/kg diet 149 mg manganese, 125 mg zinc, 17 mg iron, 7 mg copper, 1.0 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium.

## Enumeration of Clostridium Perfringens

*Clostridium perfringens* (*Cp)* was enumerated from a section of the ileum (~3''). Both, the ileum tissue and the content were weighted and homogenized in 9X volume of Fluid Thrioglycollate Medium (FTM) and were serially diluted. *Cp* was enumerated using the Tryptose Sulphite Cycloserine (FSC) Egg Yolk overlay agar, that consisted of *Cp* agar base with 5% egg yolk emulsion and D-cycloserine for 24-48h at 37°C. *C. perfringens* counts were log transformed.

**DNA preparation and sequencing**

The luminal and adhered bacteria extraction, amplification and sequencing was completed by MrDNA laboratory (www.mrdnalab.com, Shallowater, Tx, USA). The bacterial DNA was extracted using PowerSoil DNA isolation kit (QIAgen, USA). The V4 hypervariable region of the 16S rRNA bacteria gene was PCR amplified with the forward primer 515F: GTGCCAGCMGCCGCGGTAA and reverse primer 806R: GGACTACHVGGGTWTCTAAT using the HotStar Taq Plus Master Mix Kit (QIAgen, USA) following the manufacturer's instructions. The thermal cycling consisted on an initial denaturation at 98°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s, and elongation at 72°C for 1 min, followed by 72°C for 5 min. The PCR products were checked in a 2% agarose gel and then pooled in equal proportions based on the molecular concentration. The pooled samples were purified with calibrated Ampure XP beads and the Illumina library were prepared using the pooled sample and purified PCR products. Illumina sequencing was performed on a Miseq following the manufacturer's guidelines. These processes were completed by MrDNA laboratory (www.mrdnalab.com, Shallowater, Tx, USA).

**Bioinformatics analysis**

The raw sequences data was processed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline v 1.9 (Caporaso et al., 2010). The sequences were de-multiplexed and barcodes removed following the default QIIME setting for quality filtering. Chimera sequences were identified and filtered with the USEARCH 6.1 pipeline (Edgar, 2010). Operational taxonomic units, OTUs, were constructed with an open-reference approach and taxonomic assignment was performed against the Greengenes 13.8 database with a 97% cutoff (DeSantis et al., 2006). In this

process of picking OTUs with open references the reads are aligned against a reference sequence in a database and the new reads are grouped based on De novo strategy. In De novo OTUs picking the reads are grouped together without a reference genome. According to Navas-Molina et al. (2013) the recommended OTU picking approach is open-references because it provides the best trade-off between the time and the ability to discover novel diversity. Sequences assigned to chloroplast, mitochondria, and low abundance OTUs with < 0.01% abundance were removed. Rarefaction curves were constructed to reduce variability at 18,500 reads per sample. At this rarefaction depth, 4 cecum samples were excluded to conduct the downstream analysis to have access to those rare bacteria (3 ceca from sick and 1 from healthy chickens). This rarefaction level complied with the minimum recommended depth for dataset sequenced in Illumina platform (1000 sequences/sample) (Navas-Molina et al., 2013). The bacteria diversity within samples was evaluated generating alpha diversity parameters utilizing the default script of QIIME. The two alpha parameters to evaluate the community richness were Chao1 and Observed Species, and two diversity index, Shannon and Simpson, to take the abundance of species into account and evaluate the proportion and degree of concentration of the species. The comparison of the community structure between samples or beta diversity was calculated utilizing the phylogenetic-based beta diversity with UniFrac distance metric of rarified samples calculated with Weighted and Unweighted UniFrac (Lozupone and Knight, 2005), and visualized in a Principal Coordinate Analysis (PCoA) plot.

**Statistical analysis**

Productive performance parameters (body weight, **BW**, Feed intake, **FI**, feed conversion ratio, **FCR**, and Mortality) were analyzed with Least Square Means and multiple comparisons

were conducted with Tukey's honest significance in JMP (JMP Pro 13, SAS software Inc.). The transformed log10 cfu of enumerated *Clostridium perfringens* were analyzed with paired T-test in SPSS (SPSS Statistics for Windows, Armonk, NY: IBM Corp). Alpha diversity estimates and the bacterial taxa abundances were evaluated for normality with Shapiro-Wilk Test, and, due to a failed normality assumption they were assessed using Kruskal-Wallis (JMP Pro 13, SAS software Inc.). Linear discriminant analysis effect size (**LEfSe**) was used to elucidate differential abundance of bacteria taxa between sick and healthy ceca and ileum (Segata et al., 2011) with the online Galaxy workflow (http://huttenhower.sph.harvard.edu/galaxy/) with $\alpha = 0.05$ and LDA score = 3.0. This is a statistical tool to identify the differential features between groups with high-dimensionality data. LEFSe initially detects the differences in taxonomic abundance with Kruskal-Wallis sum-rank and consistency is investigated with a pairwise test Wilcoxon rank-sum test and then apply a linear discriminant analysis to estimate the effect size of each differentially abundant feature (Segata et al., 2011). The similarity in the structure of microbial communities was numerically computed with **ANOSIM** (Analysis of Similarity) tested in PRIMER 6 software (PRIMER-E ltd., Luton, UK). The non-parametric univariate test, Kruskal-Wallis test (JMP Pro 13, SAS software Inc.) compared the relative abundance of bacteria at each taxonomic level. Statistical significances were considered at $P$ value < 0.05.

## *Results*

### Animal performance

Unexpected, the productive parameters of chickens fed the LoCa diet scored the lowest compared to the Stand diet (Table 16). The groups of chickens supplemented with the feed additives A and B were not superior in body weight gain, feed intake, FCR, and mortality to

chickens fed the Stand diet. The supplement product B appeared to have a better synergy with the Stand diet than in combination to the LoCa, however, this synergism was not superior than the provision of the Stand diet alone.

**Table 16. Effect of percentage of dietary calcium and supplement A and B on BW gain, feed intake, FCR, and mortality at different growing periods**

| | Stand | LoCa | StandA | LoCaA | StandB | LoCaB | P Value[‡] |
|---|---|---|---|---|---|---|---|
| BW gain, g | | | | | | | |
| 0-14 | 362.4[a] | 301.2[c] | 361.6[a] | 322.7[b] | 355.4[a] | 323.6[b] | *** |
| 14-35 | 1483.3 | 1451.2 | 1424.2 | 1496.6 | 1463.3 | 1423.9 | 0.64 |
| 35-42 | 576.2[a] | 459.8[b] | 541.6[ab] | 499.7[ab] | 591.7[a] | 490.5[ab] | ** |
| 0-42 | 2,511.6 | 2,332.4 | 2,420.7 | 2,425.0 | 2,482.7 | 2,366.8 | 0.23 |
| Feed intake, g | | | | | | | |
| 0-14 | 481.0[a] | 408.5[b] | 479.1[a] | 425.5[b] | 469.6[a] | 428.2[b] | *** |
| 14-35 | 2,397.2 | 2,295.5 | 2,312.4 | 2,369.0 | 2,350.8 | 2,269.8 | 0.34 |
| 35-42 | 1,140.7 | 1,037.5 | 1,097.8 | 1,073.3 | 1,157.1 | 1,067.5 | 0.07 |
| 0-42 | 4,142.7 | 3,912.6 | 4,018.4 | 4,015.1 | 4,075.6 | 3,950.2 | 0.35 |
| FCR, kg:kg[1] | | | | | | | |
| 0-14 | 1.32 | 1.35 | 1.32 | 1.32 | 1.32 | 1.32 | 0.40 |
| 14-35 | 1.62 | 1.58 | 1.62 | 1.58 | 1.60 | 1.59 | 0.13 |
| 35-42 | 1.99[a] | 2.28[b] | 2.08[ab] | 2.19[ab] | 1.98[a] | 2.23[ab] | * |
| 0-42 | 1.65 | 1.68 | 1.66 | 1.66 | 1.64 | 1.67 | 0.24 |
| Mortality[2], % | | | | | | | |
| 0-14 | 4.00[ab] | 4.67[b] | 2.44[ab] | 3.57[ab] | 1.19[a] | 2.22[ab] | * |
| 14-35 | 2.06[ab] | 4.28[b] | 2.50[ab] | 4.00[ab] | 1.22[a] | 1.39[ab] | * |
| 35-42 | 0.82 | 2.43 | 1.14 | 1.67 | 0.55 | 3.11 | 0.27 |
| 0-42 | 6.67[ab] | 10.45[b] | 5.78[ab] | 8.57[ab] | 2.85[a] | 6.00[ab] | * |

[1]Mortality adjusted Feed Conversion Ratio (FCR)
[2]Mortality excluding cull
[3]Median with different superscript within a row differ P<0.05, Tukey's honest significance.
[‡]Least Square Means P value
[*]P < 0.05
[**]P < 0.01
[***]P < 0.001

## Enumeration of clostridium

No interaction between dietary calcium and feed additives was reported in the recovery of *C. perfringens* from the ileum community (P = 0.084). The administration of the supplement B in the Stand diet (StandB) reduced the $Log_{10}$cfu $g^{-1}$ of *Cp* in the ileum, P = 0.017, but there was no

reduction when combined with the LoCa, P = 0.952. Significant difference in *Cp* enumeration was found between the health and sick ileum, P = 0.007. The *C. perfringens* recovered from healthy ileum was (mean ± SD) 3.36 ± 1.12 and the sick ileum was 4.83 ± 1.78.

**Sequencing analysis**

A total of 76 samples, half ileum and half cecum, were collected to characterize the changes in the microbial structure during the onset of an outbreak of necrotic enteritis. The sequences analysis yielded 2,284,761 quality sequences (mean ± SD = 30,062 ± 7,486). Rarefaction was conducted to reduce variability among samples and it was settled at 18,500 reads per sample. At this rarefaction depth, 4 ceca samples were excluded from the downstream analysis, 3 from sick and 1 from healthy chickens.

*Alpha and beta diversity parameters based on dietary treatment*

Based on the rarefaction curve of the observed species parameter, all six dietary treatments displayed closeness in terms of the species richness (Figure 13, panel A). The alpha diversity estimates that evaluated species richness (Chao1 and observed species) and diversity index (Shannon and Simpson) were not influenced by the dietary treatments. This was statistically computed with Kruskal-Wallis test that reported all P values > 0.05. The principal coordinate analysis (PCoA) constructed with the Unweighted UniFrac distance compared the community structure of all the samples based on the type of diet showed all samples clustering, indicating high similarity between all 6 dietary treatments (Figure 13 panel B). The Weighted UniFrac also reported similarities between dietary treatments, suggesting the gut bacterial membership between dietary treatments were qualitatively and quantitatively similar.

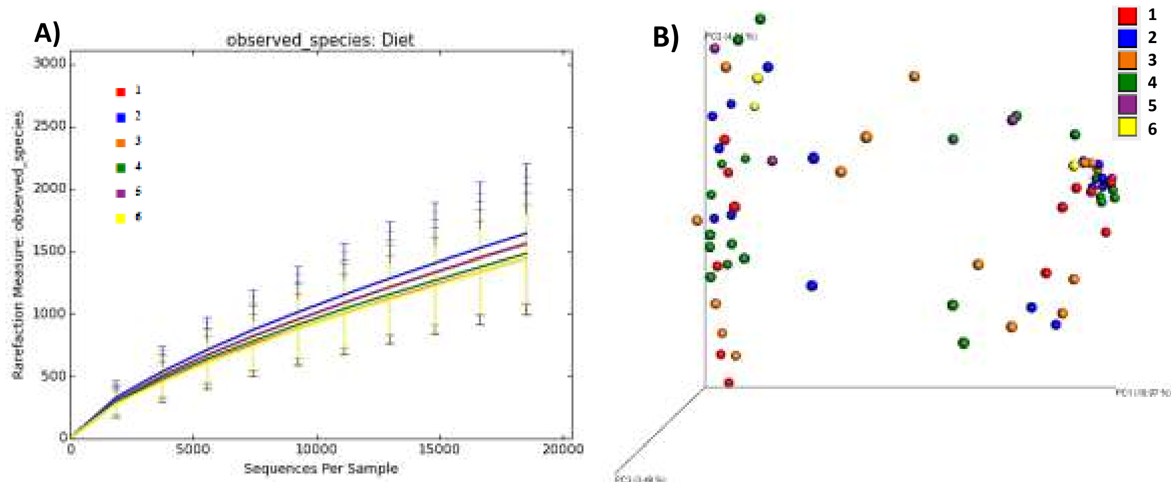


**Figure 13. Alpha and beta diversity parameters of the gut bacterial community of broiler chickens based on dietary treatments. Rarefaction analysis of 16rRN gene sequences at 18,500 reads per sample. Panel A, Alpha diversity evaluated with the number of observed species. Each line represents the mean of each dietary treatment while the error bars are the standard deviation. Panel B, beta diversity visualized with a 3D principal coordinate analysis (PCoA) of Unweighted UniFrac distance. Each point represents the community structure of the ileum bacteria of individual chickens. Dietary treatments are as follows 1 = Stand, 2 = LoCa, 3 = StandA, 4 = LowCaA, 5 = StandB, and 6 = LoCaB. Kruskal-Wallis test revealed no differences in the observed species based on the diet. No clustering is observed based on the diet with Unweighted UniFrac distance.**

*Alteration in alpha and beta diversity indices due to NE-associated symptoms*

Rarefaction curves of a random sample combining the information of the ileum and cecum from healthy and sick chickens showed the number of observed species was reduced in birds with NE symptoms (Figure 14, panel A). The multidimensional scaling principal coordinate analysis (PCoA) on Unweighted and Weighted UniFrac distances visually showed an apparent overlap of the bacterial communities between healthy and sick chickens (Figure 14, Panel B), suggesting the microbial information of pooled ileum and cecum shared similarities, in terms of phylogenetic composition.. Nevertheless, the analysis of similarity ANOSIM test numerically computed significant differences on the basis of health status with Weighted UniFrac metric ($R = 3$ $P = 0.03$), but no difference was found with the Unweighted UniFrac metric ($R = 0.04$ and $P > 0.05$). This

suggest that the combination of the ileum and cecum bacteria from healthy and sick chickens have a similar number of species while differing in the proportion of the OTUs represented on each bacterial community. Marked differences in species richness and diversity were observed between health condition based on the intestinal segments (Figure 14, Panel C and D).

**Table 17. Summary of alpha diversity estimates of the bacterial community in the cecum and ileum of healthy and sick chickens.**

| | Healthy (n=18) | Max-min | Necrotic enteritis (n=16) | Max-min | *P* Value‡ |
|---|---|---|---|---|---|
| **Tissue: Cecum** | | | | | |
| Chao1 | 5,958 ± 151 | 6,824 - 4773 | 5,078 ± 163 | 6,299 - 3,415 | ** |
| Observed species | 2,179 ± 71 | 2,535 - 1,518 | 1,773 ± 70 | 2,369 - 1,176 | ** |
| Shannon | 7.10 ± 0.23 | 8.14 - 4.29 | 5.72 ± 0.27 | 7.27 - 3.39 | ** |
| Simpson | 0.94 ± 0.01 | 0.98 - 0.74 | 0.87 ± 0.02 | 0.96 - 0.66 | ** |
| **Tissue: Ileum** | | | | | |
| Chao1 | 4,123 ± 126 | 5,054 - 3,102 | 3,825 ±110 | 4,595 - 2,927 | NS |
| Observed species | 1,165 ± 35 | 1,619 - 977 | 1,059 ± 31 | 1,325 - 805 | * |
| Shannon | 3.81 ± 0.16 | 5.70 - 2.91 | 3.19 ± 0.22 | 5.53 - 1.8 | * |
| Simpson | 0.76 ± 0.02 | 0.90 - 0.55 | 0.96 ± 0.04 | 0.90 - 0.30 | * |

[1]Numbers are given in average ± SEM, the maximum and minimum
‡*P* value determined by Kruskal-Wallis test with significance level < 0.05
NS = No significant
*$P < 0.05$
**$P < 0.01$

*Alteration in alpha and beta diversity indices, and bacteria taxa of the ceca community of chickens with NE-associated symptoms*

Several alpha diversity indices were calculated to estimate species richness including Chao1, Observed species, Shannon and Simpson diversity index. The total number of species and the species richness evaluated with Chao1 and Observed species parameters were significantly reduced in chickens with NE-associated symptoms. The alpha diversity indices that evaluated the species abundance and dominance, Shannon and Simpson, showed the microbial diversity of the cecum from sick chickens were reduced. Refer to Table 17 for the numeric values. The

comparisons of the structure of the bacterial communities between samples were visualized with PCoA with Weighted and Unweighted UniFrac distances. The 2D plot of Figure 14, panel D, showed dissimilarity of the bacterial community of the cecum between healthy and diseased chickens. This difference was additionally demonstrated with the numeric test ANOSIM of Weighted and Unweighted UniFrac, both *P* value = 0.001.

Differences in bacterial abundance were identified with the multivariate statistics LDA effect size (**LEfSe**) (Figure 15, Panel A) and the univariate Kruskal-Wallis test, Table 18. There were several bacterial taxa differentially differing in the abundance between healthy and sick cecum. The bacteria phyla that was altered due to the onset of necrotic enteritis were the *Firmicutes*, that reduced its abundance in chickens with NE symptoms, and *Proteobacteria,* that was enriched in NE birds. Based on LEfSe analysis, 17 bacterial taxa were significantly different in the abundance between health status (Figure 15, Panel A). LEfSe identified the bacteria family *Ruminococcaceae* and an unclassified bacterium from the order *Clostriales* enriched in the cecum of healthy chickens. The cecum of chickens with NE-associated symptoms were enriched in the bacteria family *Clostridiaceae* and *Enterobacteriaceae.* The univariate test, Kruskal-Wallis identified the ceca of chickens with NE were underabundant in the bacterial genera *Blautia*, *Oscillospira*, and *Ruminococcus,* but significantly increased the abundance of the bacterial genera *Clostridium* and an *Unclassified* from the family *Enterobacteriaceae,* (Table 18).

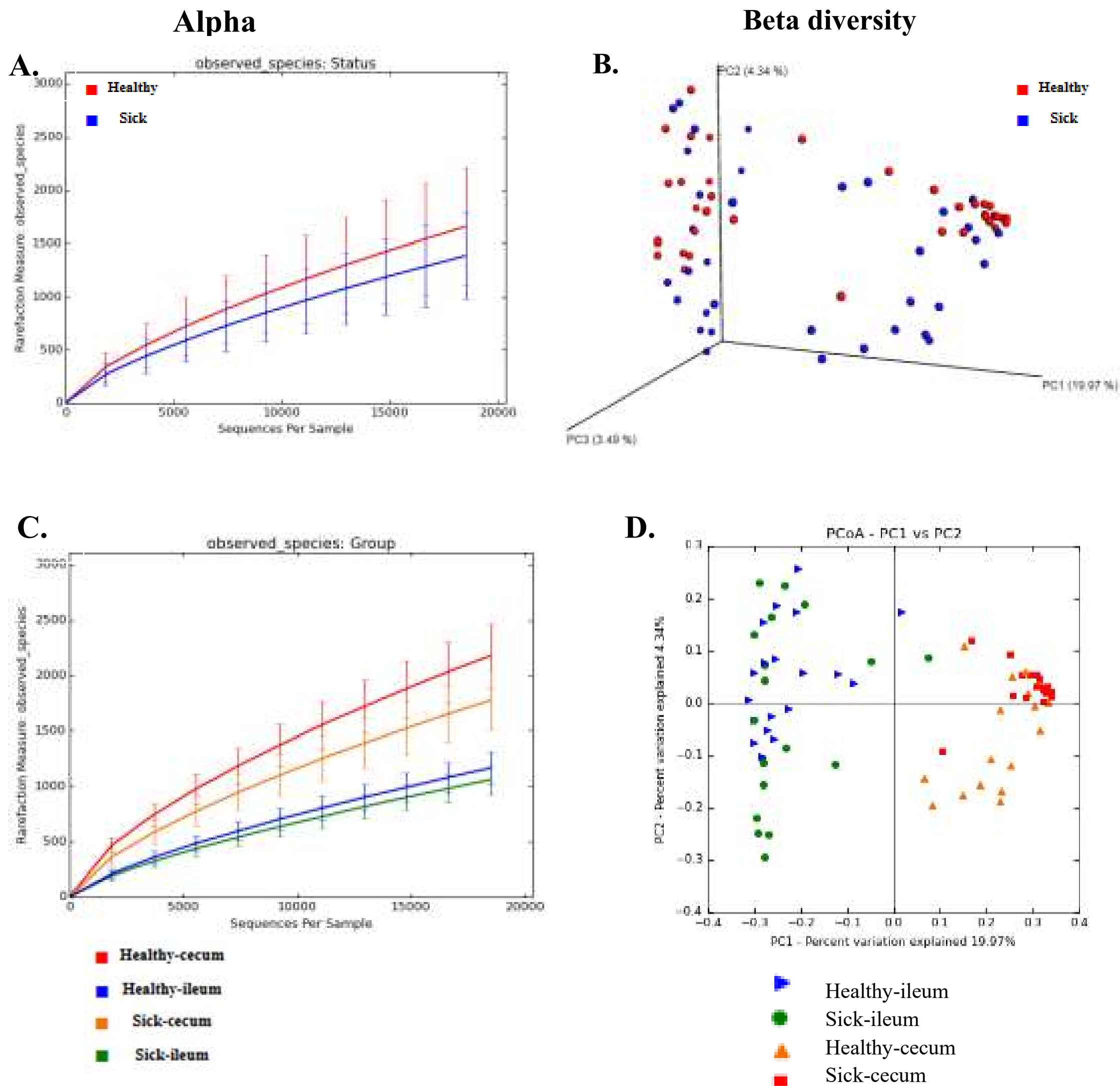


**Figure 14. Alpha and beta diversity parameters of healthy birds and chickens with NE-associated symptoms. Rarefaction analysis of 16S rRNA gene sequences at 18,500 reads per sample. Alpha diversity evaluated with the number of observed species: Panel A, rarefaction curves of numbers of Observed species by health status and Panel C, rarefaction curves of numbers of Observed species by tissue section and health status. Panel B, beta diversity visualized with a 3D principal coordinate analysis (PCoA) of Unweighted UniFrac distance, ANOSIM test revealed a significant difference in microbial population only with Weighted UniFrac metric (R = 3 P = 0.03). Panel D, community structure of each intestinal tissue by health status visualized with PC1 and PC2 by tissue section and health status.**

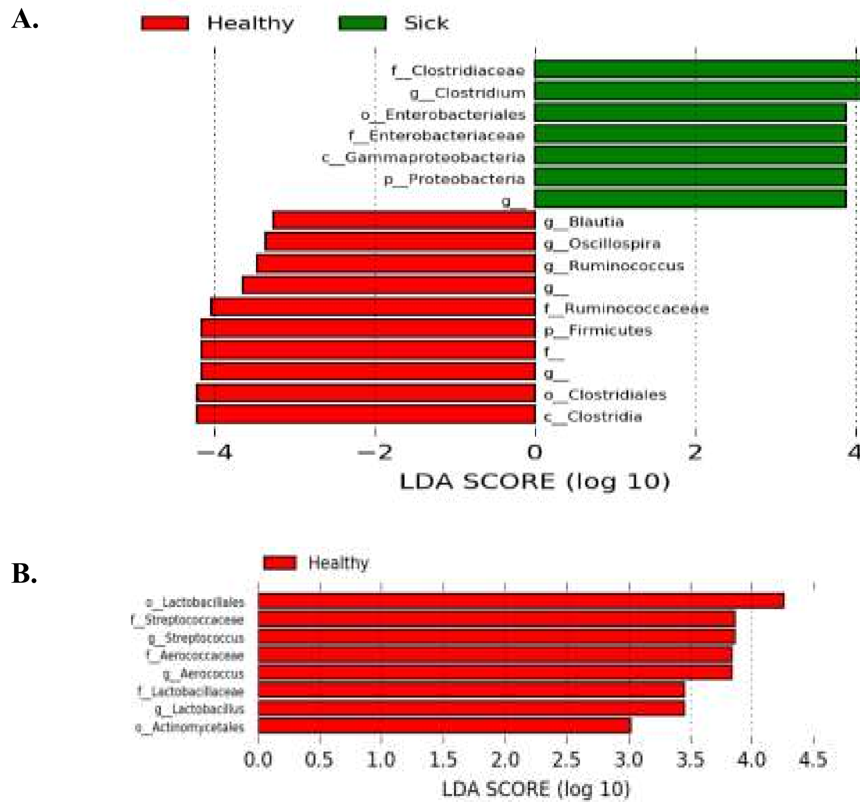


**Figure 15. Multivariate LDA effect size (LEfSe) test between healthy and chickens with necrotic enteritis. A) Differentially abundant taxa identified in the bacterial community of the ceca. B) Differentially abundant taxa identified in the bacterial community of the ileum of healthy chickens. No taxa were found enriched in ileum of birds with NE.**

*Alteration in alpha and beta diversity indices, and in the bacteria taxa of the ileum in chickens with NE-associated symptoms*

The alpha diversity parameters evaluated with Shannon and Simpson indexes showed a significant reduction of the diversity in the ileum of chickens with NE-associated symptoms. The alpha parameter calculated with Observed species, that measure the total number of species, also showed a reduction tendency in the sick ileum while Chao1 parameter was found not significant between sick and healthy ileum. The beta diversity evaluated with Principal Coordinates Analysis (PCoA) demonstrated a similarity between the community structure of the healthy and sick ileum

(Figure 14, panel D). Based on the Unweighted UniFrac ANOSIM the ileal community was dissimilar between healthy and sick chickens (R = 0.09, P = 0.01) but similar with the Weighted ANOSIM.

**Table 18. Relative abundance of the most abundant bacterial groups at different taxonomic levels in the cecum (phylum, class, order, family, genus).**

| Tissue: Cecum | Range (Min%- Maximum%) | | Medians (%) | | $P$ value[2] |
|---|---|---|---|---|---|
| | Healthy | NE[1] | Healthy | NE | |
| **Firmicutes** | 49.2 - 94.9 | 25.6 - 96.6 | 77.26 | 62.6 | ** |
| Bacilli | 3.3 - 57.0 | 1.9 - 69.3 | 15.1 | 15.0 | NS |
| Lactobacillales | 3.3 - 55.2 | 2.0 - 67.6 | 15.0 | 14.8 | NS |
| Lactobacillaceae | 0.2 - 9 | 0.1 - 18.7 | 1.7 | 0.2 | NS |
| *Lactobacillus* | 0.2 - 9.0 | 0.1 - 18.8 | 1.7 | 0.2 | NS |
| Enterococcaceae | 1.0 - 52.6 | 1.2 - 63.7 | 8.7 | 8.2 | NS |
| *Enterococcus* | 1.0 - 51.6 | 1.2 - 62.2 | 8.5 | 7.9 | NS |
| Streptococcaceae | 0 - 12.0 | 0 - 8.7 | 1.7 | 1.2 | NS |
| *Streptococcus* | 0 - 12.0 | 0 - 8.7 | 1.7 | 1.2 | NS |
| Clostridia | 18.3 - 77.1 | 22.9 - 69.8 | 15.1 | 42.2 | ** |
| Clostridiales | 18.3 - 77.1 | 22.9 - 69.8 | 62.8 | 42.2 | ** |
| Unclassified[‡] | 6.0 - 37.2 | 0.6 - 18.8 | 21.0 | 5.4 | *** |
| *Unclassified* | 6.0 - 37.2 | 0.6 - 18.8 | 21.0 | 5.4 | *** |
| Clostridiaceae | 0.2 - 2.5 | 0.3 - 65 | 0.6 | 1.33 | * |
| *Clostridium* | 0.2 - 2.4 | 0.1 - 63.1 | 0.5 | 1.1 | * |
| Lachnospiraceae | 0.5 - 29.0 | 1.0 - 13.4 | 8.4 | 6.2 | NS |
| *Blautia* | 0.1 - 11.7 | 0.2 - 1.6 | 1.1 | 0.6 | * |
| [*Ruminococcus*] [‡‡] | 0.1 - 12.0 | 0.3 - 8.6 | 3.1 | 3.5 | NS |
| Ruminococcaceae | 10.5 - 35.5 | 1.4 - 33.8 | 29.4 | 16.5 | * |
| *Unclassified* | 5.8 - 21.6 | 1.0 - 20.8 | 13.4 | 9.9 | * |
| *Oscillospira* | 1.4 - 11.8 | 0.2 - 7.4 | 3.3 | 1.6 | ** |
| *Ruminococcus* | 0.6 - 10.3 | 0 - 3.02 | 3.6 | 1.3 | *** |
| *Faecalibacterium* | 0.6 - 17.3 | 0.2 - 14.6 | 4.5 | 2.4 | NS |
| | | | | | |
| **Proteobacteria** | 0.1 - 49.6 | 0 - 36.8 | 0.2 | 3.5 | ** |
| Gammaproteobacteria | 0.1 - 49.6 | 0 - 36.8 | 0.2 | 3.5 | ** |
| Enterobacteriales | 0.1 - 49.6 | 0 - 36.8 | 0.2 | 3.5 | ** |
| Enterobacteriaceae | 0.1 -49.6 | 0 – 36.8 | 0.2 | 3.5 | ** |
| *Unclassified* | 0.2 - 49.5 | 0 - 36.8 | 0.2 | 3.5 | ** |
| | | | | | |
| **Bacteroidetes** | 0.6 - 31.3 | 1.4 - 59.6 | 18.1 | 59.6 | NS |
| Bacteroidia | 0.6 - 31.3 | 1.4 - 59.6 | 18.1 | 24.8 | NS |
| Bacteroidales | 0.6 - 31.4 | 1.36 - 59.6 | 18.1 | 24.9 | NS |
| Bacteroidaceae | 0.5 - 30.2 | 1.3 - 58.0 | 14.6 | 22.6 | NS |
| *Bacteroides* | 0.5 - 30.2 | 1.2 - 58.6 | 14.6 | 22.6 | NS |

[1]NE= chickens with necrotic enteritis
[2]P value determined by Kruskal-Wallis
[‡]Unclassified = annotation with 97% similarity to a reference sequence undefined at given taxonomic level
[‡‡]Squared brackets = suggested annotation based on Greengenes v. 13.8 database performed with QIIME v 1.9.0.
NS = No significant
$^{*}P < 0.05$
$^{**}P < 0.01$
$^{***}P < 0.001$

Most of the bacteria taxonomy differing between healthy and sick ileum were reported at the family and genera levels of the *Firmicutes* phylum. Multivariate statistic LEfSe test found 8 bacterial taxa more abundantly in the healthy ileum compared to the sick (Figure 15, panel B). The univariate test, Kruskal-Wallis, identified the bacterial family *Streptococcaceae*, *Aerococcaceae* and *Lactobacillaceae* significantly reduced in the ileum of diseased chickens (Table 19) while the healthy ileum were more abundant in the bacteria genera *Streptococcus*, *Aerococcus*, and *Lactobacillus*.

**Table 19. Relative abundance of the most abundant bacterial groups at different taxonomic levels in the ileum (phylum, class, order, family, genus).**

| | **Range (Min%- Maximum%)** | | **Medians (%)** | | |
|---|---|---|---|---|---|
| **Tissue: ileum** | Healthy | NE[1] | Healthy | NE | *P* value[2] |
| **Firmicutes** | 34.0 - 96.3 | 4.6 - 99.0 | 73.5 | 67.6 | NS |
| Bacilli | 34.0 - 92.4 | 4.3 - 98.8 | 72.0 | 43.6 | NS |
| Lactobacillales | 20.1 - 89.7 | 2.4 - 94.3 | 54.6 | 27.8 | * |
| Lactobacillaceae | 0 - 26.5 | 0 - 50.1 | 1.3 | 0.1 | ** |
| *Lactobacillus* | 0 - 26.5 | 0 - 50.1 | 1.2 | 0.1 | ** |
| Enterococcaceae | 5.6 - 72.3 | 1.8 - 88.8 | 23.8 | 17.4 | NS |
| *Enterococcus* | 4.3 - 70.7 | 1.6 - 87.1 | 23.4 | 16.8 | NS |
| Streptococcaceae | 0.1 - 43.4 | 0.1 - 5.5 | 2.0 | 0.1 | ** |
| *Streptococcus* | 0.2 - 43.4 | 0.1 - 4.5 | 2.0 | 0.2 | ** |
| Aerococcaceae | 0.2 - 47.6 | 0 - 18.5 | 1.0 | 0.4 | * |
| *Aerococcus* | 0.2 - 47.6 | 0 - 18.5 | 1.0 | 0.4 | * |
| Staphylococcaceae | 1.2 - 53.1 | 0.8 - 78.2 | 9.2 | 7.2 | NS |
| *Staphylococcus* | 1.2 - 53.0 | 0.8 - 78.2 | 9.2 | 7.1 | NS |
| Clostridia | 0.3 - 24.6 | 0.2 - 42.2 | 1.2 | 0.9 | NS |
| Clostridiales | 0.28 - 24.6 | 0.2 - 42.1 | 1.2 | 0.9 | NS |
| Clostridiaceae | 0.1 - 24.5 | 0.1 - 42.0 | 0.5 | 0.7 | NS |
| *Clostridium* | 0-0.6 | 0 - 40.5 | 0 | 0.2 | NS |
| | | | | | |
| **Proteobacteria** | 0.7 - 65.6 | 0.7 - 95.0 | 26.0 | 32.0 | NS |
| Gammaproteobacteria | 0.7 - 65.6 | 0.7 - 95.0 | 25.9 | 32.0 | NS |
| Enterobacteriales | 0.6 - 65.6 | 0.6 - 94.9 | 25.8 | 32.0 | NS |
| Enterobacteriaceae | 0.6 - 65.6 | 0.6 - 95.0 | 25.8 | 32.0 | NS |
| *Unclassified* ‡ | 0.6 - 65.5 | 0.6 - 91.9 | 24.4 | 32.0 | NS |

[1]NE= chickens with necrotic enteritis
[2]P value determined by Kruskal-Wallis
‡Unclassified = annotation with 97% similarity to a reference sequence undefined at given taxonomic level
‡‡Squared brackets = suggested annotation based on Greengenes v. 13.8 database performed with QIIME v 1.9.0.
NS = No significant
*P < 0.05
**P < 0.01
***P < 0.001

*Discussion*

The natural occurring necrotic enteritis chicken model has not been identified in regard to the shifts in the gut microbial populations. Considering that high dietary calcium concentration contributes to the pathogenesis of NE, two basal diets were formulated with high (0.90%) and low (0.70%) calcium that were supplemented with either product A or B that are feed additives for the control NE symptoms. The objective of this study was to assess the variations in the intestinal bacteria population at first signs of NE-symptoms in the natural occurring NE chicken model providing diets with two levels of calcium (high and low) and supplemented with two feed additives to ameliorate the disease. The analysis of the intestinal bacteria was explored at first signs of morbidity to evaluate possible initial alterations rather that the result of a secondary infection. The microbial information was obtained by sequencing the 16S rRNA bacteria genes of the ileum and cecum of healthy and diseased chickens.

Contrary to previous reports (Paiva et al., 2013, 2014), the low dietary calcium did not improve the negative effect of the onset of necrotic enteritis in this experiment. Additionally, the productive parameters from the low calcium diet were lower than those obtained with the standard diet which dietary calcium was proposed to be high and detrimental during a necrotic enteritis episode (Paiva et al., 2013, 2014). Similarly, no positive effect was perceived by the addition of supplement A or B regardless of the dietary calcium concentration. The final mortality was elevated in this study and the body weight was low compared to the potential body weight that a Cobb male can achieve at 42d, ~2,330g vs ~3147g (Cobb-Vantress, 2018) in all dietary treatments. This naturally occurring necrotic enteritis chicken model showed a flock with low productivity typical of a flock with necrotic enteritis outbreak.

The bacteria community of the cecum and the ileum were analyzed at 18d from a diseased chicken and a healthy pen-mate. Microbial diversity parameters were explored between dietary treatments and health conditions. The dietary calcium and the two feed additives did not affect the bacteria richness and diversity of the chickens, and the count of *Clostridium perfringens* in the ileum community were also unaffected by each dietary treatment. Rather, variations in the bacteria community were mostly influenced by the health condition and the intestinal segment of the chickens. As expected, the bacteria richness and diversity of the cecum were greater compared to the ileum, very likely to the differences on intestinal environment and substrate availability that each segment provides to the gut microorganisms. Therefore, most of our results were focused to compare the microbial population between sick and healthy chickens within each intestinal segment. Results of this experiment showed the onset of necrotic enteritis reduced the bacteria diversity in both gut segments, but more pronounced alterations were noted in the ceca microbial population than the ileum. Our results also showed that different phylotype of bacteria is affected on each intestinal tissue with the onset of NE. This naturally NE chicken model recovered greater counts of *Clostridium perfringens* from the ileum community of sick birds demonstrating the presence of necrotic enteritis disease in diseased chickens.

Marked shift in the ceca bacterial population of diseased chickens were reported by a reduction in the abundance of the bacteria family *Ruminococcaceae* (genera *Unclassified*, *Oscillospira*, and *Ruminococcus*) along with an increment in *Clostridiaceae* (genera *Unclassified* and *Clostridium*) when compared to healthy samples. The alterations in the aforementioned bacteria families were previously reported in chickens with necrotic enteritis induced with coccidia infection (Wu et al., 2014). The community of the cecum in this experiment was dominated by members of the family *Ruminococcaceae,* with a media relative abundance of 29.44 in healthy

chickens and 16.55 in diseased birds. This bacteria family has been associated with the production of butyrate short-chain fatty acid (Medvecky et al., 2018; Yang et al., 2013); thus, a reduction of its abundance may represent a reduction of butyrate concentration. Microbiome analysis of the ceca of chickens with necrotic enteritis induced with a virulent *Cp* also identified the onset of the disease reduce the population of butyrate producer bacteria (Stanley et al., 2012b). In a different study, Stanley et al. (2014) documented that the induction of necrotic enteritis with *Eimeria* and high dietary fishmeal modified the levels of various short-chain fatty acids where butyrate fatty acid greatly declined. Interestingly, the genus *Oscillospira* has also been associated with the production of butyrate fatty acid (Gophna et al., 2017; Konikoff and Gophna, 2016), and in this experiment the genus was also reduced with the onset of necrotic enteritis, from 3.3 in healthy to 1.6 in sick birds. Studies have correlated this bacterium genus with leanness, since its abundance is generally reduced in diseases that involved inflammation (Gophna et al., 2017). Therefore, it is assumed the protective effect of *Oscillospira* is through the production of butyrate short-chain fatty acid (Gophna et al., 2017; Konikoff and Gophna, 2016). The class *Clostridia* was overabundant in the cecum of chickens with NE-symptoms; it had a change in the media abundance from 15.1 to 42.2. This class of bacteria is categorized as the most abundant bacteria class in the community of the ceca of broiler chickens from 7 to 21 days post hatch (Corrigan et al., 2015). Within this class of bacteria, the family *Clostridiaceae* and the genus *Clostridium* were enriched (0.5 to 1.1) while members of an unclassified genus of the order *Clostridiale*s was reduced (21.0 to 5.4) in the ceca population of diseased chickens. Since the etiological agent of necrotic enteritis is the *Clostridium perfringens* and the enumeration of this bacterium in the ileum community was significantly greater in diseased birds compared to healthy, we attributed this enrichment may reflect an overgrowth of *Cp* in the ceca population.

The most predominant bacteria families in the ileum were the *Enterobacteriaceae,* with a median representation of 28.05 (25.88 in healthy and 32.02 in diseased birds), *Enterococcaceae* with 22.84 (23.85 healthy and 17.44 diseased) and *Staphylococcaceae* with 8.23 (9.58 healthy and 7.18 diseased). Gut bacteria analysis with bacteria fingerprinting identified that these bacteria families dominate the profile of the ileum community of broiler chickens (Yin et al., 2010). Other experiments have reported, utilizing various molecular approaches, that the bacteria family *Lactobacillaceae* is the most abundant in the ileum (Choi et al., 2014; Yin et al., 2010; Zeng et al., 2018). In this experiment the median relative abundance of *Lactobacillaceae* were in low proportion, 1.3 in the healthy ileum and 0.1 in the sick ileum which are extremely low compared to the 70% and 52% of the sequences reported in Lu et al. (2003) and Choi et al. (2014). Members of the family *Lactobacillaceae* are considered beneficial to the host because they produce antibacterial compounds (Stern et al., 2006), glucanase to degrade mixed linked β-D-glucans (Jonsson and Hemmingsson, 1991), and have bile salt hydrolases activity (Begley et al., 2006). Thus, its low abundance implies an imbalance in the gut bacteria population regardless of the health status of the chicken. Furthermore, linear discriminant analysis effect size, LEfSe, showed the ileal community of diseased birds reduced its abundance in members of *Lactobacillaceae* and *Streptococcaceae*. A reduction in these two ileal bacteria were previously reported from the mid-jejunum to mid ileum a day post-inoculation with three pathogenic *Cp* strains (Fasina et al., 2016). Chickens with subclinical necrotic enteritis significantly reduced the abundance of *Lactobacillus* spp. in the ileum (Qing et al., 2017). Various studies have concurred that a reduction of *Lactobacillus* species is a common consequence in birds with NE; in particular, the bacterium *L. johnsonii*, which is a probiotics *Lactobacillus*, has been reported to be underabundant in birds with necrotic enteritis lesions (Antonissen et al., 2015; Stanley et al., 2012b; Stanley et al., 2014; Wu

et al., 2014). Other members of the genus *Lactobacillus* that can be affected with the onset of NE are *L. ferementum*, *L. salivarius*, *L. crispatus*, and *L. pontis* (Stanley et al., 2012b). The overgrowth of *Cp* in the ileum community of diseased chickens may imply the conditions are not ideal for the growth of the aerobic *Lactobacillus* which may reduce the production of lactic acid bacteria (Fasina et al., 2016). All these observations suggest the natural occurring necrotic enteritis model reduces the abundance of lactic acid bacteria in the ileum of both healthy and diseased chickens which may be the causative of the low body weight and high mortality observed in this experiment.

Disruption on the community structure of the ceca was visually and numerically elucidated with the Weighted and Unweighted UniFrac principal coordinate analysis and ANOSIM, suggesting the community structure not only changed in the bacteria phylotypes but also in their abundance. On the other hand, the ileum community decreased in almost all calculated alpha parameters but the community structure in diseased chickens differed only with the Unweighted UniFrac. The initial onset of a necrotic enteritis outbreak is characterized by a dysbiosis in the ceca community represented by a dramatic alteration in the bacteria membership and abundance, while the ileum microbiota reduced only the species richness. The overgrowth of *Cp* in chickens with necrotic enteritis may be initiated in the anaerobic conditions of the cecum that latter colonized the proximal region of the cecum by the action of reverse peristalsis. The rarefaction level conducted in this experiment excluded three sick and one healthy cecum. An additional microbial analysis was conducted with a lower rarefaction level (15,000 read/sample) that included all 76 samples and confirmed the same tendency in alpha and beta diversity already reported in this document. Our findings shown that the bacteria alterations were mainly observed by the health condition of the host rather than dietary factors. However, based on the bacteria population found in the healthy ileum, this natural occurring necrotic enteritis model may predispose bird to environmental factors

that induced an imbalance gut microbial population. This is based on the low proportion of *Lactobacillus* spp. found in the ileum of healthy chickens which may produce less resistance to the overgrowth of *Cp*. Our initial hypothesis was that the variations in the gut microbial composition would be influenced by dietary treatments and the health status of the birds. According to our finding, the early gut microbial alterations in the spontaneous onset of necrotic enteritis in the naturally occurring model developed bacterial dysbiosis represented by a reduction of butyrate and lactic acid producer bacteria genera as previously reported by others in NE-induction models.

# CHAPTER V

# CONCLUSIONS

Avian species depend on the gut microbes for nutritional, physiological, and health reasons. The composition of the avian microbiome is influenced by several extrinsic and intrinsic factors such as diet and feed supplementation, environmental conditions, and host genetic background. Although there has been an increase in studies exploring how these different factors modulate the avian gut microbiota, most studies focus on the ability of dietary supplements to promote the growth of broiler chickens. There is little research where chicken genetics and the environment are explored as variable. Therefore, we conducted a series of studies to assess the effect of host-biology and environment related factors on the gut bacterial composition of commercial chickens. We started by characterizing the variations in the gut microbial population between the genetically divergent broiler (meat)-type and egg-type chickens in an experimental model that allowed these two breeds to hatch and live together while being fed the same diet. As these breeds of chickens differed in their metabolism, feed intake, and intestinal immunity (Han et al., 2016; Shires et al., 1987) we were able to observed host-genetic driven impacts on microbiota acquisition and development in two chickens strains within a homogenous environment. Our understanding of gut microbiota-host interaction is of relevance since the poultry industry is constantly improving the productivity through the genetic selection. In fact, some genetic programs resulted in the need to make special accommodation to extend the chicken's productivity, such as feed restriction in broiler breeder hens, which may have implications on animal welfare. Studies have allowed broiler breeder hens to consume feed until satiated and detected a number of resultant metabolic and physical compromises that limit fecundity and livability (Liu et al., 2014; Walzem and Chen, 2014). Since chicken independent studies have associated gut microbiota with the development of

metabolic disorders (Bäckhed et al., 2004; Bäckhed et al., 2007), the overfed broiler hen model motivated our second experiment to report the gut microbial changes when increasing feed intake of broiler hens without changing the macronutrients distribution of the diet fed. Our study was based on others in which Cobb 500 hens were allowed free access to feed for a 10-day period. In that strain of broiler hens overfeeding induced diabetic conditions, lipotoxicity, and impaired egg production (Chen, 2004). Finally, we assessed the influence of the environmental condition on gut bacteria composition of growing broiler in a poultry barn with a high prevalence of necrotic enteritis. The experimental design used in that last study has successfully reproduced necrotic enteritis without dietary alterations or infections with *Clostridium perfringens* and coccidia (Paiva et al., 2013, 2014). Because that model more closely resembles naturally occurring necrotic enteritis, we were able to examine microbiota during disease onset rather after secondary effects of severe intestinal damage had developed. While this naturally occurring necrotic enteritis chicken model has been used to test different dietary treatments to ameliorate the disease, information on the gut microbial associated changes is limited.

It was hypothesized that chicken genetics would determine changes in the intestinal bacteria when meat-type and egg-type chickens are exposed to the same environment. At the same time, we expected the high caloric consumption of non-feed restricted mature broiler hens, would alter the intestinal microbiota compared to the hens with the feed restriction regime due to consequences arising from genetic selection to increase growth rate and feed efficiency. Our last study hypothesized that the early gut microbial alterations in spontaneous onset necrotic enteritis conduct to an aberrant microbial composition. Our findings showed that:

- 1 First, genetically divergent meat-type and egg-type chickens develop similar gut microbial population when sharing environment and diet but the host genetic factor may influence the abundance of bacteria taxa as the chickens grew older.
- 2 Second, feeding twice the feed restriction ration (same macronutrients content) for 10 days did not affect the gut microbial diversity and induced small variations in low abundant species which may suggest a change in microbial functionality. Instead, a significant difference was reported based on the time hens were in the experimental environment.
- 3 Finally, the early gut microbial alterations in the spontaneous onset of necrotic enteritis in the naturally occurring model developed bacterial dysbiosis represented by a reduction of butyrate and lactic acid producer bacteria genera as previously reported by others in NE-induction models.

Collectively, our finding demonstrated environmental factors influencing each experimental trial. This was observed when genetically divergent chickens developed similar gut bacterial community when co-housed and fed the same diet. We also demonstrated that a change in the diet macronutrients induces alterations in the bacteria diversity in young chickens; while a surplus of dietary calories with a diet containing same macronutrients only affected low abundant bacteria in adult hens. Future research is needed to expand our comprehension on this confounding factor, especially utilizing experimental trials with similar farm conditions. This is of high industrial relevance and points to the key role of environmental exposures to flock success. Further work in host-biology should include metagenomic approach to associate bacterial functional genes with the host productive parameters. Especially targeting bacteria species of the order *Lactobacillales* that were found enriched in broiler-type chickens compared to layer (egg)-type

chickens, decreased in the feed-satiated mature broiler hens, and reduced in the naturally occurring necrotic enteritis model, suggesting an important functional role in lean meat deposition and health status in commercial chickens.

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